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RESEARCH ARTICLE

The ROS scavenger PDTC affects adaptation to treadmill running in mice: distinct effects on murine body mass, resting heart rate and skeletal muscle fiber type composition

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ABSTRACT

Regular exercise induces a broad spectrum of adaptation reactions in a variety of tissues and organs. However, the respective mechanisms are incompletely understood. In the context of their analysis, animal model systems, specifically rodent treadmill running protocols, play an important role. However, few researchers have studied different aspects of adaptation, such as cardiorespiratory and skeletal muscle training effects, within one set of experiments. Here, we analyzed physiological adaptation to 10 weeks of regular, moderate-intensity, uphill treadmill running in mice, a widely used model for endurance exercise training. To study the effects of reactive oxygen species (ROS), which have been suggested to be major regulators of training adaptation, a subgroup of mice was treated with the ROS scavenger PDTC (pyrrolidine dithiocarbamate). We found that mass gain in mice that exercised under PDTC treatment lagged behind that of all other experimental groups. In addition, both exercise and PDTC significantly and additively decreased resting heart rate. Furthermore, there was a trend towards an enhanced proportion of type 2A skeletal muscle fibers and differential expression of metabolism-associated genes, indicating metabolic and functional adaptation of skeletal muscle fibers. By contrast, there were no effects on grip strength and relative mass of individual muscles, suggesting that our protocol of uphill running did not increase skeletal muscle hypertrophy and strength. Taken together, our data suggest that a standard protocol of moderate-intensity uphill running induces adaptation reactions at multiple levels, part of which might be modulated by ROS, but does not enhance skeletal muscle hypertrophy and force.

KEY WORDS: Exercise, Reactive oxygen species, Cardiorespiratory adaptation, Metabolic adaptation

INTRODUCTION

Regular physical exercise induces characteristic adaptation reactions in a broad variety of tissues and organs. Endurance exercise, such as moderate-intensity running, particularly promotes adaptation reactions at the cardiorespiratory level. In addition, specifically in skeletal muscle tissue, metabolic adaptation reactions, such as an increase in capillary density and mitochondrial content, favoring oxidative metabolic pathways, can be observed. These adaptation reactions are orchestrated by changes in gene expression patterns, which are mainly initiated by mechanical signals, such as stretching or increased blood flow, and biochemical alterations, such as changes in calcium oscillation, energy depletion or reactive oxygen species (ROS) (for review, see Wackerhage and Woods, 2002; Powers et al., 2020).

The positive preventive, therapeutic and rehabilitative effects of regular exercise on a broad variety of medical conditions, particularly lifestyle-related diseases, have been widely acknowledged (for review, see Lavie et al., 2019). However, the underlying mechanisms are still incompletely understood. Their elucidation is a prerequisite to design and optimize training regimens and to develop exercise-associated biomarkers for different medical applications and contexts.

Animal, particularly rodent, exercise models serve as easy-to-standardize systems to assess training-induced adaptations in different tissues and organs. While particularly protocols of uphill treadmill running have been described in the literature, few studies have systematically analyzed physiological and biochemical adaptation reactions in parallel, which is a prerequisite to correlate different aspects of adaptation and eventually define causal relationships between particular biochemical signals and adaptation reactions at the physiological level.

The role of ROS in exercise is complex and has been the subject of ongoing research for the last four decades. In contracting skeletal muscle, ROS are produced primarily in the sarcolemma and T-tubules, the sarcoplasmic reticulum and the mitochondria in the context of reactions catalyzed by various enzymes, namely diverse NADPH oxidases and phospholipase A_2 (PLA_2). They might lead to skeletal muscle damage and fatigue, but also switch on signal transduction pathways that are crucial for exercise adaptation. The complex interplay between these effects, specifically the factors that determine whether a particular ROS signal at a certain time has positive or deleterious effects, has not yet been completely elucidated (for review, see Powers et al., 2020).

Here, we studied the effects of 10 weeks of uphill running on young, healthy, male mice. To evaluate the effects of the ROS pathway, a subgroup of mice was treated with the ROS scavenger PDTC. Our results indicate that both regular, moderate-intensity running and PDTC treatment exert distinct effects on physiological parameters, such as total body mass and resting heart rate, but also induce characteristic and unique metabolic adaptation reactions of individual skeletal muscles of both forelimbs and hindlimbs.

MATERIALS AND METHODS

Mice

Male C57BL/6NCrl *H-2*^{b} mice (n=16) were purchased from Charles River (Sulzfeld, Germany) at 6–7 weeks of age. They were housed and fed according to federal guidelines and regularly inspected by a veterinarian. Food (standard chow) and water were available *ad libitum*. All experimental procedures were approved by the local authorities (Regierungspräsidium Tübingen, M9/14).

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Exercise

Treadmill running was performed as previously described (Schmitt et al., 2018). Briefly, after a week of familiarization with the device, during which animals were run at 0 cm s^{-1} and 0 deg for 10 min (session 1), 10 cm s^{-1} and 5 deg for 10 min (session 2), 14 cm s^{-1} and 5 deg for 20 min (session 3) and 14 cm s^{-1} and 5 deg for 30 min (session 4), starting at an age of 8–9 weeks, mice were run over a period of 10 weeks for an hour each on Mondays, Wednesdays and Fridays. The protocol included a graded increase with regard to speed and incline: week 1: 12 m s^{-1}, 5–10 deg; week 2: 14–18 m s^{-1}, 10 deg; week 3: 19–21 m s^{-1}, 10 deg; week 4: 22/18/20 m s^{-1}, 10/15/15 deg; week 5: 22–24 m s^{-1}, 15 deg, until mice eventually exercised at 24 cm s^{-1} and 15 deg incline from the middle of week 5 onwards. Mice ran voluntarily, i.e. without electric shock stimulus. Animals that rested at the bottom of their lanes were encouraged to resume running by a gentle tip on their back. n=16 mice were employed, which were randomly clustered into four groups of n=4 animals: sedentary (not exercised); sedentary+PDTC; exercised; and exercised+PDTC.

PDTC treatment

PDTC was administered with the drinking water at an approximate daily dose of 5 mg kg^{-1} during the familiarization week, 10 mg kg^{-1} during the first week of training, and 20 mg kg^{-1} from the second week of training onwards. PDTC dosage was chosen based on literature data and previous results of our group (Schmitt et al., 2018, 2020, and references therein).

Grip strength

Grip strength of the forelimbs or all four extremities was determined using a grip strength meter (Bioseb, Vitrolles, France). Maximum force was measured immediately before (steady-state value, indicating long-term adaptation) and after the last training session (indicator of potential fatigue in response to the training session). For each animal and at each time point, five measurements were carried out and results were displayed as means of these five readings.

Determination of resting heart rate

Resting heart rate was determined 24 h after the last training of the exercised group using PhysioSuite (Kent Scientific, Torrington, CT, USA) in combination with the MouseSTAT® Pulse Oximeter & Heart Rate Monitor Module. Within a period of 10 min, five measurements were taken for each animal.

Tissue dissection

Forty-eight hours after the last training session, mice were anesthetized using isoflurane and subsequently euthanized by retrobulbar withdrawal of blood and cervical dislocation. For subsequent RNA isolation, muscle tissue of the right extremities was weighed, transferred into RNALater™ (Thermo Fisher, Waltham, MA, USA) and stored at −20°C. Tissue dissected from the left limbs was bisected. One half was immediately shock-frozen in liquid nitrogen for subsequent protein isolation; the other was transferred to a cork slide, frozen in melting isopentane in an upright position and subsequently employed in mATPase staining and immunofluorescence analysis. Both tissue isolates were then stored at −80°C until further use.

ATPase staining

mATPase staining after acid preincubation (pH 4.5) was carried out as previously described (Muscle Physiology Laboratory, 2000; Chung, 2012, and references therein; Kalmar et al., 2012).

Immunofluorescence

Immunofluorescence staining of different myosin heavy chain isoforms was carried out as previously described (Kalmar et al., 2012; Kammoun et al., 2014; Talmadge et al., 2014) using the following antibodies: monoclonal anti-bovine SC-71 (MyHC IIA) supernatant (DSHB, Iowa City, IA, USA; 1:100), monoclonal anti-bovine BA-D5 (MyHC I) supernatant (DSHB; undiluted), monoclonal anti-bovine BF-F3 (MyHC IIB) concentrate (DSHB; 1:100), Alexa Fluor 546 goat anti-mouse IgG1 (Thermo Fisher; 1:350), Alexa Fluor 350 goat anti-mouse IgG2b (Thermo Fisher; 1:100) and Alexa Fluor 488 goat anti-mouse IgG1 (Thermo Fisher; 1:250).

RNA isolation and qPCR analysis

RNA isolation and qPCR analysis were carried out as previously described (Schmitt et al., 2018). Primers were synthesized by Eurofins Genomics (Ebersberg, Germany). Primer sequences are displayed in Table 1.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Means of individual measurements were analyzed using two-way ANOVA. In the case of statistical significance, Tukey's HSD test was employed to identify significantly different experimental groups. Significance was determined as P<0.05, P<0.01 and P<0.001.

RESULTS

Body mass

Mass gain of all mice was within the normal range for strain, sex and age. Nevertheless, mice of the exercised+PDTC group lagged behind, weighing about 2 g less at the end of the experiment when compared with the other three experimental groups. When comparing exercised mice with and without PDTC treatment (i.e. exercised versus exercised+PDTC), this effect was significant (Fig. 1).

Mass of individual muscles

To determine whether this body mass discrepancy might be due to differences in individual muscle mass, a set of selected muscles, specifically hindlimb muscles m. tibialis anterior, m. gastrocnemius,

Table 1. Sequences of qPCR primers employed in this study

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
MyH7 (type 1)	CCT TGG CAC CAA TGT CCC GGC TC	GAA GCG CAA TGC AGA GTC GGT G
MyH2 (type 2A)	ATG AGC TCC GAC GCC GAG	TCT GTT AGC ATG AAC TGG TAG GCG
MyH4 (type 2B)	CAT CTG GTA ACA CAA GAG GTG	ACT TCC GGA GGT AAG GAG CA
MyH1 (type 2X)	GCT TCA AGT TTG GAC CCA CG	ACT TCC GGA GGT AAG GAG CA
Cs	GCC CAG CAG CTG TAG GAT GAC C	GGG GCC TGT CCC TGG CGT AG
Tbc1d1	GCG CAA ACA GAA CCT TGA CC	CGC AGC CTG CTT CAG CTT AC

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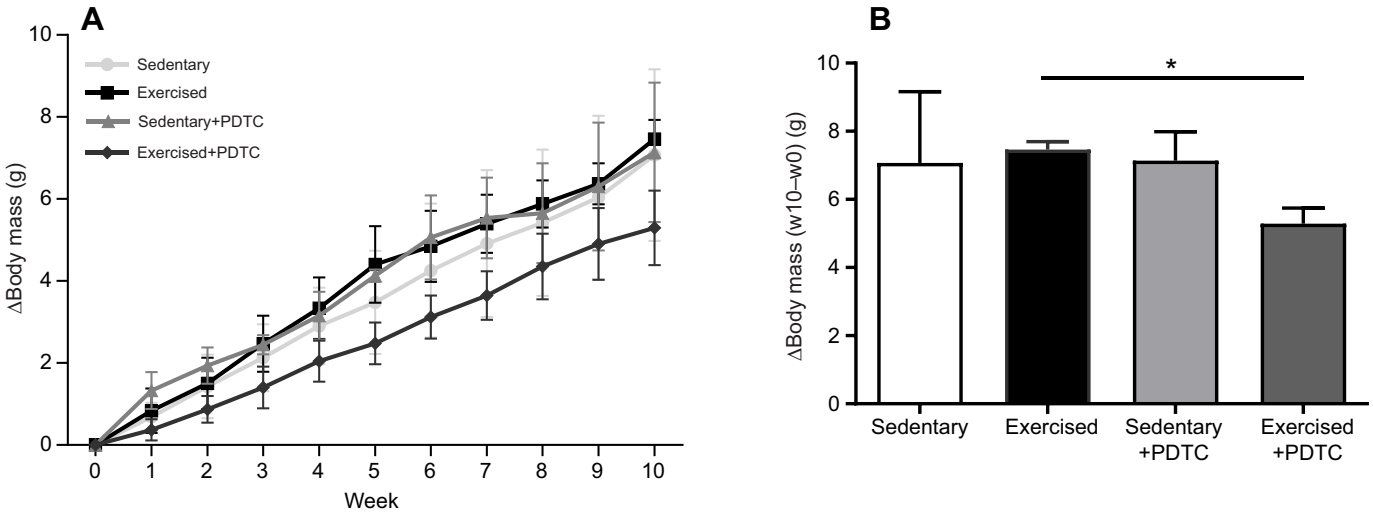

Fig. 1. Mass gain throughout the experiment. (A) Weekly mean±s.d. mass gain and (B) mean±s.d. mass gain from week 0 to week 10 in the four experimental groups. Mice were weighed before each training session. Means of individual measurements were analyzed using two-way ANOVA. In the case of statistical significance, Tukey's HSD test was employed to identify significantly different experimental groups. n=4. *P<0.05.

m. soleus, m. extensor digitorum longus and m. rectus femoris (which is part of m. quadriceps), and forelimb muscles m. triceps brachii, caput longum, m. biceps brachii, m. extensor carpi radialis, m. flexor digitorum superficialis, as well as m. pectoralis, diaphragm and heart, were dissected from all mice, weighed individually, and correlated with total body mass. However, as shown in Fig. 2, there were no significant differences, suggesting that lower mass gain in the exercised+PDTC group is unlikely to be due to a relatively lower muscle mass.

Grip strength

To test whether treadmill exercise induced gains in muscle strength, grip strength of the front limbs and of all four extremities was measured before and after the last training session of the exercise group. However, as shown in Fig. 3, apart from decreased values immediately after training, probably due to fatigue, there were no significant differences, suggesting that our protocol of uphill running did not have immediate effects on muscle strength.

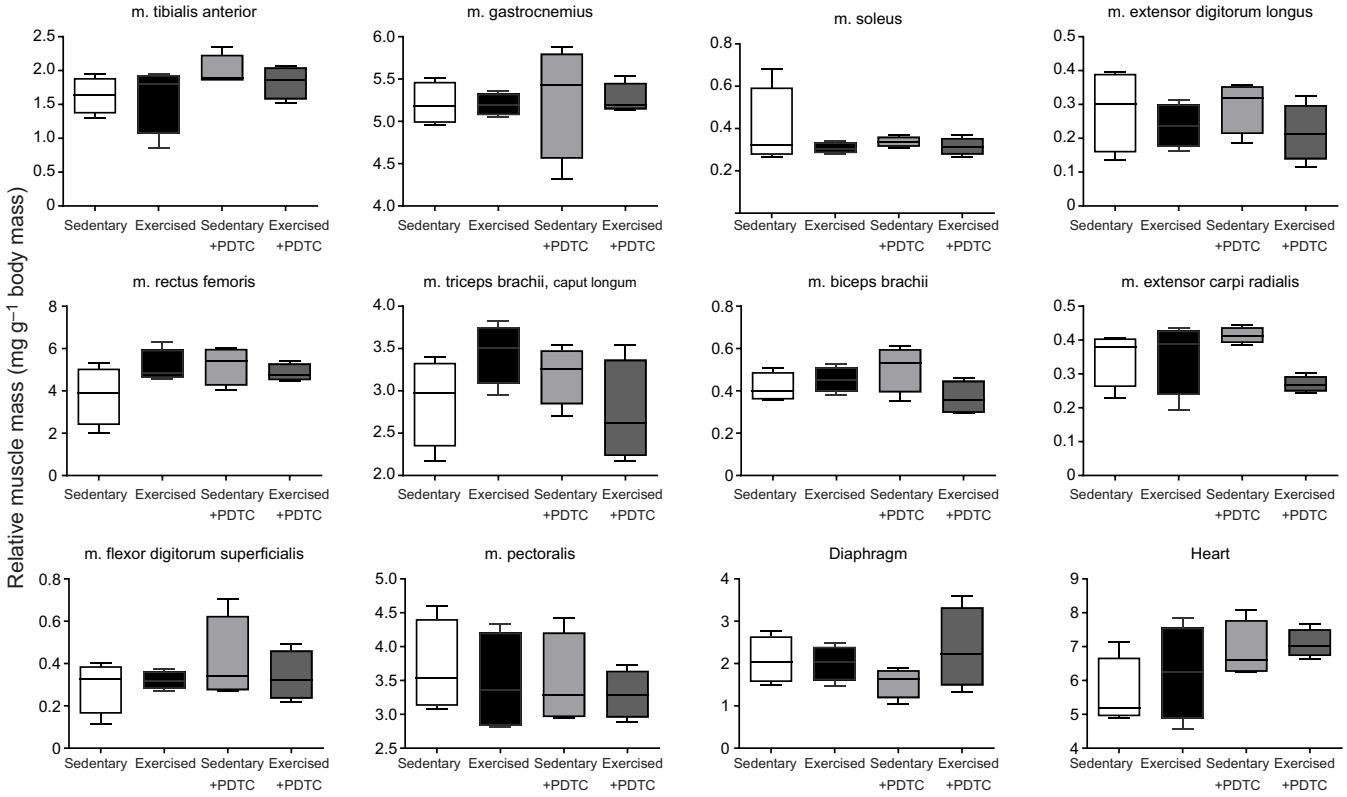

Fig. 2. Mass of individual muscles after dissection. The mean±s.d. mass of individual muscles is given relative to body mass for the four experimental groups. Means of individual measurements were analyzed using two-way ANOVA. n=4.

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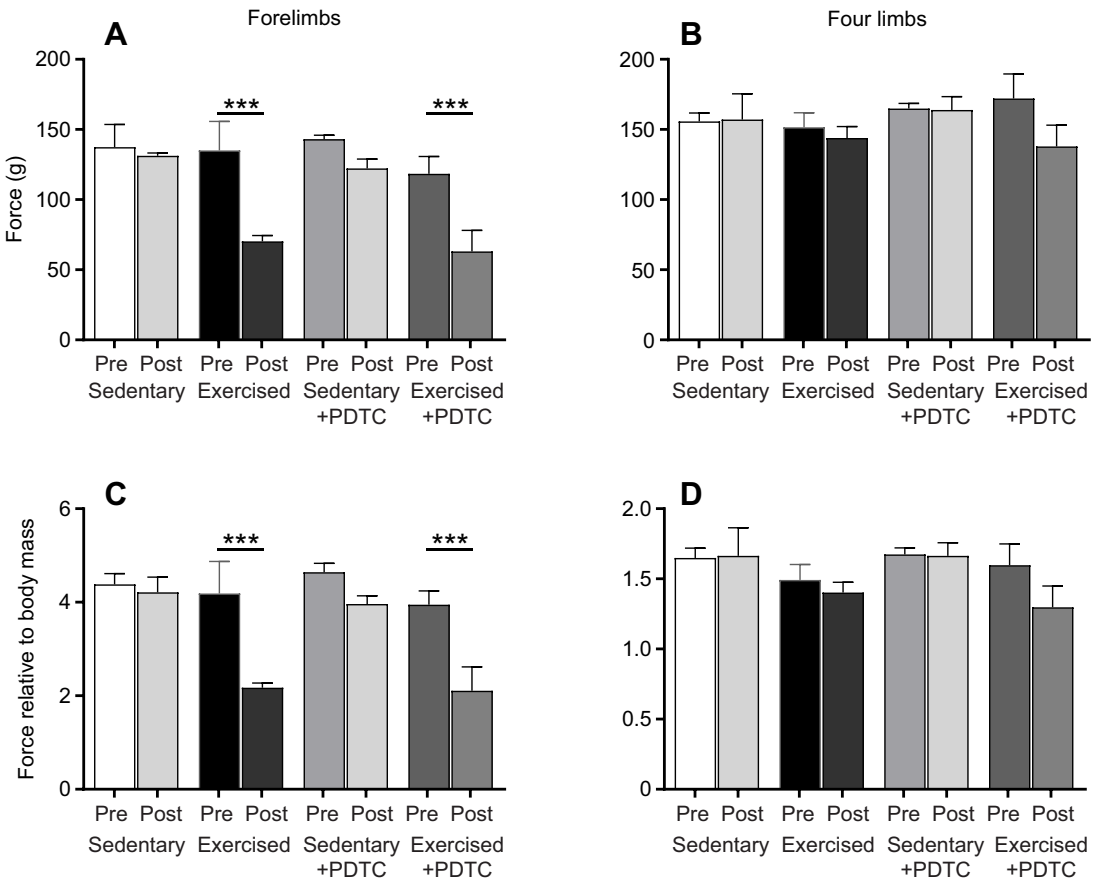

Fig. 3. Results of grip strength test. Mean±s.d. absolute (A,B) and relative (C,D) maximum grip strength, determined in the forelimbs only (A,C) or in all four limbs (B,D), before (pre) and after (post) the last training session of the exercise group. Means of individual measurements were analyzed using two-way ANOVA. In the case of statistical significance, Tukey's HSD test was employed to identify significantly different experimental groups. n=4. ***P<0.001.

Resting heart rate

To assess cardiovascular effects of training, resting heart rate of all mice was determined at the end of the experiment. Indeed, as shown in Fig. 4, there was a significant effect: both exercise and PDTC alone exerted significant lowering effects on this parameter, and there was an additive effect for the combination of the two. These data suggest that both training and PDTC induced specific adaptation reactions at the cardiovascular level.

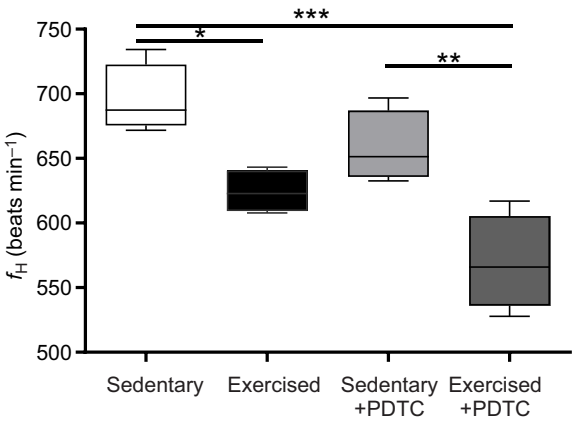

Fig. 4. Resting heart rate. Mean±s.d. resting heart rate (f_H) in the four experimental groups. Means of individual measurements were analyzed using two-way ANOVA. In the case of statistical significance, Tukey's HSD test was employed to identify significantly different experimental groups. n=4. *P<0.05, **P<0.01, ***P<0.001.

Fiber type

To test the hypothesis that regular running exercise influences muscle fiber type composition, cross-sections of m. soleus were stained for mATPase activity. This muscle was chosen because it does not contain type 2B fibers (Augusto et al., 2004), so that unambiguous identification and quantification of type 2A fibers should be possible. In addition, fiber types were visualized by immunofluorescence, using specific antibodies. As shown in Fig. 5, the two methods yielded similar results: in response to both training and PDTC, a trend towards a higher proportion of type 2A fibers was observed. Whereas after exercise, this was compensated for by a lower proportion of type 1 fibers, PDTC appeared to instead exert a lowering effect on the proportion of type 2X fibers.

Gene expression in individual skeletal muscles

To assess skeletal muscle training adaptation at the molecular level, a set of different muscles (m. gastrocnemius, m. extensor digitorum longus, m. soleus and m. triceps brachii, caput longum) was dissected from all mice and used for the determination of marker gene expression via qPCR. Specifically, expression of genes encoding fiber type-specific myosin heavy chain isoforms, metabolic markers, as well as sarcomeric and sarcomere-associated factors was analyzed, with a specific focus on potential differences between individual muscles. A similar approach has already been taken in a previous study (Schmitt

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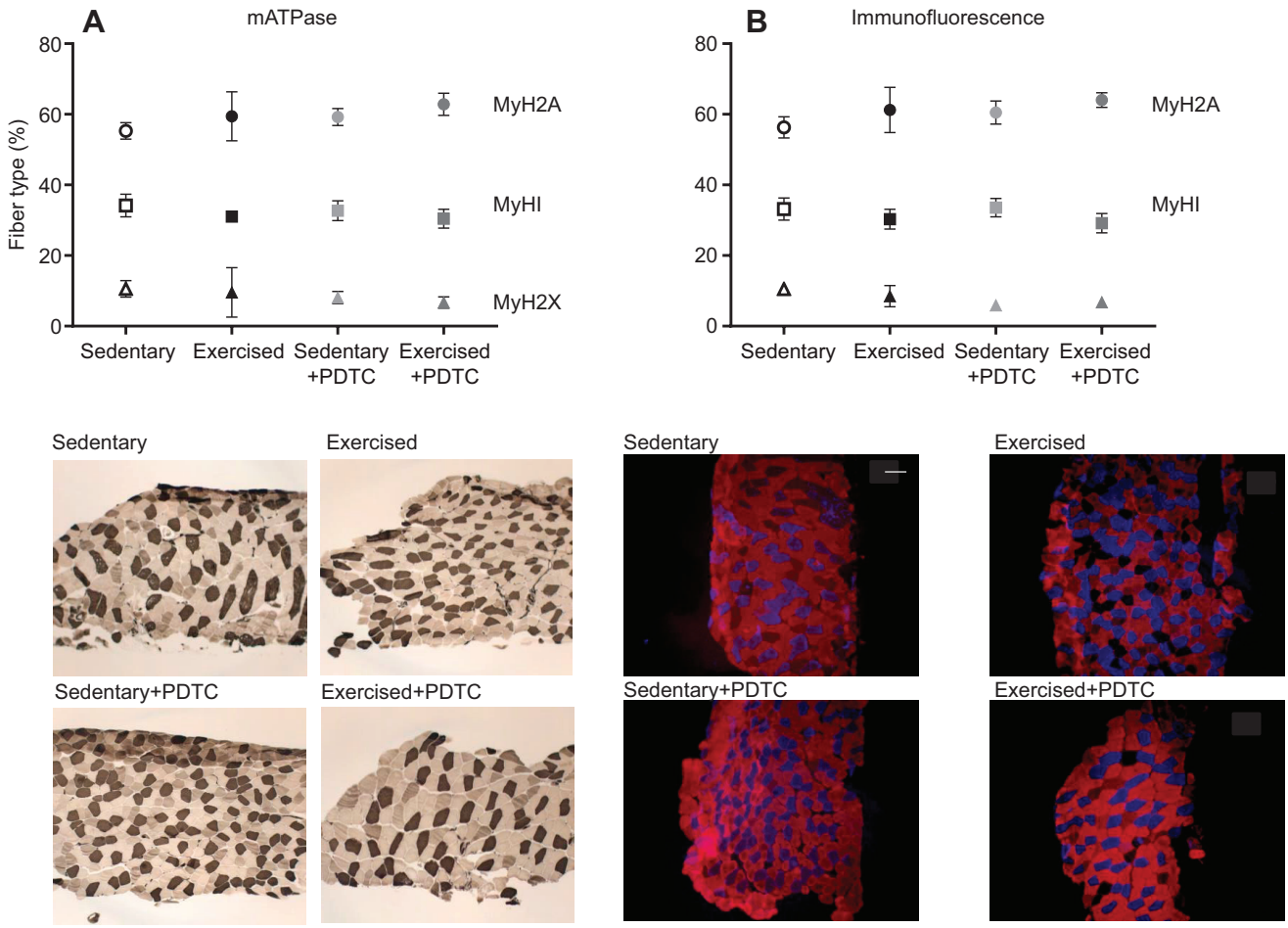

Fig. 5. Fiber type. (A,B) Cryosections of soleus were analyzed for proportions of individual fiber types. Top: mean±s.d. percentage of each fiber type. Bottom: representative micrographs. (A) m-ATPase staining: dark brown: type 1 fibers, bright: type 2A fibers, intermediate/light brown: type 2X and type 2B fibers. (B) Immunofluorescence staining: blue: type 1 fibers, red: type 2A fibers, black/unstained: type 2X fibers, green: type 2B fibers (not present in soleus). Scale bar: 100 µm. Means of individual measurements were analyzed using two-way ANOVA. *n*=4.

et al., 2018), suggesting differences between individual muscles; thus, the focus of this study was specifically the comparison of marker expression in a larger set of individual muscles.

Fiber type-specific myosin heavy chain isoforms

In m. gastrocnemius, m. extensor digitorum longus and m. triceps brachii, caput longum, we found consistent induction of *MyH2*, encoding MyH2A, by exercise. PDTC alone had no effect. Interestingly and inconsistent with the histological data, this effect was not observed in m. soleus. By contrast, there was no major effect on expression of *MyH4*, encoding the very fast MyH2B, in any of the analyzed muscles. In addition, as expected, this gene was hardly expressed in m. soleus. Similarly, expression of *MyH1*, encoding the 'fast' MyH2X, and *MyH7*, encoding the 'slow' MyH1, was not significantly different between the four experimental groups (Fig. 6).

Metabolic markers

Exercise induced a consistent induction of *Cs*, encoding citrate synthase, in almost all of the analyzed muscles, whereas PDTC had no major effect. Similar results were obtained for the *Tbc1d1* gene, at least in m. extensor digitorum longus and m. soleus, suggesting distinct effects of exercise on skeletal muscle metabolic control (Fig. 7).

Factors involved in sarcomerogenesis and sarcomere degradation

Finally, we analyzed expression of genes encoding proteins involved in sarcomere rearrangement; specifically, factors regulating folding and decay of sarcomere proteins, at both the mRNA and protein level. The respective marker set has already been described in Schmitt et al. (2018), and included genes encoding E3 ubiquitin ligases, such as atrogin-1 or MuRF1, proteases, such as the calpains, or sarcomere-associated molecular chaperones, such as the heat shock proteins Hsp70 and Hsp90. However, as already described in Schmitt et al. (2018) for m. quadriceps, m. gastrocnemius and m. tibialis anterior, in all of the analyzed muscles, there was little effect on most markers (data not shown).

DISCUSSION

Our study aimed at analyzing physiological and skeletal muscle molecular effects of a widely used mouse exercise model, regular moderate-intensity treadmill running. To study potential effects of ROS and pro-inflammatory signaling, a subgroup of mice was treated with the ROS scavenger and NFκB inhibitor PDTC.

Whereas both exercise and PDTC alone had no effect on animals' overall mass gain, body mass of exercised+PDTC mice lagged behind that of all other experimental groups throughout the experiment. As there was no difference with regard to (relative)

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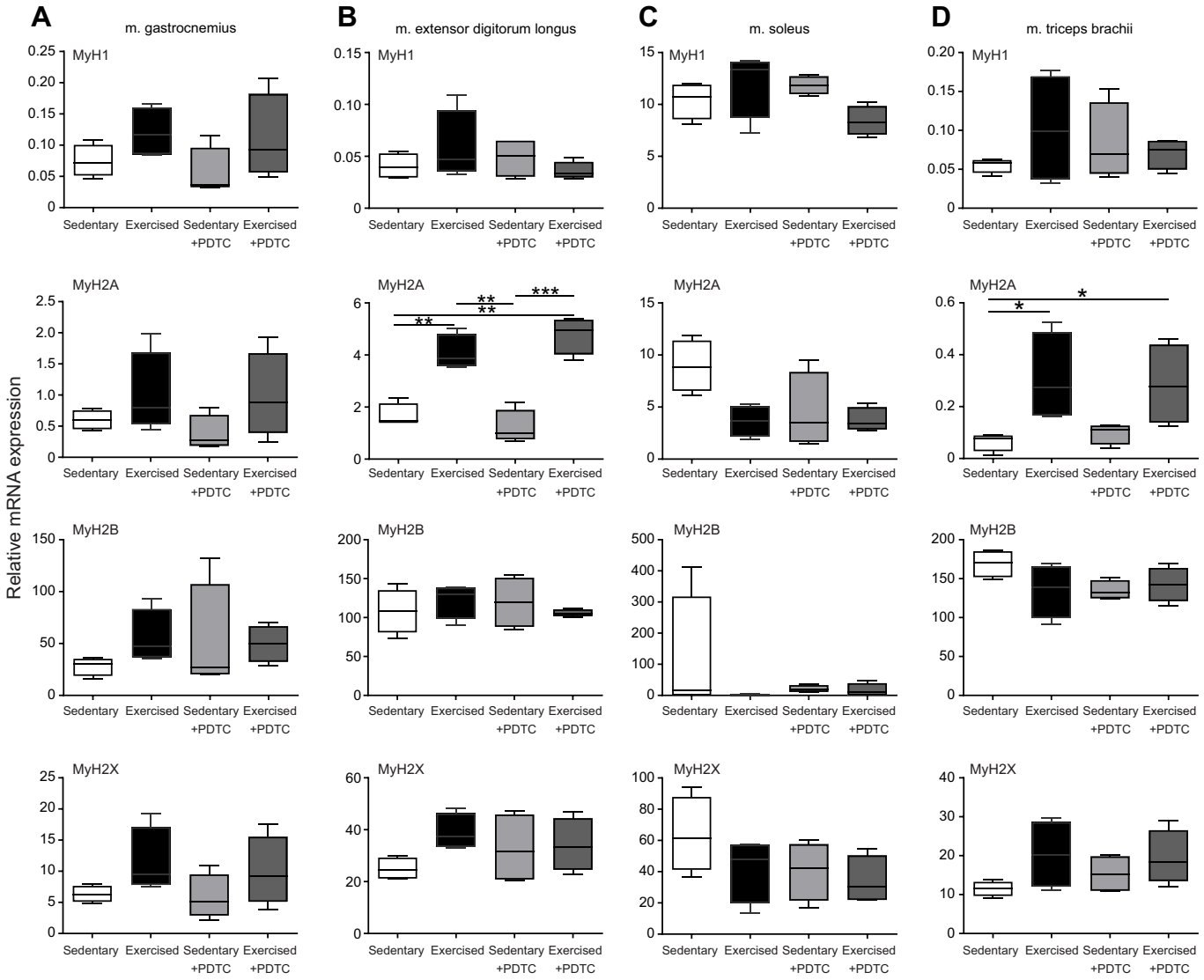

Fig. 6. Expression of genes encoding fiber type-specific myosin heavy chains. Mean±s.d. expression of genes encoding different MyH isoforms was analyzed in the indicated muscles, analyzed by qPCR. (A) MyH1 (*MyH7*), (B) MyH2A (*MyH2*), (C) MyH2B (*MyH4*) and (D) MyH2X (*MyH1*). Means of individual measurements were analyzed using two-way ANOVA. In the case of statistical significance, Tukey's HSD test was employed to identify significantly different experimental groups. n=4. *P<0.05, **P<0.01, ***P<0.001.

mass of individual muscles and, correspondingly, (relative) grip strength between any of the experimental groups, it is unlikely that this finding is due to decreased (training-induced) muscle hypertrophy. Rather, it is likely that the combination of PDTC and exercise led to a more general and probably mechanistically complex situation of developmental delay. To assess PDTC effects on training-induced muscle hypertrophy, it would be interesting to test the effects of this compound in a setting of resistance exercise, where significant gains in muscle mass should be observed.

Resting heart rate was lower in response to both training and PDTC treatment and we observed an additive effect of the two factors. Decreases in resting heart rate are a well-known phenomenon in endurance-trained subjects, despite the fact that the underlying mechanisms are not yet fully understood (for review, see Reimers et al., 2018), especially because heart rate regulation is a complex phenomenon, involving intrinsic mechanisms, such as hormonal stimuli or effects of the autonomic nervous system, and extrinsic effects, such as exercise or pharmacological interventions (for review, see Gordan et al., 2015). The fact that there was also a heart rate-depressing effect of PDTC, both alone and in combination with exercise, is interesting: given that both high levels of ROS and elevated levels of inflammatory markers have been shown to be positively associated with resting heart rate (for review, see Zhang and Zhang, 2009), it is possible that this might be due to the anti-oxidative effect of PDTC and/or blockade of downstream 'pro-inflammatory' signaling, namely the NFκB pathway. In addition, decreased mass gain specifically of exercised+PDTC mice, especially when associated with decreased food intake, points to a lower metabolic rate in these animals, which might also reduce resting heart rate (Zhang and Zhang, 2009). To test this hypothesis, further studies should monitor total caloric intake in parallel to training/PDTC treatment.

Because, for technical reasons, it was not possible to determine physiological parameters, namely resting heart rate and grip strength, before the start of the experiment, we cannot completely exclude systematic differences between animals with regard to these parameters prior to the experiment. However, this is unlikely, given that animals were of an inbred strain and of exactly the same age, were

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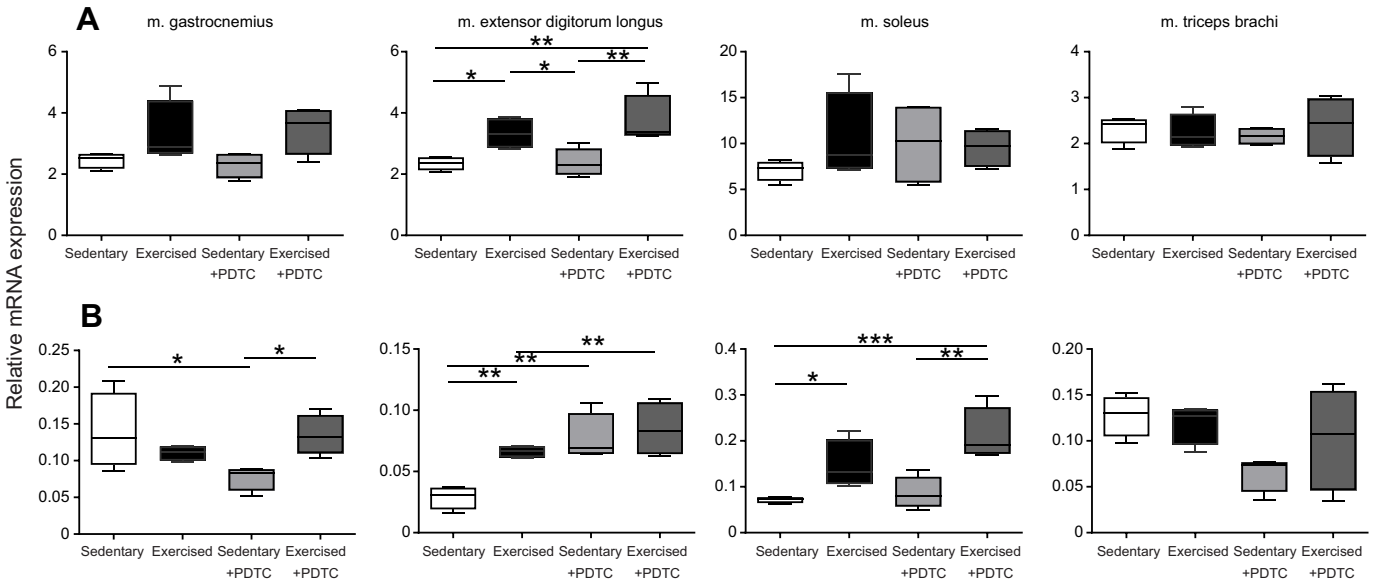

Fig. 7. Expression of genes encoding metabolic markers Cs and Tbc1d1. Mean±s.d. expression of *Cs* (A) and *Tbc1d1* (B) in the indicated muscles. Means of individual measurements were analyzed using two-way ANOVA. In the case of statistical significance, Tukey's HSD test was employed to identify significantly different experimental groups. n=4. *P<0.05, **P<0.01, ***P<0.001.

bred by the same animal facility, with some of them being littermates, and were randomly assigned to the four experimental groups. Still, this is a major limitation of our study. With regard to skeletal muscle fiber type composition and metabolism, our data point to a trend towards a higher proportion of type 2A fibers in response to both training and PDTC. Type 2A fibers are considered 'fast' and nevertheless 'oxidative' and thus represent an intermediate fiber type that is often enriched in situations of skeletal muscle adaptation to external stimuli, such as exercise (for review, see Pette and Staron, 2000). Thus, our data suggest that adaptation to training in our mice was still ongoing after 10 weeks, that adaptation capacity of most murine muscles is limited to a certain degree and/or that in most murine muscles, a substantial (slightly elevated) proportion of type 2A fibers represents the optimal adaptation state to our regimen of regular, moderate-intensity uphill running.

Despite the fact that mATPase staining and immunofluorescence yielded very similar results with regard to fiber type composition, qPCR results of *MyH* gene expression were in part, but not completely, congruent. This might be because: (1) qPCR detects MyH RNA and staining detects MyH protein (or enzymatic activity) levels, (2) fiber type counting does not take muscle fiber size/cross-sectional area into account and (3) skeletal muscle, specifically in situations of adaptation to external stimuli, such as training or pharmacological treatment, may contain a certain proportion of 'hybrid' fibers that contain several different MyH isoforms, a condition that cannot be adequately assessed via numerical quantification of fiber type proportions.

In summary, these data suggest a certain degree of skeletal muscle fiber type, i.e. metabolic adaptation. Correspondingly, we found enhanced levels of *Cs*, a marker of 'oxidative' metabolism, in almost all of the analyzed muscles. For other metabolic markers, such as the *Tbc1d1* gene, results differed more widely between individual muscles, suggesting that these markers are characterized by highly specific individual biochemical patterns of adaptation.

Against this background, it is not surprising that despite the fact that we overall found little effect on genes involved in the regulation of sarcomere rearrangement, as previously described for a small subset of muscles (Schmitt et al., 2018), there were profound effects for individual genes in certain, specific muscle types. Thus, further studies should aim at elucidating specific biochemical adaptation properties of individual muscles in the context of particular exercise regimens.

Conclusions

Our results suggest complex effects of exercise, PDTC treatment and a combination of the two on cardiovascular parameters, such as resting heart rate, as well as skeletal muscle gene expression patterns and metabolism. Future studies should aim at elucidating mechanistic effects of this in more detail. Furthermore, other ROS scavengers, as well as different training regimens, namely resistance exercise protocols, should be tested, which might have important therapeutic implications.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.S., B.M.; Methodology: F.R., A.S., A.B., A.F., B.M.; Validation: F.R., A.S., A.B., A.F., B.M.; Formal analysis: F.R., A.S., B.M.; Investigation: F.R., A.S., A.B., A.F.; Resources: A.S., A.B., A.F.; Data curation: F.R., A.S.; Writing - original draft: F.R., B.M.; Writing - review & editing: A.S., B.M.; Visualization: F.R., A.S., B.M.; Supervision: A.S., B.M.; Project administration: B.M.; Funding acquisition: B.M.

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