CARDIOVASCULAR ADJUSTMENTS AND ADAPTATIONS TO EXERCISE: FROM THE ATHLETE TO THE PATIENT

EDITED BY: Antonio Crisafulli, Massimo Piepoli, Dick H.J. Thijssen and Pier Paolo Bassareo

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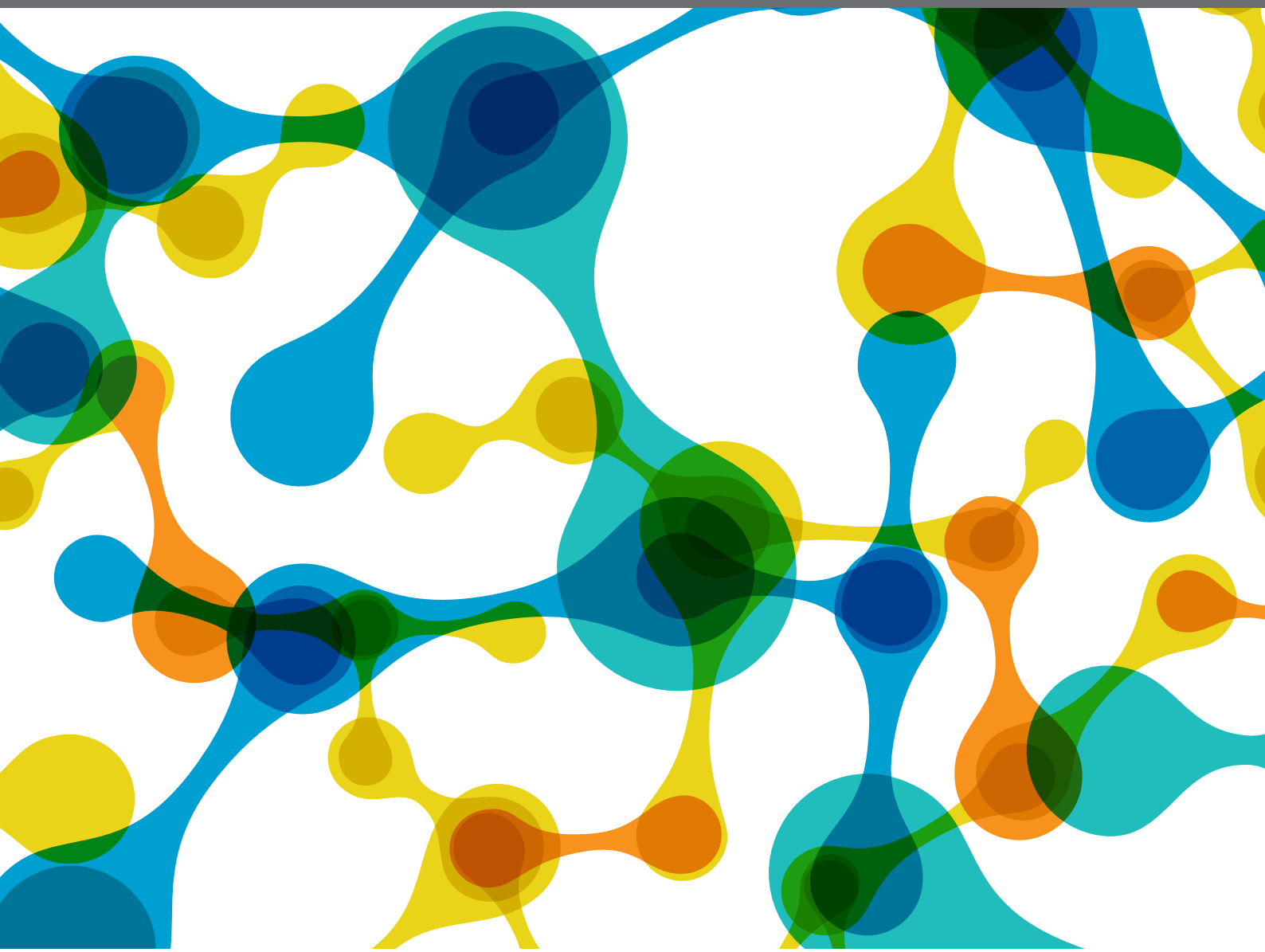

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CARDIOVASCULAR ADJUSTMENTS AND ADAPTATIONS TO EXERCISE: FROM THE ATHLETE TO THE PATIENT

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EDITORIAL
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Editorial: Cardiovascular Adjustments and Adaptations to Exercise: From the Athlete to the Patient

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Keywords: stroke volume, heart rate, blood pressure, training, cardiac output

Editorial on the Research Topic

Cardiovascular Adjustments and Adaptations to Exercise: From the Athlete to the Patient

It is well-recognized that regular exercise confers protection against cardiovascular diseases, while, conversely, sedentary lifestyle is a risk factor. Trained individuals are usually less susceptible to cardiovascular diseases and adverse events than untrained ones. Moreover, from a morphological and physiological point of view, the cardiovascular apparatus is very different when comparing athletes and sedentary individuals.

Mechanisms through which physical activity provides with cardiovascular protection however are not well-understood. A well-known phenomenon is that some cardiovascular risk factors—such as high blood pressure, blood glucose dysregulation, obesity, muscle wasting, and high cholesterol—are positively affected by an active lifestyle. Moreover, regular exercise modifies genes expression and cardiovascular regulation and improves endothelial and platelet functions.

In this Research Topic we propose a few papers dealing with some beneficial effects of physical activity on the cardiovascular morphology, function, and regulation.

It has been demonstrated that the endothelial function is a valuable marker of cardiovascular health since it refers to the ability of the body to maintain the homeostasis of vascular tone. In their contribute to this Research Topic, Bisconti et al. were able to demonstrate that 8 weeks of knee extension exercise training improved endothelial cells response only in the femoral artery of the lower limb directly involved in the exercise, without affecting the endothelial response of the brachial artery, which on the contrary was not involved in the training protocol. Moreover, Boff et al. found in patients with type 1 diabetes that 8-week high intensity interval training leads to improvement in endothelial function and physical fitness. The described effect was greater than that afforded by moderate-intensity continuous training at a similar glycemic control (Boff et al.).

A precious review by Dombrowski et al. provides a *state of the art* about the cardiovascular regulation in patients with hypertension during exercise. Specifically, they described reflexes involved in cardiovascular dysfunction during effort in these subjects and concluded that much work is needed to fully understand what happens in hypertension in terms of cardiovascular regulation. Liang et al., using an animal model of hypertension (rats), provided evidence that neural mechanisms controlling blood circulation interact during exercise. In detail, there is an interactive relationship between central command and the exercise pressor reflex, which is inhibitory in nature. However, the neural occlusion between these central and peripheral pressor mechanisms is attenuated in hypertension.

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Two studies, i.e., that by Gronwald et al. and that by Cardinale et al. dealt with the physiological effects of oxygen manipulation in inspired air. In the first investigation, by using heart rate variability, Gronwald et al. found that a hypoxic condition provoked a sympathetic activation compared to normoxia. In the second investigation, the Cardinale et al. reported that 6 weeks of hyperoxic-supplemented high intensity interval training led to only marginal gain vs. baseline in cycle performance in already trained cyclists, with no difference with respect to conventional training at sea level.

Sex-related differences in left ventricular morphology were addressed by Oláh et al. In a rat model of cardiac hypertrophy, the authors discovered that there is a more pronounced exercise-induced left ventricular hypertrophy in females as compared to males. However, only minor differences in left ventricular function were observed. Oláh et al. explained their results with molecular differences between genders.

Bernardi et al. assessed stroke volume during exercise from oxygen pulse in paralympic athletes. They reported significant differences in stroke volume as a consequence of the different health conditions. Moreover, they suggested that cardiac adaptations are possible also in paralympic athletes with spinal cord injury. Furthermore, they claimed that stroke volume can be predicted from O_2 pulse measurements in these patients.

A research by Garnacho-Castaño et al. studied the slow component of VO_2 and the exercise efficiency during resistance and endurance exercise. The authors reported a decrease in jump performance only after resistance training. Moreover, they claimed that gross efficiency could benefit from the eccentric phase of the resistance exercise.

Gajda et al. evaluated by echocardiography the heart function of swimmers after an ultramarathon relay. They observed that prolonged intense swimming did not affect left and right ventricular function, as no changes indicative of myocardial deterioration were detected 48 h after the event.

Finally, Currie et al. evaluated left ventricular structure with echocardiography in elite swimmers and runners. Their findings suggest enhanced early diastolic function in elite runners relative to swimmers. Authors attributed this result to faster left ventricular untwisting.

Taken together, all these studies strengthen the concept that exercise affects the cardiovascular system at various levels and in a complex way. Vascular reactivity, organs perfusion, nervous reflexes, cardiovascular regulation, genes expression, and molecules production by cells are all involved in the circulatory adjustments and adaptations to exercise. The regulation and the integration of many cardiovascular functions are significantly modified by exercise training.

AUTHOR CONTRIBUTIONS

AC, MP, DT, and PB conceived, wrote, and approved the editorial.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Leucine Supplementation Improves Effort Tolerance of Rats With Hyperthyroidism

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Leucine is a regulator of protein metabolism *in vivo* and information on its action on effort tolerance of both animals and humans with hyperthyroidism is scarce. The objective of the present study was to verify the influence of leucine supplementation on the effort tolerance of Wistar rats with experimental hyperthyroidism. 40 animals were divided into four groups of ten: control (C), hormone (H), leucine (L), and hormone + leucine (HL). Hyperthyroidism was induced by daily administration of 20 $\mu \cdot g100\ g^{-1}$ of levothyroxine sodium in aqueous suspension by gavage. Leucine was supplemented by adding 5% of the amino acid to the conventional feed. The animals' blood was collected by cardiac puncture to analyze TSH, T4, and T3 levels. The effort tolerance was determined by the swimming test with a 7% load attached to animals' tails. Statistical analysis was performed using the Shapiro-Wilk normality test, followed by the analysis of variance (ANOVA) of repeated measures of two factors (treatment $\times$ time) and Tukey *post hoc*, with a significance level of $p < 0.05$. Administering thyroid hormone increased the swimming performance of rats after 14 and 21 days, but with a drop in performance at 28 days. The HL group, on the other hand, had a significantly higher swimming performance compared to the other groups after 28 days of treatment. Leucine supplementation associated with the experimental model of hyperthyroidism improved the performance of rats in a swimming test after 28 days of treatment.

Keywords: creatine kinase, thyrotoxicosis, BCAA, Wistar, exercise

INTRODUCTION

The physiological effects of thyroid hormones ultimately result from the nuclear transcription of large numbers of genes, causing a generalized increase in functional activity throughout the body, metabolic rate of all body tissues, the basal intensity of O_2 consumption, and the production of heat. Thyroid hormones can raise metabolism up to 100% above normal and the effects include increased heart rate, cardiac output, and decreased systemic vascular resistance, among others

(Klein and Ojamaa, 1998). Progressive muscular weakness associated with a generalized muscular atrophy occurs in patients with hyperthyroidism, thus compromising the quality of life and the ability to accomplish daily tasks (Dillmann, 2010).

In this context, leucine is an amino acid of the branched chain amino acid (BCAA) group that regulates muscle protein metabolism *in vivo* (Kobayashi et al., 2006). Leucine supplementation has been used as a nutritional strategy to treat muscular disorders induced by several clinical disorders (Eley et al., 2007; Han et al., 2007; Fidale et al., 2018).

Pioneering studies by Shah et al. (2000) demonstrated that leucine supplementation in rats is able to markedly stimulate protein synthesis in skeletal muscle, activating the mTOR intracellular signaling pathway. Martin et al. (2017) observed in a cell culture model of skeletal muscle tissue that leucine improves mTOR signaling, associated with microtubule hypertrophy and increased maximal contractile force by electrical stimulation, providing evidence for the efficacy of leucine as an anabolic nutritional agent which may influence the functional capacity of muscle.

Leucine modulates protein synthesis by increasing post-transcription efficiency, enhancing the translation rate of mRNAs (Kimball and Jefferson, 2006; Wilkinson et al., 2017). The mechanism of synthesis is due to phosphorylation of the protein kinase p70S6k, which induces the phosphorylation of the ribosomal protein (S6), the eukaryotic initiation factor (eIF4B) and a protein involved in stretching the translation process, Eukaryotic elongation factor kinase 2 (eEF2k), which affects the initiation and elongation of other mRNA classes (Ananieva et al., 2016; Lane et al., 2017).

The hyperthyroidism chronically erating intolerance to the physiological physics in patients, however, uncertainties about the action of leucine on effort tolerance in hyperthyroidism. Therefore, the hypothesis of the present study was to test an experimental model for a period of 28 days from the time we performed a study on the performance of rats, as well as maintaining that performance in leucine supplementation. The present study aimed to verify the efficacy of leucine supplementation in an experimental model of hyperthyroidism in the effort tolerance of Wistar rats.

MATERIALS AND METHODS

The present study was approved by the Ethics Committee on the Use of Animals of the Federal University of Uberlândia (CEUA-UFU), under opinion number 193/11. For the study, 40 male Wistar rats at 10 weeks old and 370 ± 12 g mean weight were used. The experiment was carried out in the UFU experimental medicine laboratory and had a total duration of 35 days: 7 days of adaptation to the laboratory and 28 days of the experiment. During the experimental period, the ambient conditions of the laboratory were constant with respect to temperature, noise level, and brightness, with a 12-h light and dark cycle.

The animals were randomly divided into four groups of 10 animals each, control group (C), hormone group (H), hormone + leucine group (HL), and leucine group (L). The rats were kept separate in collective boxes with five animals per box.

All animals had free access to water and feed, animal body weight, feed intake, water, as well as fecal and urinary volume were observed during 2 weekly times stipulated according to laboratory standards. All experimental procedures were performed in strict accordance with the international regulation on animal welfare.

Leucine Supplementation

The standard diet presented a minimum concentration of 1.54 g·100 g (1.5%) of leucine, according to the American Institute of Nutrition (AIN-93G) which was provided to both control and hormone groups. For the leucine and leucine + hormone groups treated with a leucine-supplemented diet, a standard diet, plus 5.0 g·100 g^{-1} (6.5% total) leucine of the total dietary nutrients, which was previously used by Witham et al. (2013).

Experimental Hyperthyroidism

The animals of group H and HL received a daily dose of 20 μg·100 g^{-1} in aqueous suspension, at2 mL·kg^{-1} of 0.01% T4 during the 28-day experimental period (Engelman et al., 2001; Fernandes et al., 2007). Animals ingroups C and L received a similar dose of saline solution in the same regimen used for hormone-treated animals.

Effort Test

All animals were subjected to swimming tests and the intensity of the load was 7.0% of rat body weight, according to Prada et al. (2004). This load was attached to the tail of the animal, and it was placed in an individual tank with water in which it swam to exhaustion. The tests were performed on the last day of the adaptation week and repeated every 7 days until the end of the experiment according to **Figure 1**.

The temperature of the water was maintained between 30 and 32°C because it was considered thermally neutral in relation to the body temperature of the rat (Harri and Kuusela, 1986).

Euthanasia of Animals and Blood Collection

After the last effort test (day 28), all the animals were kept at CEBEA-UFU for 24 h, with free access to feed and water. According to Longley (2008), fasting before euthanasia is unnecessary for rodents, since they do not vomit, and present high metabolic rates in addition to storing little glycogen in the liver, which could lead to hypoglycemia associated with experimental hyperthyroidism. This practice complies with the guidelines of the National Animal Experimentation Control Council (Ministry of Science, Technology and Innovation and National Council for Control of Animal Experimentation (CONCEA), 2018). Later, the animals were anesthetized for blood collection through cardiac puncture for the determination of triiodothyronine (T3), tetraiodothyronine (T4), and thyroid

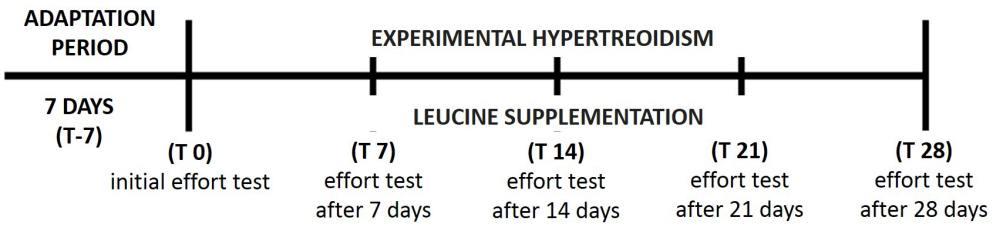

FIGURE 1 | Schematic drawing of the experimental protocol, with T-7, 7 days of adaptation to laboratory environment; HE, experimental hyperthyroidism; SL, supplementation with leucine; T0, initial effort test at last day of the adaptation week; T7, effort test after 7 days of experimental protocol; T14, effort test on the 14th day of the experimental protocol; T21, effort test on the 21st day of the experimental protocol; T28, effort test on the 28th day of the experimental protocol, weighing, blood collection, and euthanasia of the animals.

stimulating hormone (TSH) levels using INTERKIT® and for the quantification of Creatine Kinase Myocardial isoenzyme (CK-MB) was used the Liquiform 60 ml KIT of LABTEST®. The analysis was done using the enzyme-linked immunosorbent assay (ELISA) method, using the Biochemical Analyzer (LABMAX PLENNO®) Serial No. 1308.18 of the Laboratory of Veterinary Medicine of the Federal University of Uberlândia.

Statistical Analysis

Statistical analysis was performed using the Shapiro-Wilk normality test, followed by the analysis of variance (ANOVA) of repeated measures of two factors (treatment × time) and Tukey *post hoc*. Statistical analysis was done with a GraphPad statistical package Prism (5.0 version). Statistical significance was established when the value of $p < 0.05$.

RESULTS

General Observations

No deaths occurred in any of the groups during the experiment.

Effects of High Dietary Leucine on the Analyzed Blood Variables of Wistar Rats in Experimental Hyperthyroidism

Statistically significant differences in TSH and T4 were observed in groups H and HL compared to groups C and L, as shown in **Table 1** demonstrating that the experimental model effectively induced hyperthyroidism. Reduction in CKMB concentrations in the L and HL groups was observed, possibly due to reduction of autophagy and anticatabolic action.

Effects of High Dietary Leucine on Effort Test of Wistar Rats in Experimental Hyperthyroidism

Significant results in the effort tolerance of the rats were observed in the third test, performed on the 14th day of the experiment (T14); there were statistically significant differences between group H and groups C, HL, and L, with the highest swimming times presented by the group H in the three analyzed situations. In the fourth effort test (T21), performed at day 21 of the experiment, we also observed significant differences between group H and group C, with the largest swimming time presented by group H.

In the last test (T28) performed on day 28 of the experiment, upon comparing the HL group to the C group, were observed greater swimming times in the HL group, as shown in **Figure 2**.

DISCUSSION

In the present study, after 14 days of experiment, the animals of group H, in experimental hyperthyroidism, presented a longer swimming time when compared to the animals of the other groups, but with a drop in performance in 28 days, being equal to the control group C, according to **Figure 2**. The addition of leucine to the hormone, suggests a later performance increase. These results suggest a time dependent influence of the hormone on the performance of animals and an influence of leucine at that time.

Thyroid hormones act in virtually all organic systems, with a proven role in metabolism, increased contraction force, myocardial rhythm, and oxidative activity of skeletal muscle (Sun et al., 2000; Sjögren et al., 2007; Simonides and van Hardeveld, 2008). However, prolonged exposure to high doses of thyroid hormone leads to a hypermetabolic state which results in marked loss of body weight, cardiac arrhythmia, degradation of contractile proteins with increased collagen deposition, and a consequent decrease in cardiac function, as well as a marked degradation of energy substrates and skeletal muscle mass.

It was observed in the present study that experimental thyrotoxicosis in rats caused an increase in fecal and urinary volume, requiring a higher frequency in the exchange and hygiene of the animals' housings, possibly characterizing the hypermetabolic state common to hyperthyroidism. However, there was no significant difference in feed intake and total body weight at the end of the experiment in either group.

All these changes are related to the low tolerance to effort, which has been demonstrated both in animals and in humans exposed to high doses of thyroid hormone (Kahaly et al., 2002; Gonçalves et al., 2006). Martin et al. (1991) investigated the mechanism of reduced effort tolerance in an experimental model of hyperthyroidism, analyzing cardiovascular function and skeletal muscle metabolism in 18 healthy subjects. They observed that experimental hyperthyroidism, induced by daily intake of

100 μg triiodothyronine (T3) for 2 weeks, impaired short-term effort tolerance due to a decrease in skeletal muscle mass and oxidative capacity related to accelerated protein catabolism, without impairing cardiac function.

In the present study, were observed decreased effort tolerance of group H in the swimming test performed on the 28th day (T28) of treatment with thyroid hormone. However, this effect was not as pronounced when the animals' diets were supplemented with leucine (HL group). Kato et al. (2017) reported that supplementation with essential amino acids enriched with Leucine for 7 days positively modulated glycogen recovery in rat muscle tissue after exercise-induced damage through electrostimulation.

An important finding of the present study was the increased swimming time that the H group presented during the effort tests, swimming 87% more in T14, and 74% more in T21, when compared to group C, but with decreased performance at T28. The results suggest an acute increase in the tolerance to the effort, possibly by positive oxidative adaptations like increased density and mitochondrial activity previously reported by Gustafsson et al. (1965), but the performance decrease at 28 days occurring through damage (Martin et al., 1991; Fidale et al., 2013), and possible reduction of glycogen (Dos Santos et al., 2016).

Kaminsky et al. (1991) correlated thyroid hormone with the action of skeletal muscle and found that it regulates enzymatic activity in aerobic and anaerobic glucose metabolism, which directly influences mitochondrial activity and ATP supply.

TABLE 1 | Analyzed variables: Experimental model of hyperthyroidism promoted a significant increase in T4 values and a decrease inTSH values in the H and HL E groups and leucine supplementation reduced CK-MB concentrations in the L and HL groups.

Analyzed Variables		Control	Hormone	Hormone + Leucine	Leucine
Anthropometric Variables	Initial BW (g)	326,2 ± 15,89	305,4 ± 23,61	301,4 ± 9,55	312,0 ± 9,89
	Final BW (g)	348,2 ± 7,32	328,8 ± 22,86	334,8 ± 12,66	328,2 ± 10,44
Feed consumption	FC (g)	32 ± 3	36 ± 2	37 ± 4	32 ± 6
Blood variables analyzed	T3 (ng·mL^{-1})	1.98 ± 0.5	2.29 ± 0.9	2.31 ± 0.6	2.06 ± 0.5
	T4 (μg·dL^{-1})	4.76 ± 0.8	12.56 ± 3.4*†	12.46 ± 2.3*†	5.09 ± 1.2
	TSH (ng·mL^{-1})	1.45 ± 0.35	0.39 ± 0.07*†	0.42 ± 0.08*†	1.36 ± 0.29
	CK-MB (U/L)	1005,75 ± 314	934,31 ± 363	538,75 ± 112*	533,99 ± 43*

*Values expressed in average ± standard deviation for: BW, body weight; FC, feed consumption; T3, triiodothyronine; T4, tetraiodothyronine; TSH, thyroid stimulating hormone; CK-MB, Creatine Kinase Myocardial isoenzyme. *$p < 0.05$ compared to group C (ANOVA); †$p < 0.05$ compared to the L group (ANOVA).*

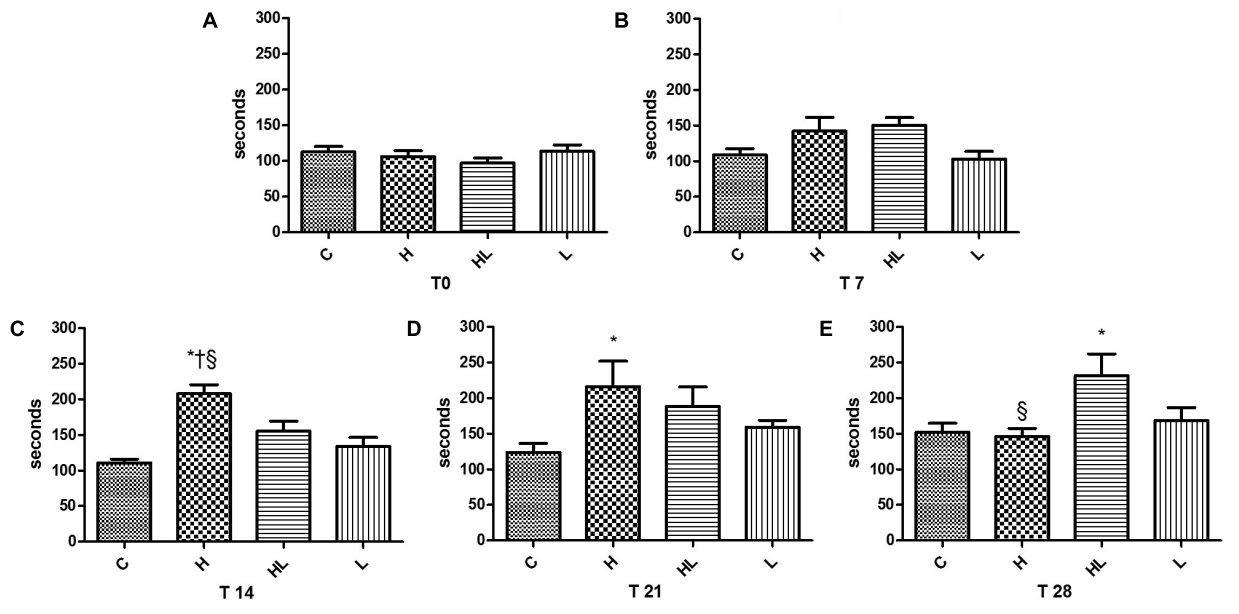

FIGURE 2 | Effort test T0, presented in Graph **A**, performed after the adaptation week; T7 effort test, presented in Graph **B**, performed after 7 days of experiment; T-14 exercise test, presented in Graph **C**, performed after 14 days of experiment, indicating an increase in swimming performance in group H; T-21 exercise test, presented in Graph **D**, performed after 21 days of experiment, evidencing the maintenance of swimming performance gains by group H; Effort test T-28, presented in Graph **E**, performed after 28 days of experiment, showing a decrease in swimming performance by group H and increased performance by the HL group. C, control group; H, hormone group; HL, hormone + leucine group; L, leucine group. *$p < 0.05$ compared to Group C (ANOVA); †$p < 0.05$ compared to Group L (ANOVA); § $p < 0.05$ in relation to the HL Group (ANOVA).

Argov et al. (1988) observed that both patients and rats with hyperthyroidism did not differ from normal controls during rest and exercise, but they had an unusually rapid recovery after exercise when compared to controls, showing the positive relationship between the hormone and muscular functional activity in this model.

Silva et al. (2015) studied rats treated with thyroxine and observed values of cross-sectional area and creatine kinase (CK) were significantly increased, the authors suggest that thyroid hormones can be used to simulate the adaptations and muscle damage that result from physical exercise.

Blood concentrations of CK are used to help diagnose progressive muscular dystrophy (Ebashi et al., 1959) and variations in CK concentration are an important clinical marker for muscle injury, which is often present during thyroid dysfunction (Rosalki, 1970; Meltezer, 1971).

In the present study were observed that in an experimental model of hyperthyroidism, serum CK-MB concentrations were significantly higher in the hormone group than in the control group, suggesting heart tissue damage possibly due to cardiac stress imposed by the excess of the hormone and myocardial susceptibility to hypoxia. This effect was ameliorated by leucine supplementation, which decreased CK-MB concentration and suggested a protective role of leucine. This protection may be linked to anabolic or anti-catabolic effects on protein metabolism, as reported in previous studies (Canedo et al., 2010; Murphy et al., 2016; Reule et al., 2017).

Despite not finding differences in CK concentrations, Reule et al. (2017) observed greater effort tolerance and strength maintenance in a group of aged, untrained, leucine-supplemented elderly subjects subjected to a fatigue protocol through exercise when compared to the placebo group. These results agree with the findings of the present study, since the group of rats supplemented with leucine did not show decreased performance even in experimental hyperthyroidism, which according to Silva (Argov et al., 1988), can simulate the muscular damage caused by physical exercise.

Intake of leucine-rich protein coupled with a physical exercise program seemed to restore the balance of skeletal muscle protein metabolism by salvaging protein synthesis (Xia et al., 2017).

STUDY LIMITATIONS

This study presents only preliminary data. Future research is needed to confirm these findings in humans and to elucidate the cellular mechanisms that link leucine to effort tolerance in hyperthyroidism.

CONCLUSION

The experimental model of hyperthyroidism increased the swimming time of rats at 14 and 21 days of treatment, but decreased performance at 28 days. Leucine supplementation in an experimental model of hyperthyroidism improved effort tolerance after 28 days of treatment.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Left Ventricular Structure and Function in Elite Swimmers and Runners

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Sport-specific differences in the left ventricle (LV) of land-based athletes have been observed; however, comparisons to water-based athletes are sparse. The purpose of this study was to examine differences in LV structure and function in elite swimmers and runners. Sixteen elite swimmers [23 (2) years, 81% male, 69% white] and 16 age, sex, and race matched elite runners participated in the study. All athletes underwent resting echocardiography and indices of LV dimension, global LV systolic and diastolic function, and LV mechanics were determined. All results are presented as swimmers vs. runners. Early diastolic function was lower in swimmers including peak early transmitral filling velocity [76 (13) vs. 87 (11) $cm \cdot s^{-1}$, $p = 0.02$], mean mitral annular peak early velocity [16 (2) vs. 18 (2) $cm \cdot s^{-1}$, $p = 0.01$], and the ratio of peak early to late transmitral filling velocity [2.68 (0.59) vs. 3.29 (0.72), $p = 0.005$]. The diastolic mechanics index of time to peak untwisting rate also occurred later in diastole in swimmers [12 (10)% diastole vs. 5 (4)% diastole, $p = 0.01$]. Cardiac output was larger in swimmers [5.8 (1.5) vs. 4.7 (1.2) $L \cdot min^{-1}$, $p = 0.04$], which was attributed to their higher heart rates [56 (6) vs. 49 (6) bpm, $p < 0.001$] given stroke volumes were similar between groups. All other indices of LV systolic function and dimensions were similar between groups. Our findings suggest enhanced early diastolic function in elite runners relative to swimmers, which may be attributed to faster LV untwisting.

Keywords: aerobic exercise, athletes, cardiovascular, diastolic function, echocardiography

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INTRODUCTION

Exercise-induced cardiac remodeling is well documented with the degree of adaptation dependent on the type of training (i.e., aerobic vs. resistance-based training) (Pluim et al., 2000; Spence et al., 2011; Utomi et al., 2013). There is also growing recognition that within aerobic or resistance-based training, different sport modalities may elicit different left ventricular (LV) adaptations (Spirito et al., 1994; Venckunas et al., 2008a; Wasfy et al., 2015). Within aerobic-based sports, the type of LV adaptation may depend on the degree of isotonic (i.e., dynamic) and isometric (i.e., static) components involved in the sport. Comparisons of land-based sport modalities have observed differences in LV structure and diastolic function between distance running (i.e., high isotonic and

low isometric) and rowing (i.e., high isotonic and high isometric) (Wasfy et al., 2015). Sport-specific differences in LV mechanics have also been described, including peak untwisting velocity which contributes to diastolic filling (Beaumont et al., 2017).

Swimming is a sport that provides a unique physiological stimulus distinct from land-based exercise modalities. It would be classified as having a high isotonic component (i.e., similar to running or rowing), but moderate isometric component which falls somewhere on the spectrum between the other two sports (Mitchell et al., 2005). Unique aspects of this exercise modality which may influence exercise-induced LV remodeling include immersion of the body in water (and the potential influence of hydrostatic pressure and water temperature), a supine posture, use of both the upper and lower limbs, and a novel requirement of breath holding (Holmer et al., 1974; Holmer, 1979; Ferrigno et al., 1986). Posture specific cardiovascular adaptations have been observed following supine and upright cycle training (Ray et al., 1990); and LV differences between rowers and runners also suggest combined upper and lower limb exercise may provide a different stimulus than lower-limb exercise alone (Wasfy et al., 2015). There is limited evidence available demonstrating similar global LV systolic and diastolic function in swimmers and other land-based athletes (i.e., runners) (Colan et al., 1987; Venckunas et al., 2008a), and no studies have examined LV mechanics, which provides more detailed information on LV systolic and diastolic function. Therefore, the purpose of this study was to extend our understanding of the extent of exercise-induced cardiac remodeling with swimming through a characterization and comparison of LV mechanics, dimensions, and global systolic and diastolic function between elite swimmers and runners. Any differences in global LV function would likely also be demonstrated through differences in LV mechanics. Based on previous evidence, however, we hypothesized global LV function would be similar between groups, and therefore LV mechanics would also be similar.

MATERIALS AND METHODS

Athletes

Inclusion criteria included males and females, 18 years of age or older, who were elite swimmers or runners, which was defined as an athlete currently competing at the Olympic or International level, or specifically identified by their national sporting group to represent their country. Swimmers were recruited from on-site advertisements and word of mouth from the 2016 FINA World Championships (25 m) in Windsor, Canada, and tested upon completion of all competitions. Runners were similarly recruited during competition season from an elite local club and tested at the University of Guelph in Canada. Sixteen athletes were included in each group, and they were matched based on age, sex, and race. Exclusion criteria included physician diagnosed cardiovascular disease, evidence of cardiovascular abnormality from a preceding 12-lead electrocardiogram (or follow-up diagnostic echocardiogram), or any language or cognitive barrier that prevented them from following English instructions. This study was carried out in accordance with the recommendations of the University of Guelph Natural, Physical and Engineering Sciences Research Ethics Board with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the University of Guelph Natural, Physical and Engineering Sciences Research Ethics Board (16OC027). All athletes provided written informed consent prior to participation and were asked to be at least 2 h fasted and 12 h post-exercise. Individuals completed a questionnaire that addressed their medical history and training history including their duration of competitive training, their average training volume, and their sporting event. With shoes removed, height was measured at end-inspiration to the nearest half centimeter using a stadiometer (SECA; Hanover, MD, United States), while weight was measured using an electronic scale (Tanita TBF-300 WA; Tanita, Arlington Heights, IL, United States). Body mass index and body surface area using the Du Bois and Du Bois (1989) formula were calculated. Athletes were then asked to rest in a supine position for 5 min, after which brachial artery blood pressure measurements were obtained in duplicate from the right arm using an automated oscillometric device (BpTRU model BPM-100, BpTRU Medical Devices; Coquitlam, BC, Canada).

Echocardiography Assessment

All LV images were collected in the left lateral decubitus position with two-dimensional echocardiography and Doppler acquisition by a single investigator and using a dedicated ultrasound (Vivid Q; GE Healthcare, Horten, Norway). The system employed a M4S Matrix Sector Array Probe (2–5 MHz), and all images were analyzed offline by a single investigator using dedicated software (EchoPAC; GE Healthcare, Horten, Norway) according to the recommendations of the American Society for Echocardiography (Quinones et al., 2002; Lang et al., 2005; Mor-Avi et al., 2011). All LV indices were determined from the average of three cardiac cycles. LV dimensions at end-diastole and end-systole were measured from parasternal long axis views. Relative wall thickness was calculated as [(2 × end-diastolic posterior wall thickness)/end-diastolic LV internal diameter], while LV mass was calculated according to the area-length formula (Lang et al., 2005) and indexed to body surface area. End-diastolic and systolic volumes and ejection fractions were derived from Simpson's biplane analysis of apical four and two-chamber views. Stroke volume was calculated from LV outflow tract diameters and velocity time integrals and multiplied by heart rate to determine cardiac output. To control for the potential differences in body size between athlete groups, end-diastolic volume, stroke volume and cardiac output were indexed to body surface area. Pulsed-wave Doppler at the tips of the mitral valve leaflet was used to determine peak early (E) and late (A) transmitral filling velocities, and the resultant E/A ratio. Pulsed-wave tissue Doppler imaging was performed at the septal and lateral mitral annulus and averaged to determine isovolumetric relaxation time and peak early (E'), late (A'), and systolic (S') mitral annular velocities.

Parasternal short axis images at the level of the mitral valve (basal) and apex (apical) were analyzed using speckle-tracking software in accordance with current guidelines (Mor-Avi et al., 2011). Raw traces were imported into customized

post-processing software (2D Strain Analysis Tool, Stuttgart, Germany), which normalizes the temporal sequence of heart rate by interpolating the data into 600 points in systole and 600 points in diastole using a standard cubic spline algorithm. Basal and apical peak rotation and rotation rate in systole and diastole were determined. Twist was determined as the maximum value obtained when subtracting the frame-by-frame basal rotation from the frame-by-frame apical rotation, while torsion was calculated by dividing twist by LV length. Peak systolic twisting rate, early diastolic untwisting rate and time to peak twisting/untwisting rates were derived in the same manner from frame-by-frame basal and apical rotation rate data.

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Science software (IBM Corporation, Armonk, NY, United States) and GraphPad Prism (GraphPad Software Inc., La Jolla, CA, United States). Data were assessed for normality using Shapiro-Wilk tests and Q–Q plot analyses. Between group differences were assessed using independent *t*-tests and Mann-Whitney *U*-tests for normally and non-normally distributed data. Given a significant difference in supine heart rates between groups, early diastolic indices (E and E'), which are influenced by heart rate, were also compared using an analysis of covariance with heart rate as the covariate. Data are presented as mean ± SD unless otherwise noted, with $P < 0.05$ considered statistically significant.

RESULTS

Athlete characteristics are presented in **Table 1**. For runners, sprint/power athletes were defined as those who competed in 100–400 m races and high jump, while distance athletes competed in races ≥800 m. Short distance swimmers comprised those competing in 50–200 m races while middle-distance/distance athletes competed in 400–1500 m races. Eleven swimmers swam freestyle, 3 swam breaststroke and 2 swam backstroke. Groups were predominantly male (81%) and white (69%) and had similar body dimensions. Swimmers participated in competitive training for a longer duration and engaged in a larger volume of weekly training, but within the swimmer and runner groups, there was no difference in these variables between distance and sprint athletes. Supine blood pressures were similar between groups, but heart rate was higher in swimmers. LV indices are presented in **Table 2**. Cardiac output and cardiac output index were larger in swimmers, while all other global LV systolic function indices and systolic mechanics were similar between groups. For global LV diastolic function, early diastolic indices were higher in runners including E and E', as well as the E/A ratio. When heart rate was entered as a covariate, E' remained different between groups ($p = 0.01$) while E was trending ($p = 0.06$). For diastolic mechanics, runners had a shorter time to peak untwisting rate and a lower apical rotation rate in diastole compared to swimmers. LV structure was similar between groups.

TABLE 1 | Athlete characteristics.

Variable	Runners	Swimmers	*P*-value
Age (year)	23 (2)	22 (3)	0.22
Sex: male, female (no)	13, 3	13, 3	–
Race: black, white (no)	5, 11	5, 11	–
Height (m)	1.86 (0.13)	1.80 (0.07)	0.12
Mass (kg)	71.3 (9.3)	74.0 (9.0)	0.41
Body mass index ($kg \cdot m^{-2}$)	22.6 (2.0)	22.9 (1.7)	0.60
Body surface area (m^2)	1.87 (0.16)	1.92 (0.15)	0.41
Supine systolic BP (mmHg)	95 (9)	100 (10)	0.14
Supine diastolic BP (mmHg)	54 (8)	55 (7)	0.76
Supine heart rate (bpm)	49 (6)	56 (5)	**0.001**
Training history			
Competitive training duration (year)	8 (3)	12 (4)	**0.02**
Average training volume ($h \cdot wk^{-1}$)	17 (5)	23 (8)	**0.01**
Sport			
Run: sprint/power	10	0	–
Run: distance	6	0	–
Swim: sprint	0	6	–
Swim: middle distance/distance	0	10	–

Data are mean (SD). BP, blood pressure. Bolded values in P-value column indicate significant between-group differences.

DISCUSSION

In a cohort of elite-level athletes, we demonstrate increased early diastolic filling in runners compared to swimmers, while all other indices of LV dimensions and systolic function/mechanics were similar between groups. This improved early diastolic filling in elite runners may be attributed to enhanced LV diastolic mechanics, increased preload due to greater blood volume (which we did not specifically examine), or a combination of both. The observations from this study suggest the exercise stimulus of running vs. swimming may be capable of producing distinct adaptations in LV diastolic, but not systolic, function.

While presently debated, there is evidence to suggest an association between aerobic exercise training and enhanced early diastolic filling, characterized using both preload-dependent transmitral filling velocity, and mitral annular velocity measurements which rely less on preload (George and Somauroo, 2012). Cross-sectional comparisons have observed enhanced early diastolic filling indices in swimmers relative to sedentary individuals (Caso et al., 2002; Venckunas et al., 2008a; Santoro et al., 2015), while only one study has examined differences between elite long and middle-distance runners and swimmers and observed no difference in transmitral filling velocities between groups (Venckunas et al., 2008a). We observed higher early transmitral filling and mitral annular velocities and a higher E/A ratio in our runner cohort, which remained significant or trending when controlling for heart rate, suggesting runners have enhanced early diastolic function relative to swimmers. While the untwisting rate was similar between groups, the time to peak untwisting rate occurred earlier in the diastole in runners. LV untwisting is an important component of early diastole as it precedes diastolic suction and subsequent filling (Burns et al., 2009). Thus, the capacity to

TABLE 2 | Indices of left ventricular structure, global function, and mechanics.

Variable	Runners	Swimmers	*P*-Value
LEFT VENTRICLE (LV) STRUCTURE			
LV internal diameter – diastole (cm)	5.3 (0.4)	5.2 (0.4)	0.37
LV internal diameter – systole (cm)	3.6 (0.3)	3.6 (0.4)	0.96
LV length – diastole (cm)	8.4 (0.6)	8.6 (0.8)	0.33
Relative wall thickness	0.33 (0.04)	0.34 (0.04)	0.22
LV mass index ($g \cdot m^{-2}$)	133.4 (23.7)	129.2 (25.8)	0.64
End-diastolic volume (ml)	126 (16)	137 (26)	0.06
End-diastolic volume index ($ml \cdot m^{-2}$)	67 (8)	71 (11)	0.29
End-systolic volume (ml)	50 (7)	57 (11)	0.32
GLOBAL LV SYSTOLIC FUNCTION			
Stroke volume (ml)	94 (22)	99 (25)	0.55
Stroke volume index ($ml \cdot m^{-2}$)	50 (11)	51 (11)	0.75
Cardiac output ($L \cdot min^{-1}$)	4.7 (1.2)	5.8 (1.5)	**0.04**
Cardiac output index ($L \cdot min^{-1} \cdot m^{-2}$)	2.5 (0.6)	3.0 (0.7)	**0.04**
Ejection fraction (%)	60 (2)	58 (2)	0.08
S' mean ($cm \cdot s^{-1}$)	9 (2)	9 (2)	0.44
GLOBAL LV DIASTOLIC FUNCTION			
E ($cm \cdot s^{-1}$)	87 (11)	76 (13)	**0.02**
A ($cm \cdot s^{-1}$)	28 (8)	29 (5)	0.12
E/A ratio	3.29 (0.72)	2.68 (0.59)	**0.005**
E' mean ($cm \cdot s^{-1}$)	18 (2)	16 (2)	**0.01**
A' mean ($cm \cdot s^{-1}$)	6 (1)	6 (1)	0.69
Isovolumetric relaxation time (ms)	74 (11)	73 (12)	0.92
E/E' mean ratio	5.0 (1.0)	4.9 (1.1)	0.69
LV SYSTOLIC MECHANICS			
Twist (deg)	11.9 (4.9)	11.8 (5.6)	0.98
Torsion ($deg \cdot cm^{-1}$)	1.4 (0.6)	1.3 (0.6)	0.75
Twisting rate ($deg \cdot s^{-1}$)	70.6 (19.4)	80.2 (29.8)	0.29
Time to peak twisting rate (% systole)	49 (8)	49 (9)	0.81
Basal rotation (deg)	−4.8 (2.9)	−5.5 (2.9)	0.52
Basal rotation rate – systole ($deg \cdot s^{-1}$)	−45.9 (16.1)	−55.1 (15.7)	0.12
Apical rotation (deg)	7.4 (2.3)	7.3 (4.1)	0.89
Apical rotation rate – systole ($deg \cdot s^{-1}$)	45.4 (20.1)	58.6 (24.2)	0.12
LV DIASTOLIC MECHANICS			
Untwisting rate ($deg \cdot s^{-1}$)	−68.4 (30.3)	−76.9 (27.5)	0.42
Time to peak untwisting rate (% diastole)	5 (4)	12 (10)	**0.01**
Basal rotation rate – diastole ($deg \cdot s^{-1}$)	35.3 (17.6)	35.6 (18.2)	0.95
Apical rotation rate – diastole ($deg \cdot s^{-1}$)	−40.4 (15.4)	−56.8 (23.7)	**0.03**

Data are mean (SD). A, peak late transmitral filling velocity. A' mean, mean mitral annular peak late velocity. E, peak early transmitral filling velocity. E' mean, mean mitral annular peak early velocity. S' mean, mean mitral annular peak systolic velocity. Bolded values in P-value column indicate significant between-group differences.

reach peak untwisting faster could prolong the early filling phase leading to increased values of early diastolic filling. No swim training studies have examined LV mechanics, but land-based aerobic exercise training in young adults has been shown to enhance untwisting mechanics (Weiner et al., 2015). The shorter time to peak untwist may also explain the observation of attenuated diastolic apical rotation rates in runners relative to swimmers. In particular, the apex may not need to untwist as much given it reaches peak untwisting sooner. The differences in diastolic apical rotation rate may also be partially explained by the differences in heart rate.

The enhanced early diastolic function observed in runners suggests this land-based exercise modality may provide a greater physiological stimulus for exercise-induced cardiac adaptation. The elevated E and E/A ratio in runners, which are both preload dependent measures, could also be attributed to a greater blood volume. Upright aerobic exercise training has been shown to elicit significant increases in blood volume in young males compared to supine aerobic exercise training (Ray et al., 1990). Additionally, female runners have been shown to have a greater blood volume than female swimmers (Parker Jones et al., 1999). Thus, the observed sport-specific improvements in diastolic function may be related to adaptations in the LV, blood volume, or both. Self-reported training history and weekly training volume were both lower in runners; thus, despite training for fewer years and fewer hours per week they still demonstrated increased resting diastolic function.

It is also possible that the diastolic function observed in runners was a necessary adaptation for their exercise modality, rather than a greater adaptation. Investigations on land have demonstrated that the supine posture promotes venous return, increasing preload, early diastolic filling and subsequently stroke volume (Sato et al., 1999; Warburton et al., 2002). Additionally, earlier work in young men demonstrated increased heart volume using x-ray following head-up immersion in water in comparison to upright and supine land postures (Lange et al., 1974). Thus, the combined supine and immersed position of swimming should function to promote diastolic filling. Previous studies have observed swimming and running at a similar oxygen uptake level have elicited similar cardiac outputs but lower heart rates during swimming, suggesting an increased stroke volume (Holmer, 1979). Thus, it is possible that swimmers do not need to further augment diastolic function to the level of a runner, because the position and water immersion aspects of their exercise modality would promote filling. While we did not compare our athletes to a sedentary control group, we would consider both groups to exhibit exercise-induced LV remodeling as they represent athletes at the elite level of their respective sports, and their values are generally higher than age-matched reference values (Lang et al., 2005; Nagueh et al., 2009). For example, the average E/A ratio is 1.88 and 1.53 for 16–20 and 21–40 year olds, respectively (Nagueh et al., 2009). This highlights that the attenuated diastolic values in swimmers relative to runners should not be interpreted as reduced diastolic function, but on a continuum of adaptations to exercise training.

The only difference in systolic function between groups was cardiac output, which can be attributed to higher heart

rates in swimmers given stroke volumes were similar. Resting bradycardia with aerobic exercise training is well characterized, and while some have observed no difference in resting heart rate between aerobic sport modalities (Colan et al., 1987; Wasfy et al., 2015), higher resting heart rates have been observed in swimmers compared to runners (Nualnim et al., 2011). For LV dimensions, cross-sectional comparisons of swimmers to sedentary controls suggest swimmers experience exercise-induced LV remodeling including increases in wall thickness, internal diastolic diameters and LV mass index (Colan et al., 1987; Caso et al., 2002; Venckunas et al., 2008a; Santoro et al., 2015). Comparisons between swimmers and runners have generally demonstrated no difference in LV internal diastolic diameter or LV mass index (Colan et al., 1987; Venckunas et al., 2008a), which is in agreement with our observations. Colan et al. (1987) argued that swimmers experience an intermediate level of LV remodeling that would fall on the spectrum between running and powerlifting athletes, and that their degree of adaptation is attributed to both the volume overload typically associated with endurance (i.e., isotonic) training and the pressure overload typically associated with resistance (i.e., isometric) training. This was based on their observation that end-systolic wall thickness in swimmers was larger than runners but smaller than power lifters. The long-standing view that isometric-based training elicits concentric remodeling, however, has recently been discredited (Spence et al., 2011). We observed no difference in relative wall thickness, which suggests both sports, regardless of their isotonic and isometric components, provide a similar physiological stimulus for remodeling.

The strengths of our study include the assessment of elite athletes during their competition season and our comprehensive assessment of LV function including both traditional measures of global function and more advanced speckle-tracking assessments of systolic and diastolic mechanics. However, we do acknowledge several limitations. Each group contained a heterogeneous mixture of sex, race, and sport distance, all of which can influence LV adaptations with exercise training (Sharma, 2003). We matched athletes in each group based on sex and race; therefore, the potential confounding effects of these factors should be minimized. Differences in LV structure have been documented between sprint and distance runners; however, there were no differences in diastolic parameters (Venckunas et al., 2008b). Within swimming, one study documented differences in LV structure and the E/A ratio between endurance (i.e., 400–800 m) and "strength" (i.e., 50–100 m) swimmers (D'Andrea et al., 2003). Strength swimmers also performed 2 h per day of land-based strength training in comparison to 1 h per week in the endurance group; therefore it is difficult to ascertain whether these differences were exclusively attributed to the swim training. Within our swimmer cohort, we are limited in our ability to compare LV parameters between sport distances given race distributions (i.e., 71% of sprint swimmers were black vs. 10% of middle distance/distance swimmers). However, comparison of diastolic indices between sprint and middle distance/distance swimmers, both with and without race as a covariate, revealed no significant differences between distance groups. We are also unable to discriminate the amount of training spent performing isotonic and isometric activities. Given the elite status of all athletes, it is safe to assume they were engaging in the best training practices for their respective event and therefore represent high-performance running and swimming athletes. For a more comprehensive understanding of cardiac differences between runners and swimmers, future investigations should consider examining the right ventricle and atria, as well as longitudinally tracking changes with training in order to draw more definitive conclusions regarding sport-specific adaptations. Furthermore, the practical implications of enhanced resting diastolic function in runners compared to swimmers is unknown. Thus, future studies should consider comparing LV performance during both supine and upright exercise in these athlete groups.

CONCLUSION

Our cross-sectional comparison of elite swimmers and runners suggest running is associated with faster LV untwisting during diastole and enhanced early diastolic filling. Further research is required to delineate whether these observations are a product of a superior exercise stimulus, or a necessary adaptation to promote filling during upright exercise. The different exercise stimuli of swimming and running did not appear to affect LV structure or systolic function, as these indices were similar between groups. Overall, our findings lend further support to the concept of sport-specific differences in LV function.

AUTHOR CONTRIBUTIONS

KC, AC, JS, RA, AW, and JB were involved in data collection. KC performed the data and statistical analysis. KC, PM, and JB interpreted the data. KC drafted the manuscript and revisions were done by KC, PM, and JB. All authors were involved in the conception and design of the study and approved the final version of the manuscript.

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REVIEW
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Neural Control of Cardiovascular Function During Exercise in Hypertension

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During both static and dynamic exercise hypertensive subjects can experience robust increases in arterial pressure to such an extent that heavy exercise is often not recommended in these patients due to the dangerously high levels of blood pressure sometimes observed. Currently, the mechanisms mediating this cardiovascular dysfunction during exercise in hypertension are not fully understood. The major reflexes thought to mediate the cardiovascular responses to exercise in normotensive healthy subjects are central command, arterial baroreflex and responses to stimulation of skeletal muscle mechano-sensitive and metabo-sensitive afferents. This review will summarize our current understanding of the roles of these reflexes and their interactions in mediating the altered cardiovascular responses to exercise observed in hypertension. We conclude that much work is needed to fully understand the mechanisms mediating excessive pressor response to exercise often seen in hypertensive patients.

Keywords: hypertension, central command, arterial baroreflex, exercise pressor response, dynamic exercise

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INTRODUCTION

Hypertension is one of the major pathological diseases of our time affecting approximately 1/3 (29.1%) of the adult population. Only 83% of these individuals are aware that they are hypertensive and of these, only 76% are medicated and despite multiple available therapeutic interventions only about one-half of treated individuals have their hypertension under control (Nwankwo et al., 2013) (**Figure 1**). Current extrapolations predict that by 2030 an additional 27 million people will be hypertensive (Kirkland et al., 2018). HTN has a myriad of risk factors: obesity, sedentary lifestyle, high salt diet, high alcohol intake, insulin resistance, low potassium intake, low calcium intake, stress, sex, and age all can contribute in concert or alone in the disease development and pathology (Carretero and Oparil, 2000). Excessive activation of the sympathetic nervous system may play a role in hypertension (Head, 1995). Although, regular moderate exercise can reduce the subsequent resting sympathetic activity in hypertensive individuals, several studies have shown that during exercise sympathetic activity may increase excessively thereby increasing risk factors for adverse events including myocardial ischemia, arrhythmia, myocardial infarctions, and sudden cardiac death (Hoberg et al., 1990; Mittleman et al., 1993). Indeed The American College of Cardiology/American Heart Association 2002 guidelines for high blood pressure warns that individuals with uncontrolled hypertension (>200 mmHg systolic and/or >110 mmHg diastolic

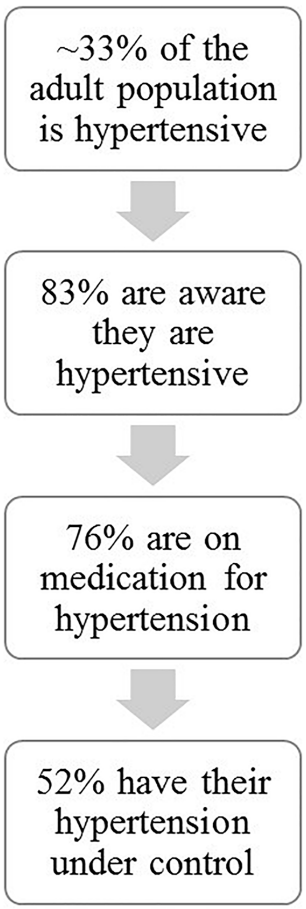

FIGURE 1 | Incidence of hypertension.

at rest) should not undergo exercise stress tests due to possible dangerously large increases in arterial blood pressure (Gibbons et al., 2002). Similarly, the Journal of Nuclear Cardiology and The American Family Physician guidelines considers systolic blood pressure above 230 mm Hg and diastolic above 115 mm Hg during exercise as contraindications to continue these tests (Darrow, 1999; Henzlova et al., 2016). Clearly, pathological increases in arterial pressure during these tests present potential risks for adverse events such as myocardial ischemia, arrhythmia, myocardial infarction, and stroke. The mechanisms mediating these exaggerated increases in sympathetic activity in hypertensive patients are not well-understood and are clinically important. This review summarizes current understanding of the roles of three major mechanisms thought to control autonomic outflow during exercise in hypertension: central command, arterial baroreflex, and skeletal muscle afferents.

CENTRAL COMMAND

The initiation of exercise elicits immediate changes in autonomic outflow which have been in part ascribed to feed-forward reflex effects of the volition to exercise, termed central command. This reflex likely contributes importantly to the immediate partial reductions in parasympathetic activity to the heart causing a rapid tachycardia. Sympathetic activity can also increase with activation of central command. To our knowledge there have been no studies investigating whether neural control of cardiovascular function during exercise by central command is altered in patients with hypertension. However, Liang et al. (2016) showed in decerebrated rats that electrical stimulation of the mesencephalic locomotor region in spontaneous hypertensive rats (SHRs) had greater pressor and heart rate (HR) responses when compared to normotensive rats indicating that "central command" maybe exaggerated in hypertension.

SKELETAL MUSCLE AFFERENTS

Skeletal muscle contains afferents that are both mechanosensitive (predominantly group III afferents) and sensitive to the metabolic environment (predominantly group IV afferents) although some afferents are polymodal (Kaufman et al., 1983, 1984; Kaufman and Rybicki, 1987). Activation of these afferents can elicit a powerful pressor response. Previous studies have concluded that the pressor, HR, and renal sympathetic nerve activity (RSNA) responses to stimulation of both mechanosensitive and metabosenstive skeletal muscle afferents are exaggerated in SHR compared to the normotensive controls (Leal et al., 2008; Mizuno et al., 2011a; Liang et al., 2016). In addition, a recent study (Barbosa et al., 2016) showed in humans that the exaggerated pressor response to leg exercise could be normalized by blockade of leg afferents via intrathecal fentanyl. Although the drug also lowered resting arterial pressure, these results strongly suggest that the exaggerated pressor response to exercise in hypertension stems, in part, from activation of skeletal muscle afferents.

Muscle Mechanoreflex

In SHR, blockade of mechanoreceptors reduced the increases in mean arterial pressure (MAP), RSNA and HR when compared to control responses (Mizuno et al., 2011b) indicating that the muscle mechanoreflex is playing an important role in the exaggerated cardiovascular responses to exercise in hypertension. These studies (Mizuno et al., 2011a,b) provide some support for the role of the mechanoreflex, however, all of these studies were done either under anesthesia or in a decerebrated animal models. In a study using conscious humans Choi et al. (2013) observed that the pressor response to static forearm contraction was exaggerated in pre-hypertensive subjects whereas the pressor response to only mechanoreflex activation (via stretching of lower leg muscles) was only seen to be greater if expressed as absolute increases in pressure, % changes were not different.

Muscle Metaboreflex

Multiple studies have been conducted in order to elucidate whether hypertension affects the strength and mechanisms of the muscle metaboreflex although the conclusions have been varied. Delaney et al. (2010) showed that hypertensive individuals had

accentuated increases in mean arterial blood pressure and muscle sympathetic nerve activity (MSNA) in response to hand grip exercise when compared to normotensive individuals, whereas the responses to the cold pressor test were not different indicating that the accentuated metaboreflex responses likely were not due to generalized increases in sympatho-excitatory reflexes. Furthermore, these exaggerated responses were maintained during post-exercise circulatory occlusion (PECO) – a setting that isolates any metaboreceptor activation during the recovery from exercise. These results indicate that the accentuated responses to hand grip exercise may be due to accentuated muscle metaboreflex activation. Furthermore, Chant et al. (2018) found that the enhanced pressor response to exercise and metaboreflex activation still occurred in medicated hypertensive patients whose pressure was deemed controlled at rest. Studies in decerebrated rats have shown that chemical activation of skeletal muscle afferents with capsaicin leads to exaggerated RSNA (Mizuno et al., 2011a) and pressor responses (Leal et al., 2008; Mizuno et al., 2011a) in SHR when compared to Wistar-Kyoto (WKY) normotensive controls. Blockade of purinergic receptors in hypertensive individuals during PECO reduced MSNA burst frequency more when compared to normotensive individuals, suggesting that the purinergic receptors are playing a role in the sympathoexcitation occurring during muscle metaboreflex activation (Greaney et al., 2015b). In addition, women with a positive family history of hypertension had greater pressor and MSNA to several stressors such as the cold pressor test, isometric hand grip, and PECO when compared to women with no family history of hypertension (Greaney et al., 2015a) suggesting there may be a genetic component contributing to these accentuated responses.

In contrast there are several studies that have shown that muscle metaboreflex induced cardiovascular responses are reduced in hypertension. Rondon et al. (2006) found that the increases in MSNA in response to moderate handgrip exercise were not sustained during PECO whereas in normal individuals this sympatho-activation is sustained in PECO. A study by Ranadive et al. (2017) showed that there was no differences in muscle metaboreflex responses between women with a history of hypertensive pregnancies when compared to women that had normotensive pregnancies. Studies in conscious dogs concluded that metaboreflex-induced increases in MAP, cardiac output (CO), stroke volume (SV) and HR were reduced in dogs after induction of hypertension (Sala-Mercado et al., 2013) and that these attenuated responses were less sustained during PECO (Spranger et al., 2015). Previous studies in canines and humans have shown that when cardiac function is impaired the mechanisms mediating the metaboreflex pressor response during submaximal dynamic exercise "switch" from primarily increases in CO to primarily peripheral vasoconstriction (Hammond et al., 2000; Kim et al., 2005a; Crisafulli et al., 2011; Sala-Mercado et al., 2013). Furthermore, a recent study in canines demonstrated that metaboreflex activation during dynamic exercise elicits beta receptor-mediated peripheral vasodilation likely via epinephrine release from the adrenal gland (Kaur et al., 2015b). However, in heart failure (HF) this response appears to be abolished (Kaur et al., 2015b). In the study by Spranger et al. (2015) the authors noted a hint of this shift in the mechanisms mediating the metaboreflex in the animals after induction of hypertension inasmuch as the small rise in peripheral vascular conductance during metaboreflex activation often noted in normal animals was abolished after induction of hypertension. This could indicate greater peripheral sympatho-activation during metaboreflex activation after induction of hypertension and/or reduced beta mediated vasodilation. In a subsequent study, Spranger et al. (2017) concluded that during metaboreflex activation in hypertensive canines there is enhanced vasoconstriction of the coronary vasculature which limits increases in ventricular function. When this restraint of coronary blood flow was blocked via prazosin, the increases in coronary blood flow and CO were restored toward normal. These data indicate that the primary reason for the attenuated metaboreflex responses seen in the canine studies was enhanced coronary vasoconstriction which limited increases in CO. Inasmuch as the rise in CO is the primary mechanism mediating the metaboreflex response when activated during exercise, when the cardiac component was decreased, the pressor response was likewise attenuated (Sala-Mercado et al., 2013; Spranger et al., 2015, 2017). Additionally, in HF previous studies have shown that muscle metaboreflex activation induces limitations in coronary blood flow resulting in a further reduced capacity to increase CO to match physiological demands (Kaur et al., 2015a). Inasmuch as the responses in hypertension coincide with those observed in HF, the mechanism ultimately resulting in the profound pressor response in hypertension may have the same functional origin. Whether the metaboreflex is increased or attenuated may be dependent on which response is used for analysis, e.g., when a shift in mechanisms occurs (from CO based pressor response to peripheral vasoconstriction as occurs in HF), the strength of the metaboreflex in the control of CO is reduced but the strength of this reflex in the ability to elicit vasoconstriction is enhanced.

ARTERIAL BAROREFLEX

The arterial baroreceptor reflex is the primary reflex for beat-by-beat control of arterial pressure. In normotensive individuals during dynamic exercise many studies have shown that the operating point of the stimulus – response relationship for the arterial baroreflex is shifted to the right but the gain remains the same (Rowell and O'Leary, 1990; Papelier et al., 1994; O'Leary, 1996, 2006; Michelini et al., 2015). At rest, hypertensive individuals have reduced baroreflex control of HR (Judy and Farrell, 1979; Souza et al., 2008). Whether baroreflex control of sympathetic input to the peripheral vasculature is altered in hypertension is controversial (Sapru and Wang, 1976; Judy and Farrell, 1979; Thames et al., 1984; Matsukawa et al., 1991a,b; Dampney et al., 2005; Rossi et al., 2010). To our knowledge no study has systematically examined the effect of exercise on the strength and mechanisms of the arterial baroreflex during exercise in hypertension. Legramante et al. (1999) did observe the effect of changes in posture on the responses to handgrip in an older cohort of normotensive and hypertensive men and

found that the effect of the static exercise on spontaneous baroreflex control of heart period was unaffected by the posture and was similar between normotensive and hypertensive groups. Inasmuch as posture changes unloads cardiopulmonary and arterial baroreceptors to variable extents, it is difficult to conclude whether the baroreflex is altered during exercise in hypertensive subjects. The authors attributed the lack of effect of hypertension on the baroreflex responses at rest to perhaps the age of the subjects. Some studies have investigated whether exercise training affects the baroreceptor reflex in hypertension but none have shown whether the arterial baroreflex strength and mechanisms are different between normotensive and hypertensive subjects during exercise. Burger et al. (1998) showed that in female exercise trained SHR, the baroreflex operating point of the systolic pressure – HR relationship was shifted to a higher point and the gain was reduced with increasing workloads, however, responses from normotensive animals were not investigated in that study. Moraes-Silva et al. (2010) demonstrated that treadmill training in during hypertension reduced absolute levels and the variability of blood pressure and HR when compared to sedentary hypertensive rats. Since a normotensive group was not utilized in the experiments previously mentioned, it is difficult to draw any conclusions about the arterial baroreflex function during exercise in hypertension. Furthermore the methods used to assess baroreceptor reflex activity is not consistent between studies, this provides a possible reason for the mismatch between the findings in the studies that have been done (O'Leary, 1996). Therefore further studies with appropriate controls are needed to fully understand impact of hypertension on arterial baroreflex control during exercise.

BAROREFLEX – METABOREFLEX INTERACTION IN HYPERTENSION

The arterial baroreflex resets during exercise to a higher set point in normotensive subjects (Rowell and O'Leary, 1990; Papelier et al., 1994; O'Leary, 1996, 2006; Michelini et al., 2015). Even though the arterial baroreflex is at a higher set point it still restrains the rise in arterial pressure caused by the muscle metaboreflex during dynamic exercise under normotensive conditions (Sheriff et al., 1990; Kim et al., 2005b). Sheriff et al. (1990) observed that sino-aortic baroreceptor denervation (SAD) increased the rise in arterial pressure in response to muscle metaboreflex activation (induced via reductions in hindlimb blood flow) during mild treadmill exercise in dogs. These results indicate that the arterial baroreflex buffers the muscle metaboreflex by about 50%. Subsequently, Kim et al. (2005b) showed that this buffering is due to arterial baroreflex attenuation of metaboreflex-induced peripheral vasoconstriction inasmuch as after SAD pronounced vasoconstriction now occurred along with the substantial increases in CO during metaboreflex activation. Therefore, the baroreflex nearly completely prevents the metaboreflex from causing substantial peripheral vasoconstriction. As workload rises, the skeletal muscle vasculature progressively becomes the largest fraction of the total vascular conductance and therefore substantial pressor responses via peripheral vasoconstriction could only occur via constriction of the active muscle (O'Leary, 1991; Kaur et al., 2016, 2018). This could engender a positive feedback, vicious cycle where further metaboreflex activation causes further skeletal muscle vasoconstriction. Indeed, recent studies have shown that there is some vasoconstriction within the active skeletal muscle during metaboreflex activation and this serves as an amplifier of the original response (Kaur et al., 2016). After induction of HF, this vasoconstriction in skeletal muscle is substantially greater perhaps due to depressed ability of the baroreflex to buffer metaboreflex-induced peripheral vasoconstriction (Kim et al., 2005a; Kaur et al., 2018). During exercise, hypertensive individuals experience accentuated increases in arterial blood pressure. Whether this is due to attenuated baroreflex restraint of pressor responses to activation of the muscle metaboreflex is unknown. Further studies are need to determine whether there is diminished arterial baroreflex function in hypertension which may be a potential mechanism allowing for the exaggerated increases in blood pressure during dynamic exercise.

CONCLUSION

In summary, it is known that central command, skeletal muscle reflexes and the arterial baroreflex are important for regulating the cardiovascular system during exercise. To what extent hypertension alters these individual mechanisms and the interaction between these reflexes is not well-understood as some studies lack appropriate controls. Also since multiple models are utilized (i.e., humans, rats, and dogs), resting and locomotive postural position may be play a role in the sometimes disparate results. Therefore, more studies are needed to understand how hypertension alters neural control of cardiovascular function which allows for the often marked increases in arterial pressure observed in these patients during exercise (Gibbons et al., 2002). In particular how hypertension affects the central neural processes involved in integrative cardiovascular control during exercise in not understood and could be a target of therapeutic strategies.

AUTHOR CONTRIBUTIONS

MD wrote the first draft of the manuscript. DO'L and JM wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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frontiers in Physiology

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The Pressor Response to Concurrent Stimulation of the Mesencephalic Locomotor Region and Peripheral Sensory Afferents Is Attenuated in Normotensive but Not Hypertensive Rats

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Central command (CC) and the exercise pressor reflex (EPR) regulate blood pressure during exercise. We previously demonstrated that experimental stimulation of the CC and EPR pathways independently contribute to the exaggerated pressor response to exercise in hypertension. It is known that CC and EPR modify one another functionally. Whether their interactive relationship is altered in hypertension, contributing to the generation of this potentiated blood pressure response, remains unknown. To address this issue, the pressor response to activation of the CC pathway with and without concurrent stimulation of the EPR pathway, and vice versa, was examined in normotensive Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats. In decerebrated, paralyzed animals, activation of the CC pathway was evoked by electrical stimulation of the mesencephalic locomotor region (MLR; 20–50 μA in 10-μA steps). Electrical stimulation of the sciatic nerve (SN, 3, 5, and 10 × motor threshold; MT) was used to activate hindlimb afferents known to carry EPR sensory information. In both WKY and SHR, the algebraic sum of the pressor responses to individual stimulation of the MLR and SN were greater than when both inputs were stimulated simultaneously. Although the blood pressure response to a constant level of SN stimulation was not significantly affected by concurrent MLR stimulation at variable intensities, the pressor response to a constant level of MLR simulation was significantly attenuated by concurrent SN stimulation in WKY but not in SHR. These findings suggest the interactive relationship between CC and the EPR is inhibitory in nature in both WKY and SHR. However, the neural occlusion between these central and peripheral pressor mechanisms is attenuated in hypertension.

Keywords: hypertension, mesencephalic locomotor region, central command, exercise pressor reflex, blood pressure, sympathetic nerve activity

INTRODUCTION

Neural drives from higher brain centers (central command, CC) and peripheral skeletal muscles (the exercise pressor reflex, EPR) contribute to the regulation of arterial blood pressure (ABP) during exercise. CC activates cardiovascular as well as locomotor control circuits simultaneously (Eldridge et al., 1985; Gandevia and Hobbs, 1990; Bedford et al., 1992; Gandevia et al., 1993; Tsuchimochi et al., 2009; Matsukawa et al., 2011; Nakamoto et al., 2011; Liang et al., 2016), playing a crucial role in mediating the circulatory responses to physical activity (Goodwin et al., 1972; Matsukawa, 2012; Michelini et al., 2015). The mechanosensitive and metabosensitive components of the EPR, which are activated by stimulating group III/IV skeletal muscle afferent fibers (Kaufman et al., 1984), also reflexively increase ABP and heart rate (HR) during exercise while the larger diameter group I/II fibers do not (McCloskey and Mitchell, 1972). Evidence suggests these central and peripheral neural signals converge both in the spinal cord as well as within cardiovascular centers in the brainstem to regulate the circulatory system in an integrative fashion (Waldrop et al., 1986; Rybicki et al., 1989; Degtyarenko and Kaufman, 2000a,b,c, 2005).

Hypertension is one of the most important causes of premature morbidity and mortality contributing to a number of cardiovascular related disorders (Kearney et al., 2005). Exercise is known to improve cardiovascular health and reduce resting blood pressure (Gibbons et al., 2002; Pescatello et al., 2004). However, circulatory hemodynamics are abnormally potentiated during exercise in this disease limiting the intensity and duration of physical activity that can be safely prescribed (Smith, 2010). The mechanisms underlying this overactive cardiovascular responsiveness are not fully understood. Recent studies in our laboratory suggest that dysfunction in both CC and the EPR contribute significantly to the exaggerated cardiovascular response to exercise in hypertension. Specifically, electrical stimulation of the mesencephalic locomotor region (MLR), a putative component of the CC pathway, induces larger ABP and renal sympathetic nerve activity (RSNA) responses in hypertensive rats compared to normotensive animals (Liang et al., 2016). Likewise, selective activation of the EPR elicits markedly greater increases in mean arterial pressure (MAP) and RSNA in hypertensive compared to normotensive rats (Smith et al., 2006; Leal et al., 2008; Mizuno et al., 2011a,b; Murphy et al., 2013). As stated, it is known that CC and the EPR interact to modulate each other's activity. Therefore, in addition to each input independently driving the abnormally enhanced cardiovascular response to exercise in hypertension, it is also plausible that alterations in the integrative relationship between the two contribute to this hyper-responsiveness.

The independent contributions of CC and the EPR to cardiovascular regulation during exercise has been studied extensively. In contrast, investigations designed to elucidate the interactive behavior of these inputs have been studied far less. The few studies that have investigated this interaction suggest that the relationship between CC and the EPR is inhibitory in nature (i.e., the sum of the pressor responses to independent activation of each is greater than the response elicited when both are stimulated simultaneously) (Degtyarenko and Kaufman, 2000a,b,c). To date, however, no studies have been conducted to determine whether this interactive relationship is altered with the pathogenesis of hypertension.

The purpose of this study, therefore, was to determine the interactive relationship between CC and the EPR in both normal, healthy rats and hypertensive animals. We hypothesized that the previously established inhibitory interaction between the two neural inputs, important for blood pressure regulation, is reduced in hypertensive animals as compared to normotensive controls. To test this hypothesis, we examined the integrative ABP response to peripheral and central neural activation in two distinct ways: (1) during electrical stimulation of peripheral skeletal muscle afferent fibers (sensory neurons known to be part of the EPR pathway) at multiple intensities throughout activation of the MLR (a component of the CC pathway) at a single, constant intensity, and (2) during MLR stimulation at multiple intensities throughout activation of skeletal muscle afferent fibers at a single, constant intensity. Both paradigms were performed in decerebrate, paralyzed normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. These paradigms were chosen as stimulation of peripheral afferent fibers and the MLR are common strategies used to investigate the contributions of the EPR and CC, respectively, to cardiovascular regulation in rodents (Bedford et al., 1992; Koba et al., 2005, 2006a,b, 2014; Liang et al., 2016).

MATERIALS AND METHODS

Experiments were performed using age-matched (13–16 weeks) male WKY (n = 11) and SHR (n = 11) rats. Animals were maintained in a temperature-controlled environment, fed ad libitum, and kept on a 12-h light-dark cycle. All studies were performed in accordance with the United States Department of Health and Human Services NIH *Guide for the Care and Use of Laboratory Animals.* The procedures outlined were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center.

General Surgical Preparation

As described previously (Smith et al., 2001; Liang et al., 2016), animals were anesthetized with isoflurane gas (4% in 100% oxygen, 1.5–2% during surgery) and intubated for mechanical ventilation. Fluid-filled polyurethane catheters were inserted into both common carotid arteries for the measurement of ABP and MAP (MLT0380/D; ADInstruments) and into the right external jugular vein for the administration of drugs. A continuous infusion of 1 M NaHCO3, 5% dextrose Ringer solution was established via the jugular vein at a rate of 3–5 ml h^{-1} kg^{-1} to stabilize fluid balance and maintain baseline ABP (Quintin, 1990). Electrocardiograph signals (ECG) were recorded by placing needle electrodes on the back of the animal, and HR was derived from the R wave of the ECG recording. ABP and HR were continuously monitored. Respiratory thoracic movement was visually observed and rectal temperature was maintained between 36.5 and 38.0 degrees Celsius with a heating pad and an

external lamp throughout the experiment. All animals were held in a stereotaxic head unit (Kopf Instruments), and a pre-collicular decerebration was performed rendering the animals insentient. Dexamethasone (0.2 mg) was given intravenously to minimize brain edema. Gas anesthesia was discontinued immediately following the decerebration procedure. Experimental protocols were performed at least 1.25 h thereafter (Kohn, 1997).

MLR Stimulation (to Mimic CC Activation)

The experimental procedures used for MLR stimulation have been described previously (Liang et al., 2016). Briefly, a concentric bipolar electrode (outer pole diameter: 200 μm, stainless steel; inner pole wire diameter: 50 μm, platinum/iridium; FHC Inc.) connected to a photoelectric stimulus isolation unit and stimulator (Grass S88, Grass Instrument Co.) was used. The tip of the electrode was placed 1.7–2.0 mm lateral, 0.3–0.8 mm anterior, and 3.5–4.5 mm deep from the surface junction of the superior and inferior colliculi (Bedford et al., 1992; Koba et al., 2005, 2006a,b, 2014; Liang et al., 2016). The motor threshold (MT) of MLR stimulation was determined by slightly increasing the current intensity until movement of the animal was observed. The site of the MLR stimulation was identified by physiological criteria as previously reported (Bedford et al., 1992; Koba et al., 2005, 2006a,b, 2014; Liang et al., 2016).

Sciatic Nerve Stimulation (to Mimic EPR Activation)

Electrical stimulation of the sciatic nerve (SN) was utilized to activate skeletal muscle afferent fibers. The left SN was exposed and separated from surrounding tissue at the knee joint. The nerve bundle was mounted on a bipolar electrode of Ag-AgCl wires, which connected to a photoelectric stimulus isolation unit and stimulator (Grass S88, Grass Instrument Co.), in a warm mineral oil pool surrounded with connective tissue and skin. The MT of SN stimulation was determined by slightly increasing the current intensity until muscle contraction was induced and movement was observed in the left hindlimb. The rat was paralyzed with pancuronium bromide (1 mg kg^{-1}, i.v.), and the lungs were artificially ventilated with a respirator after MT determination for SN and MLR stimulation.

Recording of Tibial Nerve Discharge

Tibial nerve discharge (TND) was recorded to assess motor activity induced by electrical stimulation of the MLR in one decerebrate, paralyzed WKY and SHR rat. As previously reported (Liang et al., 2016), the left tibial nerve was separated from the SN at the knee joint. To eliminate afferent discharge, the distal portion of the tibial nerve was ligated. The nerve bundle was mounted on a bipolar electrode of Ag-AgCl wires in a warm mineral oil pool surrounded with connective tissue and skin. The original TND was amplified with a band-pass filter at 100–4,000 Hz, then full-wave rectified.

Recording of Dorsal Root Nerve Activity

To identify which groups of afferent nerve fibers were activated by SN stimulation, compound action potentials of dorsal root neural activity (DRNA) were recorded in one decerebrate, paralyzed WKY and SHR rat to assess sensory nerve activity induced by electrical stimulation of the SN nerve. A laminectomy exposing the lower limb portions of the spinal cord (L_2–L_6) was performed as previously described (Smith et al., 2001, 2006; Mizuno et al., 2011a,b, 2015). The dura layers surrounding the cord were cut and reflected. The L_4 and L_5 dorsal roots were carefully isolated and sectioned. The cut peripheral ends of the roots were placed on bipolar platinum electrodes. The exposed neural tissue was immersed in mineral oil. The original compound action potentials of DRNA was amplified with a band-pass filter at 100–4,000 Hz. The distance between the stimulating and recording electrodes was assessed along with the latency of the responses in order to calculate conduction velocity. Conduction velocity at 31–120 m/s was classified as group I/II fibers, those from 2.6 to 30 m/s as group III, and those with less than 2.5 m/s as group IV (Mitchell, 1985).

Experimental Protocols

In protocols in which MLR or SN stimulation were applied alone, the following parameters were utilized. In MLR stimulation, current intensities of 20, 30, 40, and 50 μA (pulse duration of 1 ms at 60 Hz, for 30 s) were used in accordance with earlier studies (Bedford et al., 1992; Degtyarenko and Kaufman, 2005; Koba et al., 2005, 2006a,b, 2014; Liang et al., 2016). Regarding SN stimulation, current intensities equal to 3, 5, and 10 times MT (pulse duration of 0.75 ms at 20 Hz, for 30 s) were used. The latter intensities have been shown to be sufficient for activation of Group I-IV afferent fibers (McCallister et al., 1986; Harms et al., 2016).

In combined activation protocols, the MLR and SN were stimulated concurrently. In one paradigm, the current intensity used for SN stimulation was fixed at 3 × MT while MLR stimulation was applied over a range of 20–50 μA. In a second paradigm, the current intensity of MLR stimulation was fixed at 40 μA while SN stimulation was applied over a range of 3–10 × MT. When administered over a range, the application of current intensity was randomized. Moreover, the combined stimulation protocols were always performed after the sole stimulation protocols. The inter-protocol interval was at least 5 min between stimulations. All protocols were performed in paralyzed, decerebrate rats. If voluntary ventilation and/or movement were observed, supplemental doses of pancuronium bromide (0.5–0.75 mg kg^{-1}, i.v.) were administered.

At the conclusion of all experiments, the insentient animals were humanely killed by intravenous injection of saturated potassium chloride (4 M, 2 ml/kg iv). The heart and lungs were excised and weighed. In addition, the tibia was harvested and the length measured.

Data Acquisition and Analysis

ABP, MAP, HR, and stimulation pulse data were recorded and analyzed using data acquisition software (LabChart,

ADInstruments) for the Powerlab analog-to-digital convertor (Powerlab8/30; ADInstruments) at a 1-kHz sampling rate. The TND and DRNA were recorded at a sampling rate of 4-kHz. Data sets of 1 s averages for MAP and HR were analyzed. Baseline values were determined by evaluating 30 s of recorded data immediately before the MLR and/or SN stimulation. The maximum response of each variable was defined as the peak change from baseline elicited by electrical stimulation.

Statistical Analyses

Data were analyzed using Student's unpaired *t*-tests (WKY vs. SHR), two-way repeated measures ANOVA (rat group and MLR or SN stimulation intensity) with rat group (WKY and SHR) as a within-subject factor. If significant interaction and main effects were obtained with ANOVA, *post hoc* analyses were performed using a Student's unpaired *t*-test with Holm's sequential Bonferroni correction applied (Holm, 1979). The level of statistical significance was defined as $P < 0.05$. Results are presented as means ± *SE*.

RESULTS

Morphometric characteristics, baseline hemodynamics and MLR and SN motor thresholds for WKY and SHR are summarized in **Table 1**. There were no significant differences in body weight or lung weight-to-body weight ratios between groups. As previously reported (Smith et al., 2006; Leal et al., 2008; Mizuno et al., 2011a,b, 2014; Murphy et al., 2013; Liang et al., 2016), heart weight-to-body weight ratios as well as heart weight-to-tibial length ratios were significantly greater in SHR than WKY. Consistent with our previous study (Liang et al., 2016), baseline HR was significantly lower and baseline MAP was significantly higher in SHR compared to WKY. There were no statistical differences in MT for either MLR or SN stimulation between groups.

Original tracings of TND in response to MLR stimulation from one representative of both groups of animals are shown in **Figure 1A**. MLR stimulation increased TND in an intensity dependent manner as previously reported (Liang et al., 2016). Superimposed DRNA recordings (10 traces) in response to SN stimulation from one WKY and one SHR are shown in **Figure 1B**. SN stimulation significantly increased DRNA in an intensity-dependent manner in both animals, while it seemed the magnitude of the responses were somewhat smaller in SHR than WKY at all current intensities. Responses with fast conduction velocity, attributable to activation of group I/II afferent fibers, were clearly detected at current intensities of 1,3 and 5 × MT. At the higher intensity of 10 × MT, the response with a slow conduction velocity, attributable to group III/IV afferent fibers, could also be observed (conduction velocity ranged 14–23 m/s in WKY and 9–16 m/s in SHR).

Original ABP tracings in response to MLR stimulation with and without SN stimulation in representative WKY and SHR are presented in **Figure 2**. As previously reported (Liang et al., 2016), the ABP responses to MLR stimulation alone were markedly greater in SHR compared to WKY across all stimulation intensities. The responses to MLR stimulation were not appreciably affected by concomitant SN stimulation in either WKY or SHR.

Figure 3 summarizes group mean responses to MLR stimulation with or without concurrent SN stimulation in WKY and SHR. In hypertensive animals, MAP responses to stimulation of the MLR were greater compared to normotensive rats (**Figure 3A**; main "rat group" effect, $P = 0.08$ for MLR stimulation alone; main "rat group" effect, $P < 0.01$ for MLR + SN stimulation). In addition, MAP responses to MLR stimulation increased with stimulus intensity in both SHR and WKY (**Figure 3A**; main "stimulation intensity" effect, $P < 0.01$ for MLR stimulation alone; main "stimulation intensity" effect, $P < 0.01$ for MLR +SN stimulation). Importantly, in both groups of animals, the algebraic sum of the MAP response (i.e., SN stimulation alone + MLR stimulation alone) was larger than the MAP response evoked during simultaneous activation of the SN and MLR at all intensities tested indicative of an inhibitory interaction between the two inputs (**Figure 3A**). The difference in MAP calculated as the MAP evoked during MLR+SN stimulation minus the MAP evoked during MLR stimulation alone was not significantly different between WKY or SHR at any stimulus intensity (**Figure 3B**). Likewise, the difference in MAP calculated as the MAP evoked during MLR+SN stimulation minus the MAP evoked during SN stimulation alone was not significantly different between WKY or SHR at any stimulus intensity (**Figure 3C**).

Original ABP tracings in response to SN stimulation with and without MLR stimulation in representative WKY and SHR are presented in **Figure 4**. The pressor responses to SN stimulation increased in an intensity-dependent manner in both animal groups. Importantly, as compared with SN stimulation alone, the pressor responses became smaller with increased SN stimulation intensity when combined with MLR stimulation in WKY but became surprisingly larger in SHR.

Figure 5 summarizes group mean responses to SN stimulation with or without simultaneous MLR stimulation in WKY and SHR. In SHR, MAP responses to stimulation of the SN were significantly greater compared to WKY (**Figure 5A**; main "rat

TABLE 1 | Morphometric characteristics, baseline hemodynamics and motor threshold.

	WKY	SHR
N	11	11
Body weight, g	328 ± 3	327 ± 4
MAP, mmHg	72 ± 4	107 ± 7*
HR, beats min^{-1}	465 ± 11	415 ± 11*
Heart weight/body weight, mg/g	3.0 ± 0.1	3.3 ± 0.1*
Heart weight/tibial length, mg/mm	25.0 ± 1.2	27.9 ± 0.4*
Lung weight/body weight, mg/g	5.3 ± 0.3	5.9 ± 0.3
MLR stimulation motor threshold, μA	21 ± 2	23 ± 2
SN stimulation motor threshold, μA	49 ± 5	50 ± 4

*Values are means ± SE WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; MAP, mean arterial pressure; HR, heart rate; MLR, mesencephalic locomotor region; SN, sciatic nerve. *P < 0.05 compared with WKY rats.*

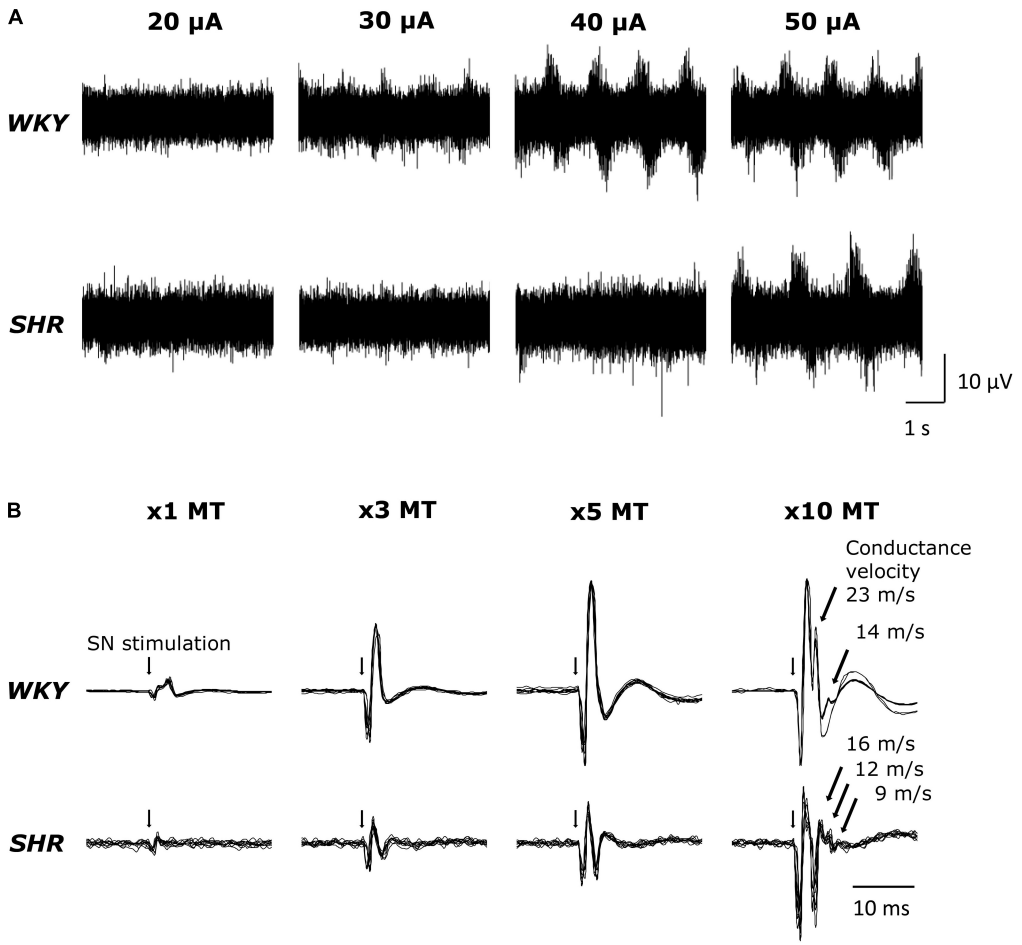

FIGURE 1 | (A) Original tracings demonstrating tibial nerve discharge (TND) in response to mesencephalic locomotor region (MLR) stimulation (20–50 μA) in WKY (MT: 18.3 μA) and SHR (MT: 18.9 μA). **(B)** Original tracings (superimposed 10 trials for each condition) of dorsal root nerve activity (DRNA) in response to stimulation of the sciatic nerve (SN) (1–10 × MT) in WKY and SHR.

group" effect, $P < 0.05$ for SN stimulation alone; main "rat group" effect, $P < 0.01$ for SN + MLR stimulation). In addition, the pressor responses to SN stimulation increased with each elevation in stimulus intensity in both SHR and WKY (**Figure 5A**; main "stimulation intensity" effect, $P < 0.01$ for SN stimulation alone) while decreasing step-wise when combined with MLR stimulation (**Figure 5A**; main "stimulation intensity" effect, $P < 0.01$ for SN+MLR stimulation). As before, in each group of animals, the algebraic sum of the MAP response (i.e., SN stimulation alone + MLR stimulation alone) was larger than the pressor response evoked during concurrent activation of the SN and MLR at all intensities tested. Evidence again that an inhibitory interaction exists between the two inputs (**Figure 5A**). The difference in MAP calculated as the MAP evoked during SN+MLR stimulation minus the MAP evoked during SN stimulation alone tended to be greater in SHR compared to WKY (**Figure 5B**; $P = 0.08$). Additionally, the differences in MAP calculated as the MAP evoked during SN+MLR stimulation minus the MAP evoked during MLR stimulation alone were significantly higher in SHR than WKY (**Figure 5C**, $P < 0.05$) with the differences actually below baseline in WKY. The latter finding suggests that the inhibitory relationship between the two inputs is maintained in normotensive animals but compromised in hypertensive rats.

Using data from **Figures 3A**, **5A**, the blood pressure responses to combined stimulation of the MLR and SN were calculated as a percent of the algebraic sum of each input alone (i.e., SN stimulation only + MLR stimulation only). The MAP responses evoked during SN stimulation (fixed at 3 × MT) combined with MLR stimulation (ranging from 20 to 50 μA) tended to be a larger percentage of the algebraic sum in SHR compared to WKY at most intensities tested although statistical significance was not reached (**Figure 6A**). The responses elicited during SN stimulation (ranging from 3 to 10 × MT) combined with MLR stimulation (fixed at 40 μA) were a significantly greater percentage of the algebraic sum in hypertensive compared to normotensive animals (**Figure 6B**) indicative of a change in the interactive relationship in SHR. In both paradigms, the responses produced during combined stimulation were less than 100% of the algebraic sum indicating an inhibitory relationship existed.

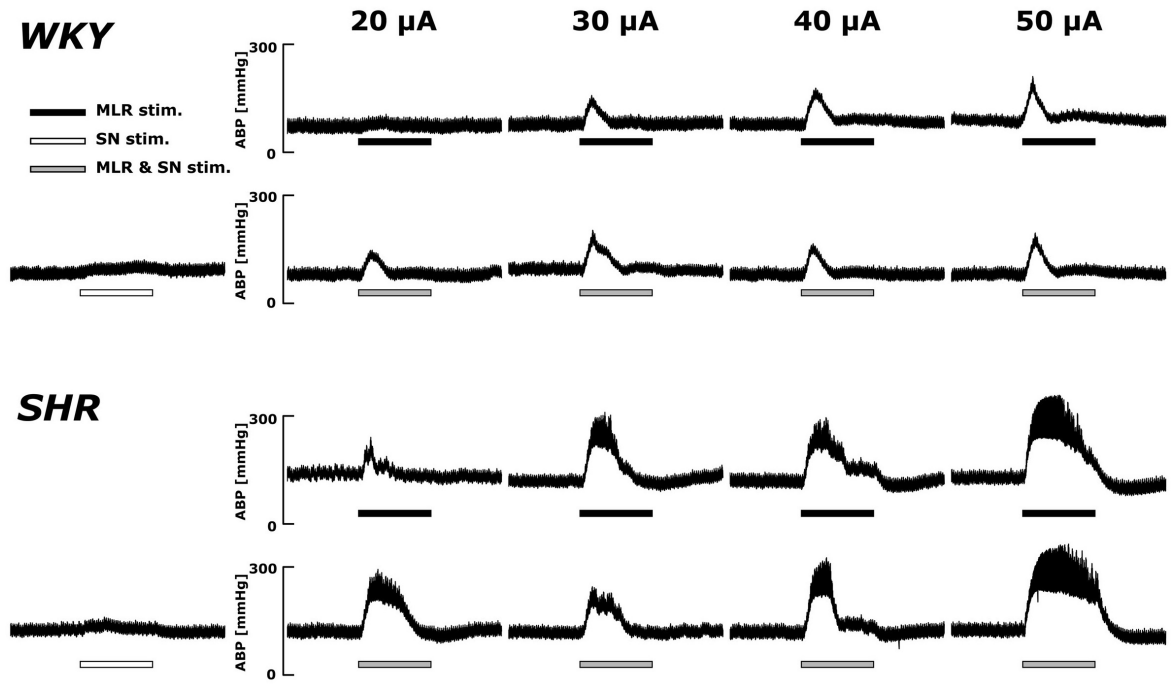

FIGURE 2 | Representative recordings of the arterial blood pressure (ABP) response to MLR stimulation with or without concurrent SN stimulation (3 × MT) in WKY (MLR stimulation MT: 37.5 μA) and SHR (MLR stimulation MT: 24.1 μA). Horizontal bars indicate the 30-s period of each stimulation; black: MLR stimulation alone; white: SN stimulation alone; gray: MLR and SN stimulation.

DISCUSSION

The present study was designed to investigate the interactive relationship between CC and the EPR in the regulation of blood pressure in normotensive and hypertensive animals. Activation of the CC pathway was induced by electrical stimulation of the MLR, while activation of skeletal muscle afferent fibers (components of the EPR pathway) was evoked by electrically stimulating the SN. In normotensive, healthy rats, the findings complement previous reports that the EPR and CC integrate in an inhibitory fashion such that the response to their combined stimulation is less than the algebraic sum of their individual responses. Some reports have described this as inhibition whereas others occlusion (Degtyarenko and Kaufman, 2000a,b,c, 2005; Eldridge et al., 1985; Waldrop et al., 1986; Rybicki et al., 1989). Constituting a major new finding, the data suggest that this inhibitory relationship is compromised in hypertensive animals. It has been previously reported that the independent function of the EPR and CC are exaggerated during exercise in hypertension. The findings from this investigation suggest that this overactivity may result, at least in part, from a reduction in the inhibitory relationship between the two inputs. Stated simply, CC overactivity in hypertension may be due, at least in part, to a reduction in the ability of the EPR to buffer its function and vice versa. This loss of inhibition may partially underlie each inputs abnormal contribution to the exaggerated cardiovascular response to exercise in hypertension.

TND Response to MLR Stimulation

Central command simultaneously activates neural circuits modulating locomotion as well as cardiovascular function. To preferentially assess CC in the absence of input from the EPR in the current study, it was necessary to apply MLR stimulation after the induction of neuromuscular blockade. This allowed activation of circuits in the CC pathway independent of actual muscle contraction which would, if allowed to occur, concurrently stimulate the EPR. The production of "fictive" locomotion in this manner is a common strategy used in animals to assess CC function (Bedford et al., 1992). To ensure that MLR-induced "fictive" locomotion was equivalent between WKY and SHR, the TND response to MLR stimulation was assessed. Consistent with our previous investigation (Liang et al., 2016), MLR stimulation increased TND in an intensity dependent manner in both groups (**Figure 1A**). Moreover, as previously reported (Liang et al., 2016), there was no difference in the MT during MLR stimulation when comparing WKY and SHR (**Table 1**). These data suggest that the motor command evoked by stimulation of the MLR was not different between WKY and SHR.

DRNA Response to SN Stimulation

Sciatic nerve stimulation at 3, 5, and 10 × MT was utilized to activate skeletal muscle afferent fibers associated with the EPR in this study. To confirm that the afferent fibers were activated by the current intensities used, we recorded DRNA in both WKY and SHR during SN stimulation (**Figure 1B**).

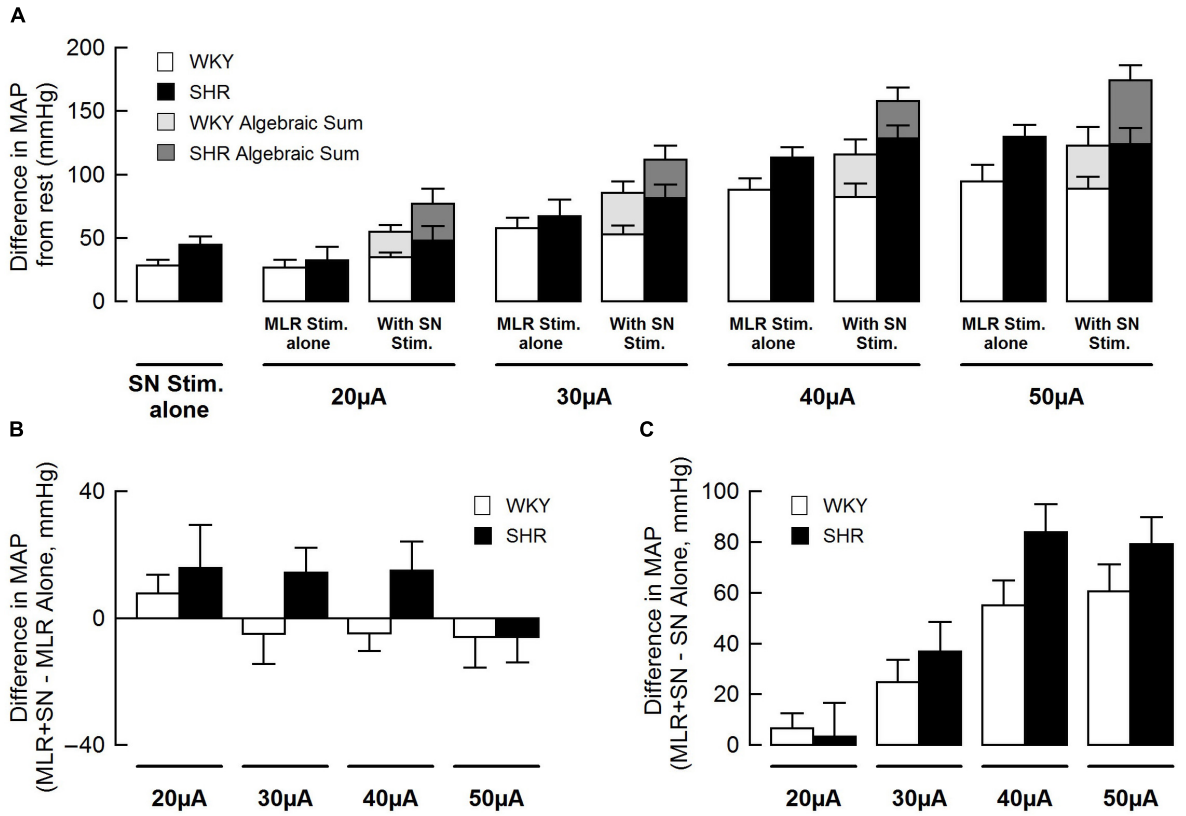

FIGURE 3 | (A) Peak changes in mean arterial pressure (MAP) associated with MLR and/or SN stimulation in WKY (white bars, n = 11) and SHR (black bars, n = 11). The intensity of the SN stimulation was fixed at 3 × MT with the MLR stimulation ranging from 20 to 50 μA. Light (WKY) and dark (SHR) gray bars depict the algebraic sum of MAP responses to SN stimulation alone + MLR stimulation alone. **(B)** Differences in the MAP response between combined stimulation and MLR stimulation alone. **(C)** Differences in the MAP response between combined stimulation and SN stimulation alone.

Since sensory threshold is considerably lower than MT in general, a small but distinct response was detected in the DRNA recording with the current intensity at 1 × MT (an intensity used solely to establish MT). Although the magnitude of the DRNA responses at each current intensity was somewhat smaller in SHR than in WKY, DRNA increased in an intensity-dependent manner in both groups. Importantly, it was determined that the responses with fast conduction velocity (characteristic of group I/II afferent fibers) were detectable during SN stimulation of 1−5 × MT (50–100 m/s), while those with slow conduction velocity (characteristic of group III/IV afferent fibers) appeared with 10 × MT in both WKY and SHR (9–23 m/s). This result is consistent with recent studies demonstrating that 5 × MT stimulation activates group I/II or III afferent fibers but not group IV (Harms et al., 2016) in rats. In addition, there was no difference in the latency of responses between WKY and SHR. These data suggest that SN stimulation-induced equivalent afferent fiber activation at all levels of intensity in both groups.

Impact of SN Stimulation on the Pressor Responses to MLR Stimulation

Consistent with our previous report (Liang et al., 2016), intensity-dependent pressor responses to MLR stimulation were greater in SHR compared to WKY with or without concurrent SN stimulation. Although an inhibitory interaction between the two inputs was evident and tended to be greater in magnitude in WKY compared to SHR (**Figures 3B**, **6A**), the differences were not statistically significant. This might be explained by the intensity of SN stimulation used in this particular protocol (i.e., SN stimulation of 3 × MT). Given this trend, use of a greater SN stimulus than 3 × MT may have elicited a larger occlusive response in WKY compared to SHR. Future investigation is warranted.

Impact of MLR Stimulation on the Pressor Responses to SN Stimulation

The present study also examined the effects of peripheral afferent input of multiple intensities on the pressor response to activation of the CC pathway (at a constant level) in both hypertensive and normotensive rats. In this paradigm, intensity-dependent pressor responses to SN stimulation were again larger in SHR compared to WKY with or without concurrent MLR stimulation. Importantly, the inhibitory interaction between the two inputs was clear (established by virtue of the pressor response to combined activation of each input being less than the algebraic sum of the response to each input stimulated individually) with

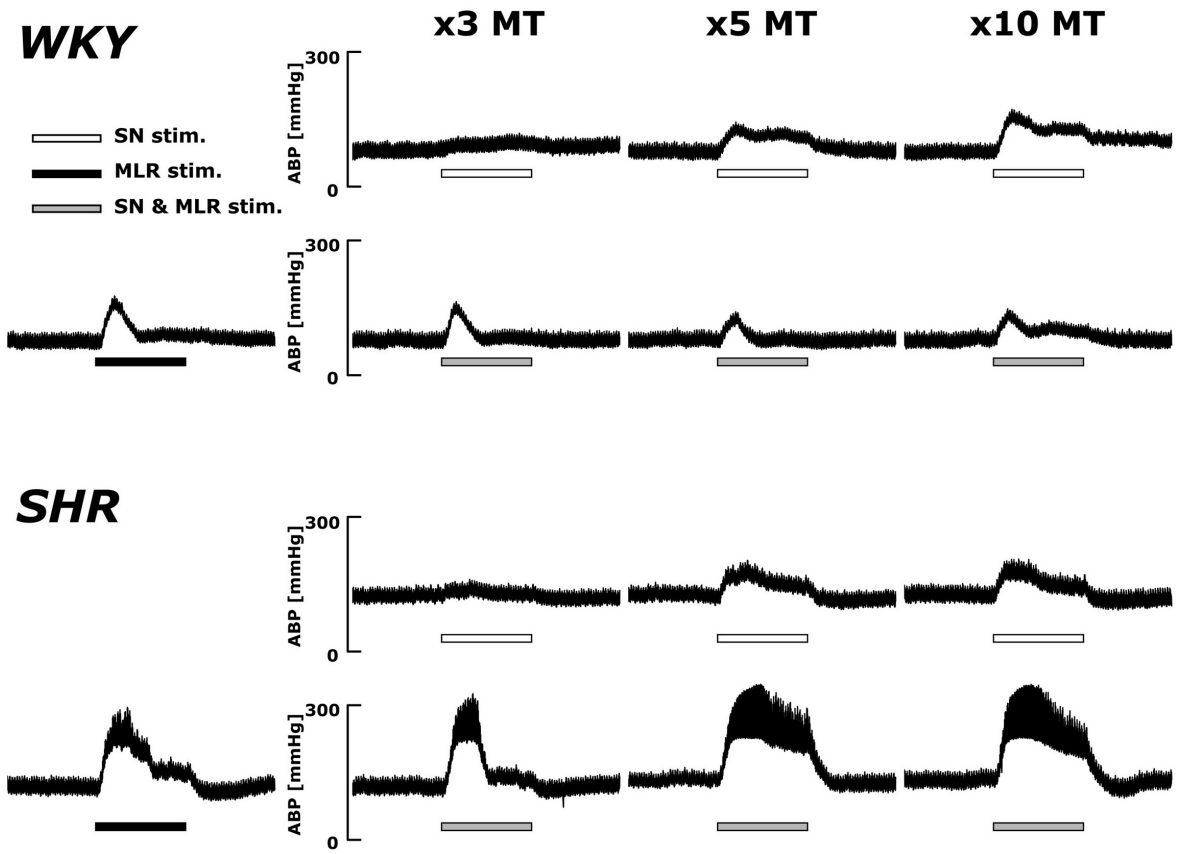

FIGURE 4 | Representative recordings of the blood pressure response to SN stimulation with or without concurrent MLR stimulation (40 μA) in WKY and SHR (identical rats as shown in the **Figure 2**). Horizontal bars indicate the 30-s period of each stimulation; white: SN stimulation alone; black: MLR stimulation alone; gray: MLR and SN stimulation.

the magnitude of the inhibition being significantly greater in WKY compared to SHR (**Figures 5B**, **6B**). As more evidence, the pressor response with combined MLR and SN stimulation remained unchanged or was larger than responses with MLR stimulation alone in SHR but was significantly smaller in WKY (especially at higher SN current intensities). Combined, these analyses suggest that the inhibitory relationship between the EPR and CC was significantly compromised in SHR as compared to WKY.

There is a substantial body of information in normal, healthy animals which suggests that the present results are consistent with prior observations. Stimulation of the MLR is known to produce an inhibition of the activity of cells receiving input from afferents mediating the pressor response to muscle contraction (Degtyarenko and Kaufman, 2000a,b,c). It is probable that this inhibition is not based on presynaptic occlusion of group III/IV afferent terminals but rather postsynaptic inhibition of interneurons (Rudomin and Schmidt, 1999). It is likely that MLR stimulation also inhibits responses due to the tonic activity of high threshold afferents resulting in less overall activity. Given the findings of the investigation, it seems probable that these mechanisms for inhibition were operative in normotensive WKY to a greater extent than in hypertensive animals. Alternatively, it is also possible that stimulation of peripheral afferent neurons in the EPR pathway directly inhibit neurons in the CC pathway centrally contributing to the responses obtained. This is purely speculative, however, as the current study was not designed to make this determination. What is clear from this study is that when the two pathways are stimulated concurrently, the interaction between these central and peripheral blood pressure mechanisms is occlusive in nature albeit attenuated in hypertensive animals.

Methodological Considerations

Autonomic adjustments regulating the cardiovascular system during exercise are determined by integrating input from the arterial baroreflex as well as the EPR and CC. Moreover, the baroreflex is known to modulate the activity of the EPR and CC. In the current study, the baroreflex remained intact and was not experimentally controlled. Moreover, it has been shown that the sensitivity of the baroreflex is reduced in hypertension (Moreira et al., 1992; Lanfranchi and Somers, 2002; Minami et al., 2003). That being acknowledged, previous studies have demonstrated that the cardiovascular responses to SN stimulation are enhanced in SHR compared to WKY and independent of impairments in baroreflex function

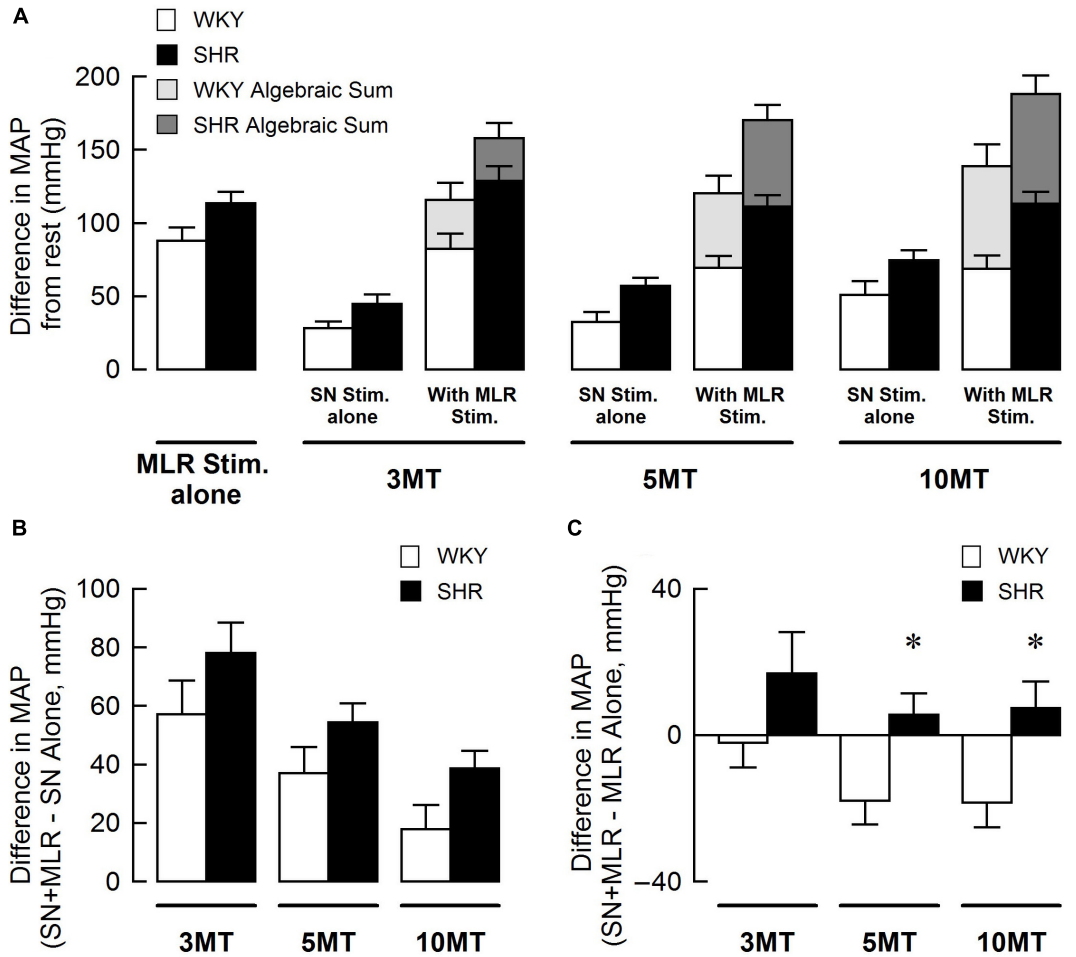

FIGURE 5 | (A) Peak changes in mean arterial pressure (MAP) in response to MLR and/or SN stimulation in WKY and SHR. The intensity of the MLR stimulation was fixed at 40 μA with SN stimulation ranging from 3 to 10 × MT. Light (WKY, $n = 11$) and dark (SHR, $n = 11$) gray bars depict the algebraic sum of MAP responses to SN stimulation alone + MLR stimulation alone. **(B)** Differences in the MAP response between combined stimulation and SN stimulation alone. **(C)** Differences in the MAP response between combined stimulation and MLR stimulation alone. $^*P < 0.05$ significant difference between WKY and SHR.

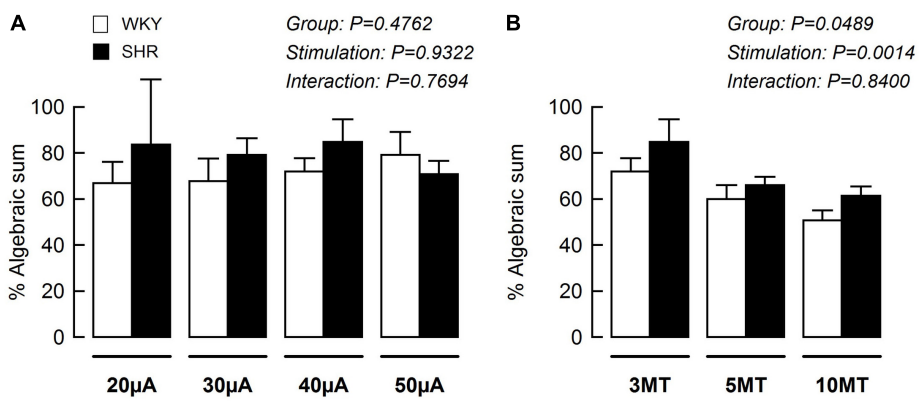

FIGURE 6 | The mean arterial pressure (MAP) response to concurrent MLR and SN stimulation expressed as a percentage of the algebraic sum of the MAP responses to SN stimulation alone + MLR stimulation alone in WKY (white bars, $n = 11$) and SHR (black bars, $n = 11$). Representations calculated from **Figures 3A**, **5A**. **(A)** The intensity of the SN stimulation was fixed at 3 × MT while MLR stimulation ranged from 20 to 50 μA. **(B)** The intensity of the MLR stimulation was fixed at 40 μA while SN stimulation ranged from 3 to 10 × MT.

(Ruggeri et al., 2000). As such, although an intact baroreflex may have influenced the results observed, its impact would be expected to be minimal.

CONCLUSION

The blood pressure response to exercise is abnormally exaggerated in hypertension. Due to the dangers inherent with such an enhanced pressor response, the prescription of physical activity as a safe treatment for this disease is often limited to exercise of short duration and mild to moderate intensity. Determining the mechanisms underlying this exaggerated responsiveness may lead to the development of therapies aimed at reducing this limitation allowing the benefits of exercise to be more fully realized in this patient population. To this end, previous studies have demonstrated that, when activated individually, stimulation the CC pathway and activation of the EPR pathway contribute significantly to the potentiated blood pressure response to exercise in this disease. Importantly, findings from the current study suggest that the CC and EPR overactivity manifest in hypertension is not solely due to alterations in the neural pathways of each input but also from alterations in the manner in which the inputs interact. Specifically, this investigation demonstrated for the first time, that the ability of each input to buffer the activity of the other is compromised in hypertension. This type of reduction in inhibitory influence with the pathogenesis of hypertension is likely to mediate, in part, the exaggerated blood pressure response to activation of both CC and the EPR during physical activity.

AUTHOR CONTRIBUTIONS

NL, GI, JM, SS, and MM decided on the conception and design of the research, and interpreted the results of experiments. NL, GI, RD, and MM performed the experiments. NL and MM analyzed the data, prepared the figures, and drafted the manuscripts. NL, GI, RD, JM, SS, and MM approved final version of the manuscripts.

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Evaluation of the Heart Function of Swimmers Subjected to Exhaustive Repetitive Endurance Efforts During a 500-km Relay

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Aim: Knowledge of the human body's ability to adapt to repeated endurance efforts during swimming is limited. We echocardiographically assessed the impact of an exhausting and repetitive swimming effort on cardiac activity.

Materials: Fourteen well-trained amateur swimmers (8 female swimmers aged 16–43 years and 6 male swimmers aged 13–67 years old) participated in an ultramarathon relay. Over 5 days, swimmers swam 500 km in the Warta River (in 5-km intervals). Each swimmer swam seven intervals, each within 44:46 to 60:02 min. Objective difficulties included low water temperatures, strong winds, rain, and night conditions.

Methods: Transthoracic echocardiography (TTE) was performed three times: at baseline (the day before exertion), at peak effort, and during recovery (48 h after the event). The heart rate (HR) of each swimmer was monitored.

Results: Swimmers completed the ultramarathon relay within approximately 91 h. The average HR value at the end of each interval was 91% HRmax. TTE test results showed no significant changes indicative of deterioration of myocardial function at peak effort or after 48 h. Significant increases in left ventricular (LV) ejection fraction, LV fractional shortening (LVFS), LV myocardial systolic velocity, and right ventricular (RV) fractional area changes observed on day 2 after swimming were compared to baseline values and peak effort values. No significant changes in diastolic heart function were observed.

Conclusion: Echocardiography assessment indicated that prolonged intense swimming does not affect LV or RV function. Supercompensation of the post-event RV function and increased global LV systolic function demonstrated ventricular interaction after prolonged intense swimming.

Keywords: endurance swimming, echocardiographic assessment, open-water swimming, exhaustive exercise, transthoracic echocardiography, myocardial function

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INTRODUCTION

Knowledge regarding the adaptations of the human body following long hours of exhaustive swimming is limited (Alexiou et al., 2005; Drygas et al., 2014; Stepien et al., 2017). However, in many countries, including Great Britain and the United States (Nikolaidis et al., 2018), Switzerland, Italy (Rüst et al., 2014b), Canada (Rüst et al., 2014a), Australia, and Poland, long-distance races and swimming marathons, such as the Manhattan Island Marathon Swim (Knechtle et al., 2014a), the Catalina Channel Swim (Knechtle et al., 2015), and the Zurich Lake Marathon Swim (Eichenberger et al., 2013), are becoming increasingly popular (Knechtle et al., 2014b). Every year, many swimmers undertake extremely difficult challenges such as attempting to cross extremely long distances in seas, lakes, and rivers (Khodaee et al., 2016; Valenzano et al., 2016). More than 1800 swimmers, including 571 females from more than 40 countries, successfully completed swimming the English Channel (approximately 32.2 km) prior to 2013 (Knechtle et al., 2014b). Unfortunately, long-distance swimming is associated with high risks to the health of the swimmer. Open-water swimming in seas, lakes, and rivers is particularly associated with distinct threats to health (Nelemans et al., 1994; Castro et al., 2009; Tipton and Bradford, 2014). According to information provided by the media, some swimmers who undertake extremely long, uninterrupted efforts must stop due to extreme fatigue, hypothermia, hyperthermia, or injuries caused by dangerous sea creatures (Keith, 2014; Halliday, 2018; Amazon Swim, 2019; Caldas, 2019). Grünig and co-authors reported 38 cases of swimming-induced pulmonary edema (SIPE) selected from 17 works (Grunig et al., 2017). The literature also describes dozens of sudden deaths and cardiac arrests of triathletes while participating in the swimming part of competitions (Harris et al., 2017). Deaths among high-level competitors during swimming marathons have also been reported (Harris et al., 2017; Smith et al., 2017). In our recently published studies, we presented detailed changes in biochemical and hematological indicators and clotting and fibrinolysis parameters experienced by a 61-year-old swimmer who swam solo for a distance of 120 km over the course of 27 h without leaving the river (Drygas et al., 2014; Stepien et al., 2017).

Although the benefits of systematic training for physiological capabilities and the health status of athletes are indisputable, numerous studies have indicated that multi-hour, exhausting physical effort may cause heart rhythm disturbances, heart attacks, and even sudden death during or after exertion (La Gerche et al., 2012; O'Keefe et al., 2012; Vitiello et al., 2013; Eijsvogels et al., 2016). Determining whether systematic, long-lasting, multi-hour endurance efforts can lead to dysfunction and even permanent damage to the heart is among the most important and most controversial problems in sports medicine. Previous studies involving ultramarathon runners showed that, in some cases, worsening of the heart function was observed by echocardiography at the peak of effort (Spirito et al., 1994). Typically, these changes are related to the right ventricular (RV) function and are transient (Leischik and Spelsberg, 2014; Rimensberger et al., 2014). Several authors have hypothesized that acquired arrhythmogenic RV cardiomyopathy develops as a result of repeated extreme and long-term endurance efforts (La Gerche et al., 2012, 2017; Heidbuchel, 2018). Other authors, citing their own long-term research, have strongly denied this possibility (Knackstedt et al., 2015; Leischik, 2015). Undoubtedly, health status based on age and sex is significant, but the level of training of athletes and the type and duration of the physical effort are also important. In addition, ruling out the potential contribution of illegal pharmacological assistance used in some sports by both professional athletes and leisure sports participants is difficult.

The aim of our study was to evaluate several vital functions during and after exhausting, repetitive endurance swimming of a group of well-trained, but not elite, swimmers. The swimmers included females and males aged 13–67 years who participated in a 500-km ultramarathon swim relay in open water. Our study included several anthropometrical, biochemical, hematological, and hemostatic parameters and evaluated endothelium function and intraocular pressure. We describe data related to the influence of endurance swimming on cardiac function and structure. We hypothesized that repeated exhausting effort, such as long-distance swimming in a relay swimming ultramarathon, may adversely affect cardiac function, as assessed by echocardiography.

MATERIALS AND METHODS

Our study involved a group of swimmers who undertook an extraordinary physical effort: swimming 500 km in a relay race in the Warta River in Poland. The event began on July 15, 2016, at 9:00 pm, between kilometers 292 and 297 of the Warta River. The distance was repeated 90.5 times, and the final 48 km ended in Poznań at kilometer 244 of the Warta River on July 17, 2016, at 4:00 pm. Swimmers swam a total distance of approximately 500 km. Fourteen well-trained non-elite swimmers, including eight females 16–43 years old and six males 13–67 years old, participated in the relay. The athletes alternated turns swimming during the relay; each time they started, they jumped into the water from the boat after the previous swimmer finished a 5-km shift. The competitors swam each shift within 44:46 to 60:02 min depending on the time of day, water temperature, and atmospheric conditions (i.e., strong wind, rain, etc.). Only athletes who completed the relay and for whom we possessed echocardiographic data of all three measurements (12 swimmers; 7 females, and 5 males aged 13–67 years) were included in the statistical analysis. One 15-year-old girl was unable to continue the event on the third day due to acute symptoms of airway inflammation. She was examined by experienced physicians and withdrawn from the relay. Notably, she was free of serious health-related symptoms within 2 days. Another participant, a 43-year-old woman, successfully completed the event, but was unable to participate in the final echocardiography examinations, which were performed 48 h after the event, due to professional obligations. The detailed demographic, clinical, and echocardiographic characteristics of the subjects are reported in **Table 1**. **Table 1** also presents

TABLE 1 | Baseline demographic, clinical, and echocardiographic data of the study participants.

Participant	Age (year)/ sex	Height (cm)/ weight (kg)	BMI (kg/m^2)	BSA (m^2)	HR (beats/min)	Systolic blood pressure (mmHg)/ diastolic blood pressure (mmHg)	VO_2 max (mL/kg/min)	LVEDD (cm)/ LVESD (cm)	IVS (cm)/ PW (cm)	LV EF (%)	LAD (cm)	RVEDD (cm)
1	67/M	177.3/63.5	20.2	1.8	46	140/82	42.2	5.0/3.5	1.0/1.1	66.0	3.7	2.9
2	38/M	181.0/76.0	23.2	2.0	72	120/75	37.5	4.8/3.0	1.0/0.8	66.6	3.2	2.6
3	31/F	171.0/54.5	18.6	1.6	52	105/70	39.0	4.9/3.1	0.9/0.6	66.0	3.3	2.8
4	43/F	166.6/58.2	21.0	1.6	55	100/65	38.9	4.8/3.2	0.8/0.8	63.4	2.9	2.9
5	16/F	158.5/48.0	19.1	1.5	60	115/70	39.8	4.8/3.0	0.8/0.9	67.0	3.0	2.6
6	29/M	180.3/75.0	23.1	1.9	83	120/77	36.6	5.3/3.4	0.9/1.0	65.0	3.1	2.2
7	18/F	170.5/61.0	21.0	1.7	62	75/62	43.1	5.1/3.2	0.8/0.8	68.0	3.4	2.8
8	16/M	158.0/63.0	25.2	1.7	63	147/68	52.2	5.0/3.1	0.9/0.7	67.9	3.0	3.0
9	23/M	163.5/63.0	23.6	1.7	65	110/70	42.4	4.7/3.3	0.8/0.8	60.0	3.1	2.6
10	24/F	176.5/66.6	21.4	1.8	55	130/80	46.5	4.7/2.9	1.0/0.8	66.8	3.3	2.7
11	39/F	162.7/55.0	20.8	1.6	45	145/95	50.5	5.2/3.4	1.0/0.8	63.9	3.5	2.9
12	13/M	157.8/54.5	21.9	1.6	82	125/75	36.7	4.3/3.0	0.9/1.0	58.8	3.1	2.7

F, female; M, male; BMI, body mass index; BSA, body surface area; HR, heart rate; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; IVS, interventricular septum; PW, posterior wall; EF, ejection fraction; LAD, left atrial diameter; RVEDD, right ventricular end-diastolic diameter.

basic anthropometric and physiological data characterizing the participants in this swimming relay event. Notably, there was a wide range of ages (13–67 years; mean, 30 ± 15 years). Furthermore, all relay participants had a slim body structure, low fat content, and, in most cases, lower HR (<60/min) at rest. The VO_2max values obtained during a stress test performed on a moving treadmill indicated that the swimmers were not high-performance athletes with very high aerobic capacity (Bhat and Shaw, 2017).

The organizers of this extraordinary swimming ultramarathon carefully prepared the entire event while considering the physical and psychological preparations of the relay participants and their safety during the many hours of swimming. All participants had been regularly swimming for at least 5 years, usually 2 to 3 times per week for 45 to 60 min each time. Importantly, the swim ultramarathon participants were mostly well-trained, but non-competitive, athletes. During the 6 months preceding their participation in the ultramarathon, swimmers increased both the frequency and duration of their swimming training. Several training sessions were conducted in natural water reservoirs (lakes and rivers) at low air and water temperatures and at night to adequately prepare them for the upcoming ultramarathon. During the last 5 days before the relay event, the swimmers only participated in light training in the form of warm-up exercises to fully rest before the swimming ultramarathon. During the 2 to 3 weeks before the swimming ultramarathon, all swimmers participated in specialized sports medical tests, including detailed interviews and a medical examination, electrocardiogram (ECG), spirometry test, measurement of maximum oxygen consumption (VO_2max) during a mobile treadmill test, biochemical blood tests, and an eye examination, at the Department of Sports Medicine of the Medical University of Łódź.

Weather and Living Conditions

Notably, during the relay, all participants lived together as a group in a tent under harsh conditions. The participants slept for a maximum of 3 to 4 h between their swimming shifts; during each shift, they were driven from the base to the boat, where they started and finished their shift. The subjects were instructed to abstain from consuming alcohol and to eat a light meal, such as boiled chicken breasts with steamed vegetables and rice, before the event and during the breaks of the swim relay.

Water and air temperatures during these 5 days drastically changed. The air temperature ranged from 8 to 22°C and the water temperature ranged from 14 to 24°C, depending on the time of day and place of measurement. Additionally, very unfavorable weather conditions in the form of heavy rains and strong winds occurred during the event. Due to the large waves caused by the strong winds, the competitors repeatedly choked on swallowed water, particularly during the night shifts. Therefore, the swimmers' bodies underwent serious physical and mental stress. Data from other research showed that calorific deficits and low ambient temperatures had significant adverse effects on the body's functions (Planer et al., 2012; Schnitzler et al., 2018). Weather conditions during the relay are shown in **Table 2**.

Transthoracic Echocardiography

Each participant underwent an echocardiogram three times as follows: the day before the effort, 48 h after the effort at an echocardiographic laboratory, and during peak effort (i.e., after the final exit from the water, on a boat, in a cabin particularly adapted for this purpose). Each test was performed using the same equipment. All studies were performed and interpreted by a single experienced investigator.

Participants underwent standard Doppler echocardiography using an ultrasound imaging system (Digital Portable Color Doppler SonoScape S8 Exp [S8EXP/S9PRO]; SonoScape Medical, Corp., Shenzhen, China) with a 2.5-MHz transducer and Doppler tissue imaging. Echocardiographic variables, including the left ventricular (LV) end-diastolic diameter, LV end-systolic diameter, septal and posterior wall thicknesses, RV end-diastolic diameter, and left atrial diameter (LAD), were recorded in the parasternal

TABLE 2 | Weather conditions during the swim relay.

Data	Hour	Water temperature (°C)	Air temperature (°C)	Day	Night
Tuesday, July 12	21:00	24	23	Sun	Clear, starry
Wednesday, July 13	09:00	23.5	21	Sun, overcast	
	21:00	22	20	Overcast, rain, periods of sun	Overcast and starless
Thursday, July 14	09:00	18	16	Heavy rain, overcast	
	21:00	17	14	Heavy rainfall, overcast, gusty winds, waves 20 cm	Dark and starless
Friday, July 15	09:00	16	17	Heavy rain, overcast, periods of sun, windy	
	21:00	16	12	Very cloudy, rain	Windy all night, waves 50 cm
Saturday, July 16	09:00	16	18	Rain, periods of sun, windy	
	21:00	15	10	Showers, overcast	Starless, cold wind
Sunday, July 17	09:00	14	8	Periods of slight sun, overcast, weak rainfall	Dense fog with limited visibility
	16:00	15.5	22	Periods of sun from 2:00 pm, weakening wind	

view. LV fractional shortening (FS) was calculated as the percentage change in the LV systolic and diastolic dimensions. The LV ejection fraction (LVEF) was calculated using Simpson's biplane method. The Doppler-derived LV diastolic inflow was recorded in the apical four-chamber view by placing the sample volume at the level of the leaflet tips. In the same view, the RV end-diastolic and end-systolic areas were measured, and the RV fractional area change (FAC) was calculated. Additionally, the tricuspid annular plane systolic excursion (TAPSE) was acquired using the conventional M-mode method at the lateral tricuspid annulus. The LV myocardial tissue Doppler peak systolic (S_m), early diastolic (E_m), and late diastolic (A_m) velocities were measured by placing the sample volume at the septal and lateral angles of the mitral annulus, and the average values of each velocity were calculated. Similarly, pulsed tissue Doppler was used with the sample volume positioned at the lateral corner of the tricuspid annulus to assess its velocity (RV S_m).

Heart Rhythm Analyses

Heart rate (HR) measurements during the exercise were performed using Polar V800 HR monitors (POLAR Electro, Kempele, Finland). The results were analyzed by a cardiologist with extensive experience.

Statistical Analyses

Significant differences between consecutive measurements using echocardiography (first, baseline; second, peak effort; and third, during recovery) were analyzed by an analysis of variance (ANOVA) Friedmann test followed by a Friedmann *post hoc* test. Parameter characteristics are presented as the average value (± standard deviation). All statistical calculations were performed using STATISTICA 12 software. The significance level was set as $p < 0.05$.

Ethical Issues

The swimming ultramarathon was organized for charity. Both young and very experienced amateur swimmers participated to raise a considerable amount of money for the Oncology, Hematology, and Pediatric Transplantology Clinic in Poznań.

The athletes provided written consent to participate in the swimming ultramarathon, to participate in the aforementioned study, and to have their results published. Parents provided written consent for the teenage swimmers to participate in the swimming ultramarathon and all biomedical tests. Approval was obtained from the Bioethics Committee of the Medical University of Łódź to perform biomedical monitoring of the Warta Marathon participants.

RESULTS

The average HR value at the end of each shift was 91% HRmax (169 bpm). The average HR during the effort was 157 bpm. This was 84% of the average maximum HR of all athletes. The maximum HR was the value reported by the athletes after previous maximum swimming efforts, such as tests or competitions (in two cases). The HR values of the individual competitors are shown in **Table 3**.

Echocardiographic studies showed no obvious LV wall hypertrophy, with a mean septal thickness of 0.88 ± 0.08 cm and a mean posterior wall thickness of 0.84 ± 0.14 cm

TABLE 3 | Heart rate values of individual competitors obtained during the ultramarathon relay event and the percentages in relation to the maximum heart rate.

				Mean and maximum HR values during the swim relay	
Participant	Age (year)	Sex	Maximum HR of the athlete	Mean HR (% of maximum HR of the athlete)	Maximum HR (% of maximum HR of the athlete)
1	67	M	158	131 (83)	145 (92)
2	38	M	186	148 (80)	165 (89)
3	31	F	197	164 (83)	172 (87)
4	43	F	176	152 (86)	164 (93)
5	16	F	189	177 (94)	197 (104)
6	29	M	195	156 (80)	176 (90)
7	18	F	194	161 (83)	170 (88)
8	16	M	182	155 (85)	160 (88)
9	23	M	197	167 (85)	172 (87)
10	24	F	169	146 (86)	162 (96)
11	39	F	194	158 (81)	165 (85)
12	13	M	200	166 (83)	180 (90)
			Mean 186.4 ± 12,4	157 (84)	169 (91)

TABLE 4 | Left ventricular systolic function.

Echocardiographic parameter	Baseline	Peak effort	Recovery	*p*-Value
LVEDD (cm)	4.87 ± 0.27 [4.88]	4.90 ± 0.59 [4.74]	4.87 ± 0.47 [4.77]	NS
LVESD (cm)	3.16 ± 0.18 [3.13]	3.17 ± 0.39 [3.04]	2.92 ± 0.41 [2.76]	<0.05*
LV EF (%)	64.9% ± 3.0 [66.0]	64.2% ± 5.1 [64.2]	71.6% ± 3.5 [72.3]	<0.05*
LV SF (%)	36.3% ± 2.1 [36.9]	35.2% ± 3.9 [35.3]	41.1% ± 2.9 [41.7]	<0.05*
Myocardial systolic velocity, S_m (cm/s)	13.17 ± 1.71 [13.2]	13.13 ± 1.48 [13.3]	14.64 ± 1.27 [14.4]	<0.01*

Mean ± SD (median value).
**Recovery vs. peak effort and recovery vs. baseline.*
LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; EF, ejection fraction; SF, shortening fraction.

TABLE 5 | Left ventricular diastolic function.

Echocardiographic parameter	Baseline	Peak effort	Recovery	*p*-Value
Mitral peak E velocity	103.0 ± 18.3 [102.5]	91.2 ± 13.7 [88.8]	104.6 ± 25.0 [10.1.3]	NS
Mitral peak A velocity	64.4 ± 13.5 [62.4]	62.6 ± 10.3 [59.7]	68.5 ± 11.2 [72.0]	NS
Mitral peak E/A ratio	1.64 ± 0.32 [1.60]	1.49 ± 0.31 [1.43]	1.56 ± 0.42 [1.54]	NS
Myocardial early diastolic velocity, E_m (cm/s)	17.2 ± 2.8 [17.2]	16.6 ± 3.9 [17.3]	16.8 ± 4.3 [18.0]	NS
Myocardial late diastolic velocity, A_m (cm/s)	9.72 ± 2.18 [9.68]	10.44 ± 1.85 [10.64]	9.62 ± 2.98 [8.50]	NS
E_m/A_m ratio	1.86 ± 0.51 [1.99]	1.67 ± 0.60 [1.61]	1.93 ± 0.73 [2.05]	NS
E/E_m ratio	6.08 ± 1.23 [5.91]	5.68 ± 1.04 [5.43]	6.46 ± 1.42 [6.25]	NS
LAD (cm)	3.21 ± 0.22 [3.12]	3.25 ± 0.25 [3.27]	3.22 ± 0.27 [3.14]	NS

Mean ± SD (median value).
LAD, left atrial diameter.

TABLE 6 | Right ventricular systolic function.

Echocardiographic parameter	Baseline	Peak effort	Recovery	*p*-Value
RVEDD (cm)	2.71 ± 0.20 [2.74]	2.63 ± 0.39 [2.56]	2.64 ± 0.27 [2.61]	NS
RV myocardial systolic velocity, S_m (cm/s)	18.4 ± 3.9 [17.0]	16.9 ± 2.7 [16.7]	18.24 ± 3.4 [16.9]	NS
TAPSE (mm)	24.3 ± 2.8 [24.2]	24.3 ± 3.3 [23.7]	25.6 ± 4.5 [25.0]	NS
FAC (%)	51.5% ± 3.4 [51.3]	56.6% ± 7.4 [58.4]	58.6% ± 6.9 [57.8]	<0.05*

Mean ± SD (median value).
**Recovery vs. baseline.*
RVEDD, right ventricular end-diastolic diameter; TAPSE, tricuspid annular plane systolic excursion; FAC, fractional area change.

(Lang et al., 2015). All individuals displayed preserved LV systolic function, with a mean LVEF of 64.9 ± 2.9%, mean LV end-diastolic diameter of 4.87 ± 0.27 cm, and end-systolic diameter of 3.16 ± 0.18 cm.

Regarding the global systolic LV function, compared to measurements obtained at baseline and at peak effort, significant increases in the LVEF and LV shortening fraction were observed during recovery. Similarly, the myocardial systolic peak velocity (Sm) was higher 2 days after swimming (**Table 4**).

Left ventricular diastolic function, as assessed by the standard transmitral flow, was not affected by the extreme exercise (**Table 5**). Similarly, compared to the baseline assessment results, the left atrial diameter, early and late myocardial peak velocities, and E_m/A_m and E/E_m ratios did not change during or after swimming.

The RV diameters and non-geometric parameters describing the RV function (TAPSE and S_m) did not significantly differ between consecutive measurements (**Table 6**). Moreover, compared to the baseline measurement, an increase in the RV FAC was observed 48 h after exercise.

TABLE 7 | Division of the intensity of physical effort depending on the achieved heart rate.

	Intensity					
	Very low	Low	Medium	High	Very high	Maximum
%HRmax	<35	35–59	60–79	80–89	>90	100

Kozłowski S, Nazar K. Introduction to clinical physiology. PZWL 1995.

While separately analyzing the individual echocardiographic data of all ultramarathon participants, we did not detect any changes that could be interpreted as signs of cardiac "fatigue," dysfunction, or any other cardiac pathology. Full echocardiographic data of the study participants are presented in **Appendix 1**.

DISCUSSION

The increasing popularity of endurance and ultra-endurance swimming in many countries is an interesting and intriguing phenomenon (Knechtle et al., 2014b). Unfortunately, despite many excellent studies analyzing factors related to performance, speed, and energy, information regarding the adaptation of cardiac function to endurance or ultra-endurance swimming are scarce (Costa et al., 2015). Moreover, we were unable to find any studies related to repeated exhaustive endurance swimming in open water.

During our study, 14 competitors participated in an ultra-marathon swim relay event comprising a distance of 500 km. Three echocardiographic examinations were performed for seven females and five males who completed the ultramarathon and participated in all three tests. Each competitor swam approximately 35 km. Each competitor was assumed to have considered the shift "very intense training." The average HR value at the end of each shift was 91% HRmax. Some competitors even reached 95 to 104% of their maximum HR (considered maximum until the event) during individual sections of the swimming ultramarathon. Based on these values, we determined whether the intensity of the effort was high, very high, or maximum (Kozłowski and Nazar, 1995) (**Table 7**). The HR values of the athletes during swimming were considered accurately measured. The records of the HR monitors that we analyzed indicated no unexpected fluctuations in values that could indicate the occurrence of artifacts. In such situations, we can assume that HR monitors used by athletes correctly indicate both the average and maximum HR values (Gajda et al., 2018).

Swimmers completed the ultramarathon relay within approximately 91 h. Considering the length of the swimming sections (5 km), the duration of the effort, the number of repeated trials (seven times), the special conditions (the need to swim at night and in low water and air temperatures), and the HR values reached during each shift, the relay was considered exhaustive or extremely difficult (Kozłowski and Nazar, 1995).

The data from our study strongly demonstrate that intense prolonged repeated bouts of swimming in open water in difficult environmental conditions did not negatively influence the heart function.

Echocardiography assessment indicated that prolonged intense swimming does not affect LV or RV function.

In the group of swimmers included in this study, atrial enlargement or deterioration of the RV or LV systolic function was not observed during the echocardiographic evaluations of the heart.

Transthoracic echocardiography (TTE) test results showed no significant changes indicative of deterioration of myocardial function at peak effort or after 48 h. Regarding the systolic function of both chambers, compared to the baseline values and the values at peak effort, significant increases in the EF and FS of the left ventricle, myocardial LV systolic velocity, and RV FAC were observed on day 2 after completing swimming. No significant changes in the diastolic function of the heart were observed.

We did not observe a significant decrease in the echocardiographic parameters when assessing RV function during the post-swimming examination. TAPSE and RV systolic velocity illustrating the function of RV longitudinal fibers did not change; moreover, compared to assessments made at baseline and at peak effort, the RV FAC significantly increased during recovery. A possible explanation for this phenomenon might be that the FAC describes not only longitudinal but also oblique and circular myofibers that appear to enhance its function to address the exercise-induced increased contractility demand. Therefore, our study showed that the right ventricle copes with the increase in load during swimming even if the intense exercise is maintained for a long duration and is repeated during a short period. The supercompensation of the post-event RV function and the increase in the global LV systolic function demonstrated the ventricular interaction after high-intensity swimming.

Moreover, while analyzing separately the individual echocardiographic data of al 500-km relay participants, we did not detect any changes that could be interpreted as signs of cardiac "fatigue" dysfunction, or any other cardiac pathology.

The observed changes did not suggest that long-distance swimming caused changes in the echocardiographic parameters of the tested athletes, thus demonstrating no harmful effect on the hearts of athletes.

Studies investigating changes in LV function following prolonged intense exercise have yielded inconsistent data, and studies involving swimmers are scarce (Douglas et al., 1986). Cahill et al. (1979) measured LV dimensions of 14 athletes, including 7 international swimmers, before and immediately after submaximal exercise. They noted significantly larger left ventricular muscle mass and end-diastolic dimensions in swimmers as compared to controls and decrease in LV end-systolic dimension both in swimmers and controls after exercise. According to our results, the echocardiography-derived global LV function was not only unaffected during intense swimming but also significantly increased during recovery. Santoro et al. (2016), who studied competitive water polo players, observed a significant increase in the LVEF immediately after maximal exercise. As mentioned, the different types of exercise might explain why our observations were not made immediately after the effort, but later during recovery. In contrast to our results, Alexiou et al. (2005) observed a reduction in LVFS and LVEF after exhaustive open-water swimming. Studies using Doppler tissue imaging to investigate the LV systolic function of swimmers are lacking. Similar findings of enhanced LV myocardial systolic velocity after racing were observed by Vitiello et al. (2013) when investigating a small group of runners who participated in an extreme mountain ultra-marathon. However, due to the differences in the study groups, environmental conditions, and types of exercise analyzed in the discussed works and our study, it is challenging to make comparisons and draw conclusions.

Regarding the Doppler-derived diastolic parameters, no significant changes were observed in the LV filling pattern in this study. A previous study showed that after ultra-endurance exercise, the peak early transmitral filling velocity significantly decreased (Hassan et al., 2006). In contrast, full-body immersion led to the opposite diastolic filling

changes (increase in early velocity) (Marabotti et al., 2013). Furthermore, Santoro et al. (2016) observed a significant increase in both early and late diastolic transmitral velocities immediately after maximal exercise in competitive water polo players. Notably, our observations were obtained under unique exercise conditions (i.e., prolonged intense exercise in the water).

Previous studies have revealed that intensive endurance exercise may lead to RV dysfunction, and that the degree of RV functional impairment depends on the duration of the exercise (La Gerche et al., 2012; Heidbuchel, 2018).

Numerous studies have shown that certain cardiac arrhythmias and their preceding morphological and functional changes in the heart are more common in high-performance athletes than in their inactive peers. These arrhythmias include paroxysmal atrial fibrillation and atrial flutter (Castro et al., 2009), and atrial overload enlargement and/or fibrosis resulting from ischemic damage during extreme long-term effort are considered the cause (Everett et al., 2011; Nielsen et al., 2013). However, no works have specifically found an increased risk of AF or RV arrhythmias specifically in swimmers (Guasch and Mont, 2017). The idea that arrhythmogenic RV cardiomyopathy develops in endurance athletes is controversial (La Gerche et al., 2012, 2017). Numerous studies have shown that immediately after intense, long-lasting physical effort, a significant increase in pressure occurs in the right ventricle that recedes during rest (La Gerche et al., 2014). Transient worsening of RV function in the form of deterioration of the EF and other changes in the right ventricle parameters, both morphological and functional, were also observed (La Gerche et al., 2011). In general, the more prolonged the effort, the more intense the cardiac changes (La Gerche et al., 2011, 2012; Sanz-de la Garza et al., 2017). According to several works, repeated dysfunction of the right ventricle after repeated extreme exertions may eventually lead to acquired arrhythmogenic RV cardiomyopathy (La Gerche et al., 2014; Heidbuchel, 2018).

It should be noted that, in our study, the effort was performed during approximately 45- to 60-min sessions. The resting time between sessions may have allowed partial RV recovery (and possibly LV recovery). In practice, the time from leaving the water by the swimmer until the next shift was 9 h. Accordingly, a repetitive exercise pattern might yield a different cardiac adaptation than swimming 25 km consecutively (Alexiou et al., 2005), swimming a marathon, swimming a triathlon (La Gerche et al., 2012), or swimming for 24 consecutive hours (Drygas et al., 2014). However, there was no reason to assume that a 9-h break between 45- and 60-min efforts repeated seven times was enough to fully rest. Moreover, we instructed our swimmers to perform only very light workouts during the last 5 days before the ultramarathon. Thus, the baseline echocardiographic examinations on the day before the ultramarathon relay were performed for the "rested" heart.

The main strengths of our study were the unique exercise protocol and comprehensive monitoring of various important vital functions during and after the ultramarathon. Each athlete performed several intensive swimming endurance exercise sessions in open water during the day and night under difficult environmental conditions. Moreover, other than two very experienced master athletes who were champion endurance swimmers, most swimmers had not been previously involved in competitive swimming training, although all had experience with long-distance swimming. Despite the age groups used in other studies, we included both females and males of various ages in our study. Echocardiography was performed by the same cardiologist with extensive experience with similar studies. A member of our study team was constantly with the swimmers to monitor the necessary physiological parameters and report potential adverse effects. The biochemical, hematological, anthropometric characteristics, intraocular pressure, and endothelium function analyses are currently being prepared by our research group for publication in another article.

The main limitation of this study was that monitoring of the cardiac, hematological, and biochemical adaptations to prolonged repetitive swimming was performed for a relatively small group of athletes. As previously mentioned, we obtained echocardiography data of 12 males and females. The echocardiographic analysis was limited to standard two-dimensional, Doppler, and tissue Doppler studies. Regional myocardial deformation was not performed. We analyzed a heterogeneous group of swimmers that included adolescents (13- to 16-year-old participants) and adults (males and females). However, cardiac adaptations and remodeling may significantly change in adolescents and between sexes (Finocchiaro et al., 2017). The effort was performed during seven 45- to 60-min sessions. Resting time between sessions may have allowed partial recovery of the right ventricle (and possibly the left ventricle). Despite these limitations, our study was the first devoted to morphological and functional cardiac changes following repeated exhaustive endurance swimming in open water. Therefore, although our observations were limited to a relatively small group of swimmers, the results of our study are relevant to a large group of professional athletes involved in endurance swimming. The data from our study are also interesting in the context of whether prolonged endurance exercise may cause transitory or even permanent pathological changes in the function or structure of the right or left ventricle (all data supporting this study are provided as supplementary information in **Appendix 1**).

In summary, the results of this study indicated that prolonged intense swimming does not affect LV and RV function, as assessed by echocardiography.

CONCLUSION

Echocardiography assessment indicated that prolonged intense swimming does not affect LV and RV function. Supercompensation of the post-event RV function and the increase in the global LV systolic function demonstrated ventricular interaction after prolonged intense swimming.

DATA AVAILABILITY

All datasets generated for this study are include in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Resolution No. RNN/230/16/KE of July 12, 2016, Bioethics Commission at the Medical University of Łódź with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Bioethics Commission at the Medical University of Łódź.

AUTHOR CONTRIBUTIONS

RG and WD conceived the study. WD, MK, ER, and MN designed the study details and supervised the data collection. SR, ER, RG, MK, MN, and WD contributed significantly to data collection. RG, EK, PH, and WD participated in writing the paper and checking the draft for errors. WŚ was the statistician and was involved in designing the study. RG, WD, and EK written by the final version of the manuscript. All authors read and accepted the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00296/full#supplementary-material

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The Slow Component of Oxygen Uptake and Efficiency in Resistance Exercises: A Comparison With Endurance Exercises

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Introduction: There is a lack of information regarding the slow component of oxygen uptake (VO_2sc) and efficiency/economy in resistance exercises despite the crucial role played in endurance performance.

Purpose: this study aimed to compare the VO_2sc, efficiency/economy, metabolic, cardiorespiratory responses, rating of perceived effort and mechanical fatigue between cycling and half-squat (HS) exercises during a constant-load test at lactate threshold (LT_1) intensity.

Methods: Twenty-one healthy men were randomly assigned in a crossover design to perform cycle-ergometer or HS tests. The order of the two cycle ergometer tests was an incremental test for determining load-intensity in watts (W) at LT_1, followed by a constant-load test at the LT_1 intensity. For the three HS tests, the order was a 1RM test to determine the load (kg) corresponding to the 1RM percentages to be used during the second test, incremental HS exercise to establish the load (kg) at the LT_1 intensity, and finally, a constant-load HS test at the LT_1 intensity. A rest period of 48 h between each test was established. During the HS and cycle-ergometer constant-load tests, cardiorespiratory and metabolic responses were recorded. Lower limbs fatigue was determined by a jump test before and after the constant-load tests.

Results: A significant exercise mode × time interaction effect was detected in VO_2, heart rate, energy expenditure (EE), gross efficiency (GE), and economy ($p < 0.05$). A significant and sustained VO_2 raise was confirmed in HS exercise ($p < 0.05$) and a steady-state VO_2 was revealed in cycle-ergometer. A higher GE and economy were obtained in HS test than in cycle-ergometer exercise ($p < 0.001$). In both exercises, a non-significant decrease was observed in GE and economy ($p > 0.05$). Lower limbs fatigue was only detected after constant-load HS test.

Conclusion: Although the VO_2, heart rate and EE responses were higher in cycling exercise, the constant-load HS test induced a greater VO_2sc and EE raise than the cycling test in a predominantly aerobic metabolism. These results could explain a decrease observed in jump performance only after HS test. GE and economy could benefit from the eccentric phase of the HS exercise.

Keywords: oxygen uptake kinetics, gross efficiency, energy expenditure, lactate threshold, mechanical fatigue

INTRODUCTION

Laboratory testing of respiratory exchange using a breath-by-breath open-circuit gas analyzer have become a fundamental practice for measuring oxygen uptake (VO_2) kinetics during constant-load endurance exercises. Pulmonary VO_2 tends to rise slowly for a given power output beyond ∼3 min during prolonged constant-load endurance exercise, involving sustained lactic acidosis; this surpasses the primary component initiated at exercise onset. This ventilatory phenomenon is known as the slow component of VO_2 (VO_2sc) (Gaesser and Poole, 1996).

As some authors suggest, the VO_2sc could be affected by the behavior of various parameters such as the power-load, VO_2, and lactate threshold (LT), conditioning cardiorespiratory performance and efficiency (Burnley and Jones, 2007). The power output developed above, below or at the LT will determine the amplitude of the VO_2sc response. Therefore, LT intensity plays a key role in the assessment of VO_2sc. According to three-phase model (Skinner and McLellan, 1980), two LTs (LT_1 and LT_2) are recognized during cardiopulmonary exercise testing (Binder et al., 2008). LT_1 is considered as "aerobic threshold" at 40–60% of VO_{2max} (light exercise), and LT_2 is discerned as "anaerobic threshold" at 60–90% of VO_{2max} (moderate to heavy exercise). Obviously, the VO_2sc at LT_2 intensity will increase to a greater extent than at LT_1 intensity during constant-load exercise.

Despite the important role of VO_2sc in endurance performance (Lucía et al., 2002), respiratory exchange tests for evaluating power output or VO_2 at the LT_1 intensity are not usually applied to resistance exercises in laboratory conditions and, therefore, there is a surprising lack information about VO_2sc. To date, only one recent study has focused on VO_2sc in resistance exercises at the LT_1 intensity (Garnacho-Castaño et al., 2018a). Two findings of this study draw the attention. Firstly, the authors reported a slightly higher VO_2sc in absolute values (153.8 mL.min^{-1}), during 31 min of constant-load HS testing at the LT_1 intensity in healthy young practitioners, compared to that reported in another study with professional cyclists (130 mL.min^{-1}) during 20 min of constant-load test at an intensity above LT_1 (80% VO_{2max}) (Lucía et al., 2000). This detected response of VO_2sc usually occurs at intensities above the LT_1 in endurance exercises (Burnley and Jones, 2007). It could be that the VO_2sc in HS exercise, in a mainly aerobic metabolism (LT_1), is comparable to the VO_2sc observed in endurance exercises at intensities above the LT_1. It has been shown that VO_2sc is lower in a leg cycle compared to an arm crank exercise (Koppo et al., 2002) and higher in cycling than in running exercise (Billat et al., 1998). This difference between the exercise modes was chiefly associated with the amplitude of response (Carter et al., 2000), which in turn was conditioned by loading intensity during constant-load test (Carter et al., 2002). So, the VO_2sc is exercise- and intensity-dependent.

Secondly, the authors demonstrated that the continuous increase in VO_2 and energy expenditure (EE) was linked to a decrease in gross efficiency (GE) (Garnacho-Castaño et al., 2018a). In addition, lower limbs fatigue was detected after constant-load HS test. The VO_2sc could be explained, at least partly, by the variation in GE which assesses the effects of blood alkalinization on the gradual loss of muscle efficiency (Gaesser and Poole, 1996) and progressive fatigue (Garnacho-Castaño et al., 2018a).

Keeping these two premises in mind, it appears reasonable to suggest a greater increase in VO_2sc and EE, whereas the efficiency decrease, during HS exercise than during a constant-load cycling test at the LT_1 intensity. In theory, the power output or load equivalent to the LT_1 intensity means the highest power output or load that will not elicit VO_2sc (Burnley and Jones, 2007) during constant-load endurance tests. However, to the best of our knowledge, no studies have compared VO_2sc, GE, EE, and mechanical fatigue between resistance and endurance exercises during long-term constant-load test at the same aerobic metabolic intensity (LT_1).

To compare VO_2sc and efficiency between resistance and endurance exercises could provide relevant information for clarifying the underlying physiological mechanisms that related VO_2sc and EE to efficiency and fatigue in resistance exercise and, therefore, to determine whether resistance or endurance exercises are more efficient in a predominantly aerobic metabolism. This study aimed to compare VO_2sc, efficiency/economy, metabolic responses and mechanical fatigue between cycling and HS exercises during a constant-load test at an intensity corresponding to LT_1.

MATERIALS AND METHODS

Participants

Twenty-one healthy participants were recruited among the male students of the Physical Activity and Sports Sciences Department (age: 21.4 ± 1.5 years, height: 180.2 ± 5.4 cm, weight: 81.8 ± 8.6 kg, body mass index: 25.2 ± 2.0). All participants had at least 6 months of experience in resistance training and were accustomed to HS exercise.

Four exclusion criteria were established: (1) any cardiovascular, metabolic, neurological, pulmonary or orthopedic disorders that could limit exercise performance,

(2) the use of any medication, supplements, or performance-enhancing drugs, (3) a one-repetition maximum (1 RM) of less than or equal to 150 kg in HS exercise, (4) being an elite athlete.

Eligible participants were informed of the tests they would be taking, and provided their signed written consent to participate. The participants were instructed to refrain from other exercises or resistance training during the course of the study. The study protocol adhered to the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of TecnoCampus-Pompeu Fabra University (Mataró, Barcelona, Spain).

Experimental Design

Subjects were required to visit the laboratory on five occasions at the same time each day under similar environmental conditions (temperature 21–25°C, atmospheric pressure 715–730 mm Hg, relative humidity ∼45%). The protocols were implemented according to the procedures previously established by our research group (Garnacho-Castaño et al., 2015a). Participants were randomly assigned in a crossover design to perform cycle ergometer or HS tests.

The order of the two cycle ergometer tests was an incremental test for determining load-intensity in watts (W) at LT_1, followed by a constant-load test at the LT_1 intensity. For the three HS tests, the order was a 1RM test to determine the load (kg) corresponding to the 1RM percentages to be used during the second test, incremental HS exercise to establish the load (kg) at the LT_1 intensity, and finally, a constant-load HS test at the LT_1 intensity.

A rest period of 48 h between each test was established. During the HS and cycle ergometer constant-load tests, acute cardiorespiratory and metabolic responses were recorded. Timing of blood lactate sampling was the same in both tests. Before and after the constant-load tests, mechanical fatigue in the lower limbs was determined by a counter movement jump (CMJ) test.

Cycle Ergometry Tests

Incremental and constant-load cycle ergometer tests included a 5-min warm-up on a cycle ergometer (Monark ergomedic 828E, Vansbro, Sweden) at an initial pedaling cadence of 50 rev.min^{-1} and work rate of 50 W, followed by 5 min of dynamic joint mobility drills and stretching exercises. After 2-min rest time, the cycle ergometer tests commenced. In both tests, blood lactate concentrations were measured using a portable lactate analyzer (Lactate Pro LT-1710, Arkray Factory Inc., KDK Corporation, Siga, Japan). The reliability of this device has been previously evaluated (McNaughton et al., 2002).

The incremental test was carried out in a ramp protocol starting with a load of 50 W, which was increased in steps of 25 W.min^{-1} until completing 8 min at a pedaling cadence of 50 rev.min^{-1}. Blood samples (5 μL) were attained by finger pricking at rest and every 2 min during the incremental test. The LT_1 was determined according to three-phase model (Skinner and McLellan, 1980), following the guidelines established by Binder et al. (2008). The LT_1 was detected by inspecting blood lactate concentrations plotted against workload according to the protocol described by Weltman et al. (1990). The LT_1 was defined as the highest exercise load completed when a 0.5 mmol.L^{-1} rise over baseline is detected in at least 2 instances.

The constant-load cycle ergometer test involved continuous pedaling at a cadence around 70–80 rev.min^{-1} at an intensity (W) equivalent to the LT_1 previously determined in the incremental test. The test duration was 31 min. Blood lactate samples were obtained at the start of the test and at the following minutes of cycling: min 4, min 8.5, min 13, min 17.5, min 22, min 26.5, and min 31. Respiratory exchange data were recorded during the constant-load test using a breath-by-breath open-circuit gas analyzer (Vmax spectra 29, Sensormedics Corp., Yorba Linda, CA, United States), which had been previously calibrated. VO_2, minute ventilation (VE), carbon dioxide production (VCO_2) and respiratory exchange ratio (RER) were monitored. Heart rate was checked every 5 s by telemetry (RS-800CX, Polar Electro OY, Finland).

Half Squat Tests

In HS tests, a Smith machine (Matrix Fitness, Johnson Health Tech, Cottage Grove, MN, United States) was used to ensure controlled movements. Each HS test started with a warm-up consisting of 5 min of low intensity running and 5 min of joint mobility. This was followed by a specific warm-up consisting of 1 set of 3–5 repetitions (HS) at a relative intensity of 40–60% of the maximum perceived effort. After 2-min, HS test protocols commenced.

Establishing the 1RM involved 3–5 lifting attempts using increasing weight. The 1RM was defined as the last load lifted by the subject, completing a knee extension to the required position. The rest period between each attempt was 4 min (Garnacho-Castaño et al., 2018a).

The incremental HS test was carried out in 5 sets at relative intensities of 10, 20, 25, 30, 35, and 40% 1RM as previously described (de Sousa et al., 2012; Garnacho-Castaño et al., 2015a,b, 2018a). Each set lasted 1 min and involved 30 repetitions of 2 s each (1 s for both eccentric and concentric muscle actions). This rhythm was checked with a metronome while a researcher provided visual and verbal cues. A passive rest period of 2 min between sets (Garnacho-Castaño et al., 2015a,b) was provided while blood samples were collected for LT_1 and the load was augmented. The test was terminated voluntarily by the participant or when he was powerless to continue performing repetitions at the set cadence or did not correctly execute repetitions. Blood samples (5 μL) were obtained by finger pricking 30 s after the end of each set, and lactate levels were measured using the same portable lactate analyzer.

The LT_1 was recognized by means of the algorithm adjustment method based on Orr et al. (1982) as the load-intensity at which blood lactate concentrations start to increase in an exponential manner (Wasserman and McIlroy, 1964). The LT_1 was detected through computerized 2-segment linear regression by fixing the 2 linear regression equations emerging for each segment at the point of intersection between a plot of blood lactate concentration and relative intensity. Data analysis was performed using Matlab version 7.4 (MathWorks, Natick, MA, United States).

The constant-load HS test was conducted as 21 sets of 15 repetitions of 2 s each (1 s for both eccentric and concentric phases) guided by metronome, visual, and verbal cues. The duration of each set was 30 s and the rest period between sets was 1 min. These guidelines were established in preliminary trials and, subsequently, in previous studies (Garnacho-Castaño et al., 2015a,b). In the constant-load test, it was not possible to perform HS sets in a time longer than 30 s. Furthermore, a recovery period of less than 60 s between sets could not be standardized because in both cases the blood lactate concentrations increased exponentially.

The whole constant-load test took 31 min. Respiratory exchange and heart rate data were recorded as previously described (Garnacho-Castaño et al., 2015a,b). Blood samples were obtained at rest and 30 s after the end of the HS sets (S) S3, S6, S9, S12, S15, S18, and S21, when lactate concentrations were obtained as described above for the incremental test.

VO_2 Slow Component, Efficiency/Economy in Constant-Load Tests

In both HS and cycle constant-load tests, the VO_2sc was identified as the difference between end-of-exercise VO_2 and the VO_2 at the end of the third minute of constant-load exercise (Δ VO_2, in $mL.min^{-1}$). The latter was taken as the average VO_2 from 2 min 30 s to 3 min 30 s (set 2 to set 3); end-exercise values were taken as the average of the last 2 min of the tests (29 min 0 s to 31 min 0 s, set 20 to set 21). Mean cycling-(CE) and HS-economy (HSE) was expressed in $W.L^{-1}.min^{-1}$. GE was calculated as the ratio of work accomplished per minute (i.e., W in $kcal.min^{-1}$) to energy consumed per minute (i.e., in $kcal.min^{-1}$) as follows:

$$GE\ (\%) = (\text{Work accomplished}/EE)\ \times\ 100.$$

The mean power output during the same period as the respiratory exchange collection was recorded in order to determine "Work accomplished," which was converted into $kcal.min^{-1}$ as follows:

$$\text{Work accomplished}\ (kcal.min^{-1}) = \text{Power output}\ (W) \times\ 0.01433.$$

Energy expenditure was calculated from VO_2 and the RER. The calorific equivalent of O_2 was determined from the corresponding RER, using the tables provided by Peronnet and Massicotte (1991).

$$EE\ (kcal.min^{-1}) = VO_2\ (L.min^{-1})\ \times\ Kcal.L^{-1}\ \text{of}\ O_2.$$

The power output to quantify HSE and GE during HS test was calculated by means of a reliable and validated linear position transducer (Tendo Weight-lifting Analyzer System, Trenèín, Slovakia) (Garnacho-Castaño et al., 2015c). The power output was computed in each repetition based on bar velocity (Lake et al., 2012). The mean power output was calculated as the mean of all repetitions.

Lower Limbs Mechanical Fatigue

Lower limbs fatigue was evaluated in a CMJ test using a force plate (Quattro Jump model 9290AD; Kistler Instruments, Winterthur, Switzerland), as previously described (Garnacho-Castaño et al., 2015a,b). Jump height, mean power, and peak power were recorded before the start and at the end of both constant-load tests, immediately after the last blood lactate reading. Participants carried out 3 jumps and the mean height, mean power, and peak power output were used in the data analysis. A recovery period of 30 s between each jump was established.

Perceived Effort

The Borg scale was used to monitor the rating of perceived effort (RPE) (Borg, 1978). Scores were recorded by each subject at the blood collection time points for blood lactate determination during incremental and the constant-load tests.

Statistical Analysis

The Shapiro–Wilk test was used to check the normal distribution of data, provided as means, standard deviation (SD), confidence intervals (95% CI) and percentages. To identify significant differences between HS and cycle ergometer exercises in VO_2 kinetics, lactate levels, and economy/efficiency variables during constant-load tests, a general linear model with a two-way analysis of variance (ANOVA) for repeated measures was performed. The two factors were exercise mode (HS and cycle ergometer) and time (corresponding to 7 checkpoints performed in both tests). When appropriate, a Bonferroni *post hoc* adjustment for multiple comparisons was implemented. To determine mechanical fatigue, an ANOVA for repeated measures was performed. A Student's *t*-test was used to compare heart rate, VO_2, RPE and blood lactate concentrations at LT_1 intensity during incremental test in cycle ergometer and HS exercises.

Partial eta-squared (η_p^2) was computed to determine the magnitude of the response to both exercise modes. The statistical power (SP) was also calculated. Intraclass correlation coefficients and coefficients of variation percentage were used to determine

TABLE 1 | Data related to 1RM- and incremental-load tests.

Variables	HS	CYC
1RM (Kg)	200.3 (39.7)	–
HS load at LT_1 (kg)	49.6 (16.2)	–
Relative intensity at LT_1 (%1RM)	23.9 (4.8)	–
Load at LT_1 (W)*	242.6 (86.9)	168.1 (38.2)
VO_2 at LT_1 ($mL.kg^{-1}.min^{-1}$)$^{\delta}$	2.08 (0.32)	1.96 (0.37)
Lactate at LT_1 ($mmol.L^{-1}$)$^{\delta}$	2.51 (0.59)	2.21 (0.51)
HR ($beats.min^{-1}$)$^{\delta}$	134.95 (16.84)	125.43 (17.16)
HR (%)$^{\delta}$	63.14 (8.53)	67.96 (8.60)
RPE (6–20)$^{\delta}$	10.62 (1.80)	9.81 (2.09)

*Data are presented as mean and standard deviation (SD). 1RM, one-repetition maximum; CYC, cycle-ergometer; HR, heart rate; HR (%), percentage regards theoretical maximum heart rate; HS, half-squat; LT_1, lactate threshold one; RPE, rating of perceived exertion; VO_2, oxygen uptake. *Significant differences between HS and cycle ergometer. $^{\delta}$No significant differences between HS and cycle ergometer.*

the relative and absolute reliability. All statistical methods were performed using the software package SPSS Statistics version 23.0 for Mackintosh (SPSS, Chicago, IL, United States). Significance was set at $p < 0.05$.

RESULTS

Descriptive data related to incremental-load test in cycle ergometer and HS exercises are presented in **Table 1**. Differences in VO_2, heart rate, metabolic, RPE and economy/efficiency responses between HS vs. cycle ergometer during constant-load tests are shown in **Table 2**. Mean intraclass correlation coefficient and mean coefficient of variation for all VO_2, metabolic and economy/efficiency variables was 0.982 (0.966–0.991) and 5 ± 2%, respectively.

VO_2, Lactate, RPE and Heart Rate Responses at LT_1 During Incremental Tests

No significance differences were found between cycle ergometer and HS exercises in VO_2, lactate, RPE and heart rate responses at LT_1 during incremental tests ($p > 0.05$).

VO_2, Heart Rate, Respiratory Exchange Ratio, Lactate and RPE During Constant-Load Tests

In VO_2, a significant exercise mode x time interaction effect was observed [$p = 0.001$, $F_{(6,120)} = 4.05$, $\eta_p^2 = 0.17$, SP = 0.97]. A significant time effect [$p < 0.001$, $F_{(6,120)} = 25.06$, $\eta_p^2 = 0.56$, SP = 1.00], and exercise mode effect were detected [$p < 0.001$, $F_{(1,20)} = 35.14$,$\eta_p^2 = 0.64$, SP = 1.00]. After Bonferroni adjustment of multiple comparisons, a significant and sustained VO_2 raise was confirmed from S3 in HS exercise ($p < 0.05$) and a steady-state pulmonary VO_2 was revealed from M4 in cycle ergometer. Higher VO_2 was found in cycle ergometer than HS exercise at each checkpoint ($p < 0.001$) (**Figure 1**).

In heart rate, a significant exercise mode x time interaction effect was detected [$p < 0.001$, $F_{(6,120)} = 8.30$, $\eta_p^2 = 0.29$, SP = 1.00]. A significant time effect [$p < 0.001$, $F_{(6,120)} = 34.69$, $\eta_p^2 = 0.63$, SP = 1.00], and exercise mode effect were detected [$p < 0.001$, $F_{(1,20)} = 30.14$, $\eta_p^2 = 0.60$, SP = 0.99]. Bonferroni test determined a higher heart rate in cycle ergometer than HS exercise at each checkpoint ($p < 0.001$) (**Figure 2**).

No significant exercise mode x time interaction effects or time and exercise mode effects were detected in lactate concentrations ($p > 0.05$) (**Figure 3A**). It was only detected a time effect in RER [$p < 0.001$, $F_{(6,120)} = 15.89$, $\eta_p^2 = 0.44$, SP = 1.00] (**Figure 3B**) and RPE [$p < 0.001$, $F_{(6,120)} = 32.88$, $\eta_p^2 = 0.62$, SP = 1.00].

VO_2sc, Energy Expenditure, Gross Efficiency and Economy During Constant-Load Tests

In VO_2sc at each checkpoint, a significant exercise mode × time interaction effect was observed [$p = 0.027$, $F_{(6,114)} = 2.48$, $\eta_p^2 = 0.11$, SP = 0.82], along with a significant time effect [$p < 0.001$, $F_{(6,114)} = 10.61$, $\eta_p^2 = 0.35$, SP = 1.00]. Bonferroni adjustment of multiple comparisons confirmed a greater VO_2sc

TABLE 2 | Differences in VO_2, heart rate, RPE, metabolic and economy/efficiency responses between half-squat vs. cycle-ergometer during constant-load test at LT1 intensity.

	HS (95% CI)	CYC (95% CI)	P^1 ES/SP	P^2 ES/SP	P^3 ES/SP
VO_2 ($L.min^{-1}$)	1.60	2.26	**<0.001**	**<0.001**	**0.001**
	(1.51–1.68)	(2.06–2.46)	0.56/1.00	0.64/1.00	0.17/0.97
HR ($beats.min^{-1}$)	125.91	143.58	**<0.001**	**<0.001**	**<0.001**
	(119.40–132.41)	(134.85–152.31)	0.63/1.00	0.60/0.99	0.29/1.00
RER	0.94	0.92	**<0.001**	0.224	0.879
	(0.92–0.95)	(0.90–0.94)	0.44/1.00	0.07/0.22	0.02/0.16
Lactate ($mmol.L^{-1}$)	3.04	2.92	0.670	0.581	0.168
	(2.69–3.39)	(2.35–3.48)	0.03/0.26	0.02/0.08	0.07/0.58
VO_2sc ($L.min^{-1}$) (at each checkpoint)	0.09	0.05	0.10	**<0.001**	**0.03**
	(0.05–0.12)	(0.03–0.08)	0.13/0.37	0.35/1.00	0.11/0.82
EE ($Kcal.min^{-1}$)	7.93	11.19	**<0.001**	**<0.001**	**0.001**
	(7.48–8.37)	(10.19–12.19)	0.58/1.00	0.63/1.00	0.17/0.97
GE (%)	43.49	17.66	**<0.001**	**<0.001**	**<0.005**
	(37.65–49.33)	(15.62–19.69)	0.53/1.00	0.75/1.00	0.14/0.92
EC ($W.L^{-1}.min^{-1}$)	150.78	60.91	**<0.001**	**<0.001**	**<0.013**
	(130.46–171.11)	(69.07–86.73)	0.49/1.00	0.76/1.00	0.13/0.88
RPE	9.93	10.49	**<0.001**	0.141	0.92
	(9.11–10.76)	(9.65–11.33)	0.622/1.00	0.11/0.31	0.17/0.14

CYC, cycle-ergometer; EC, economy; EE, energy expended; ES, effect size; GE, gross efficiency; HR, heart rate; HS, half-squat; L, liter; LT1, lactate threshold one; min, minute; RER, respiratory exchange ratio; RPE, rating of perceived exertion; SP, statistical power; VO_2, oxygen uptake; VO_2sc, slow component of oxygen uptake; W, watt. P^1 Significant differences for time effect. P^2 Significant differences for exercise mode effect. P^3 Significant differences for exercise mode × time interaction effect. Data are provided as mean and 95% confidence intervals (95% CI).

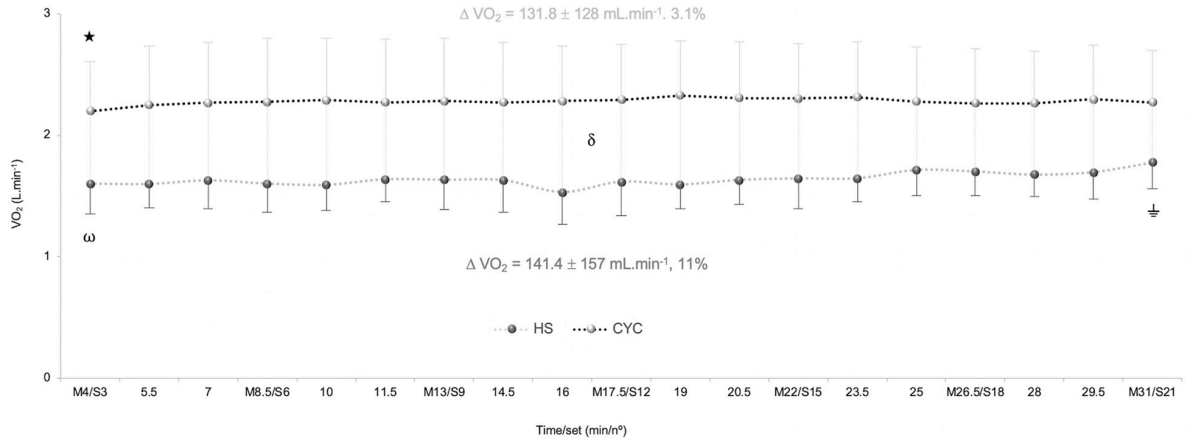

FIGURE 1 | Differences in slow component of oxygen uptake (VO_2sc) between half-squat (HS) exercise and cycle ergometer (CYC) during constant-load test. ω Significantly different from S6 ($p = 0.027$), S18 ($p = 0.001$), and S21 ($p = 0.001$). ⏚ Significantly different from S3 ($p = 0.001$), S6 ($p = 0.043$), and S12 ($p = 0.003$). ★ Significantly different from M8.5, M13, M17.5, M22, M26.5, and M31 ($p < 0.001$). δ Significant differences between cycle ergometer and HS exercise at each checkpoint ($p < 0.001$).

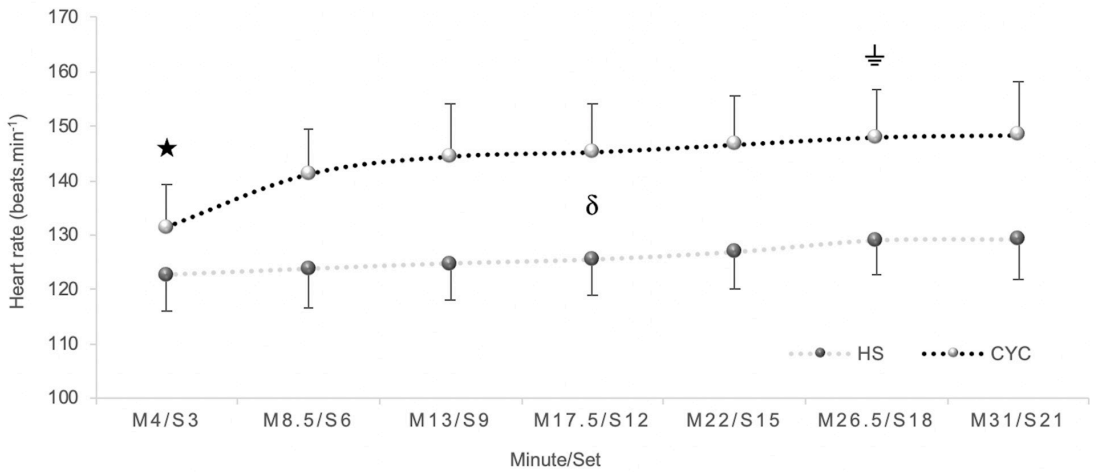

FIGURE 2 | Differences in heart rate between half-squat (HS) exercise and cycle ergometer (CYC) during constant-load test. ★ Significantly different from M8.5, M13, M17.5, M22, M26.5, and M31 ($p < 0.001$). ⏚ Significantly different from M3, M8.5, M17.5, and M22 ($p = 0.05$). δ Significant differences between cycle ergometer and HS exercise at each checkpoint ($p < 0.01$).

in HS than in cycle ergometer testing at the end of exercise (M22/S15, M31/S21) ($p < 0.05$) (**Figure 4A**).

In EE, a significant exercise mode × time interaction effect was discovered [$p = 0.001$, $F_{(6,120)} = 3.96$, $\eta_p^2 = 0.17$, SP = 0.97]. A significant time effect [$p < 0.001$, $F_{(6,120)} = 27.10$, $\eta_p^2 = 0.58$, SP = 1.00] and exercise mode effect were identified [$p < 0.001$, $F_{(6,120)} = 34.25$, $\eta_p^2 = 0.63$, SP = 1.00]. Bonferroni *post hoc* analysis confirmed a higher EE in cycle ergometer than HS exercise at each checkpoint ($p < 0.001$). A slight and continued EE increase was detected from S3 in HS exercise ($p < 0.05$). A stable EE was observed from M4 in cycle ergometer ($p > 0.05$) (**Figure 4B**).

In GE, a significant exercise mode x time interaction effect was discovered [$p = 0.005$, $F_{(6,120)} = 3.31$, $\eta_p^2 = 0.14$, SP = 0.92]. In addition, a significant exercise mode and time effect was found [$p < 0.001$, $F_{(1,20)} = 61.41$, $\eta_p^2 = 0.75$, SP = 1.00; $p < 0.001$, $F_{(6,120)} = 22.65$, $\eta_p^2 = 0.53$, SP = 1.00, respectively]. After Bonferroni multiple comparisons, a higher GE was perceived in HS than in cycle ergometer exercise ($p < 0.001$). There were significant differences between M4/S3 vs. all checkpoints in both exercises ($p < 0.05$). However, a non-significant but sustained decrease was produced from M4/S3 in both exercise modalities (∼13%) during constant-load tests ($p > 0.05$) (**Figure 5A**).

In economy, a significant exercise mode x time interaction effect was found [$p = 0.013$, $F_{(6,120)} = 2.85$, $\eta_p^2 = 0.13$, SP = 0.88]. A significant exercise mode and time effect was found [$p < 0.001$, $F_{(1,20)} = 61.66$, $\eta_p^2 = 0.76$, SP = 1.00; $p < 0.001$, $F_{(6,120)} = 18.84$,

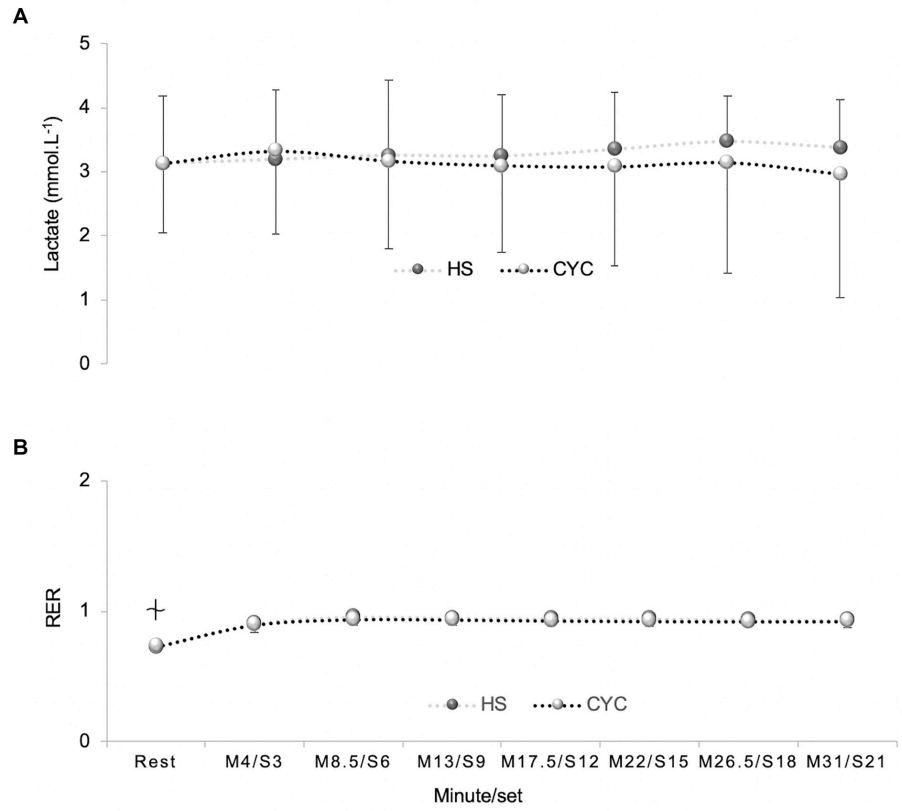

FIGURE 3 | Differences between half-squat (HS) exercise and cycle ergometer (CYC) during constant-load test in: **(A)** Blood lactate. **(B)** Respiratory exchange ratio (RER). No significant differences between cycle ergometer and HS exercises ($p > 0.05$). ┼ Significantly different from M8.5, M13, M17.5, M22, and M26.5 in cycle ergometer ($p < 0.05$) and significantly different from M8.5, M13, and M17.5 in HS exercise ($p < 0.01$).

$\eta_p^2 = 0.49$, SP = 1.00, respectively]. Bonferroni test determined a higher economy in HS than in cycle ergometer exercise ($p < 0.001$). There were significant differences between M4/S3 vs. all checkpoints in both exercises ($p < 0.05$). However, a non-significant but continued decrease was observed from M4/S3 in both exercise modalities ($p > 0.05$) (**Figure 5B**).

Lower Limbs Fatigue

In CMJ test, a significant exercise mode x time interaction effect was observed in jump height [$p = 0.004$, $F_{(1,20)} = 10.76$, $\eta_p^2 = 0.35$, SP = 0.88], mean power [$p = 0.003$, $F_{(1,20)} = 11.82$, $\eta_p^2 = 0.37$, SP = 0.91], and peak power [$p < 0.001$, $F_{(1,20)} = 23.61$, $\eta_p^2 = 0.54$, SP = 0.99]. In Bonferroni test, significant losses were produced between pre- and post-test in jump height ($p < 0.001$), mean power ($p = 0.001$), and peak power ($p < 0.010$) only in HS exercise. Peak power was increased after cycle ergometer test ($p < 0.05$) (**Figure 6**).

DISCUSSION

In support of our initial hypothesis, the main novel finding of this study was that the VO_2sc and EE increased slowly only in HS constant-load test at LT_1 intensity. As expected, during cycle-ergometer exercise at a constant work rate, a steady-state in pulmonary VO_2 and EE was reached. These outcomes could justify, at least in part, a decrease observed in jump performance (height and power) only after HS test. Contrary to our expectation, GE/economy in HS exercise did not reduce to a greater magnitude than in cycle ergometer test at the same LT_1 intensity. In addition, there was a higher response in VO_2 and heart rate during the constant-load test in cycle ergometer than in the HS exercise.

The results of VO_2sc obtained in HS exercise (absolute values 141.4 mL in 28 min, relative values 5.05 mL.min^{-1}) were slightly higher than in the cycling test (absolute values 131.8 mL in 28 min, relative values 4.7 mL.min^{-1}). HS results were reinforced by our previous investigations (Garnacho-Castaño et al., 2018a) that found similar VO_2sc values in HS exercise (absolute values 153.8 mL in 28 min, relative values 5.49 mL.min^{-1}), slightly higher in absolute values (130 mL in 17 min) and slower in relative values (7.6 mL.min^{-1}) than that obtained by professional cyclists at intensities clearly above the LT_1 (80% VO_{2max}) (Lucía et al., 2000). These results visibly differed from those reported in well-trained triathletes during constant work rate at 90% of VO_{2max} in cycling (absolute values 269 mL in 10 min 35 s, relative values ∼25 mL.min^{-1}) and running (absolute values 21 mL in 10 min 54 s, ∼2 mL.min^{-1}) (Billat et al., 1998). These variances

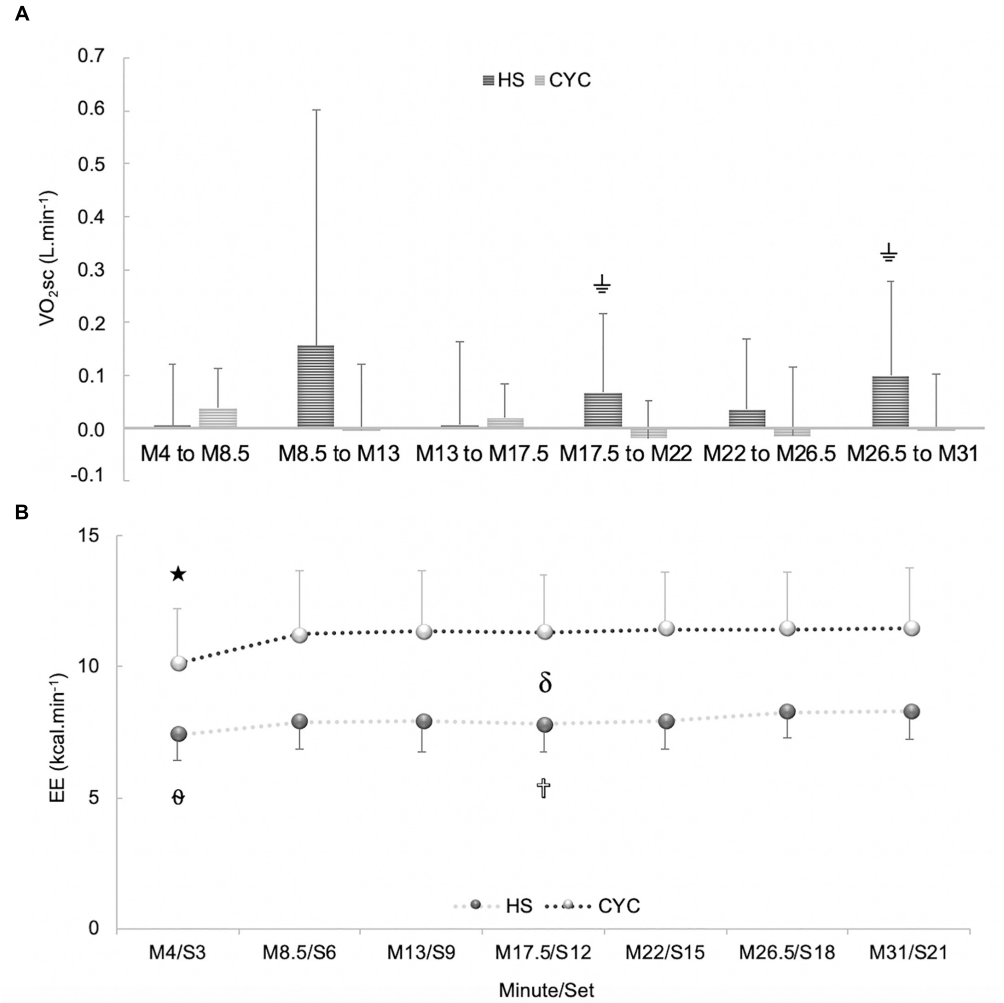

FIGURE 4 | Differences between half-squat (HS) exercise and cycle ergometer (CYC) during constant-load test at each checkpoint: **(A)** Slow component of oxygen uptake (VO_2sc). **(B)** Energy expenditure (EE). ★ Significantly different from M8.5, M13, M17.5, M22, M26.5, and M31 ($p < 0.001$). θ Significantly different from S6, S15, S18, and S21. ǂ Significantly different from S21. δ Significant differences between cycle ergometer and HS exercise at each checkpoint ($p < 0.001$). ⏚ Significantly different from cycle ergometer ($p < 0.05$).

of VO_2sc are not fully understood, though they could be related to the difference in the magnitude of VO_2sc between exercise modes and load intensity (Carter et al., 2000; Koppo et al., 2002), training status (Burnley and Jones, 2007), and prolonged constant-load tests (Hopker et al., 2017).

The physiological mechanisms that cause the increase of VO_2sc during constant-load HS test are uncertain because the power output or load equivalent to the LT_1 intensity means, in theory, the highest power output or load that will not elicit VO_2sc (Burnley and Jones, 2007). The VO_2 kinetics observed during constant-load cycling test justified a steady state in VO_2 and EE at the LT_1 intensity. In consequence, the blood lactate increased above the resting values, but did not accumulate over time as occurred during both constant-load tests (Garnacho-Castaño et al., 2015a). If VO_2 continued to increase, especially at the end of the constant-load HS test, it could be assumed that the VO_2sc is associated with fatigue and a decrease in muscular efficiency, so blood lactate should accumulate at a constant or increasing rate in response to the transition toward a predominantly anaerobic metabolism (O'Connell et al., 2017). The only hypothesis that was confirmed was an increase in VO_2sc and EE linked to lower limbs fatigue at the end of the HS test. Blood lactate was oxidized in a mainly aerobic metabolism and exercise intensity was considered as being at or below the anaerobic or LT (Svedahl and MacIntosh, 2003).

This detected response of VO_2sc in HS exercise usually occurs at intensities above the LT_1 in endurance exercises. Unlike the cycle ergometer test, performing 31 min (21 sets) of HS exercise at the LT_1 intensity would only be conceivable with a recovery time between each set. Although break durations of 30 s have indicated negligible effects on lactate kinetics during discontinuous protocols (Gullstrand et al., 1994), our HS protocol caused a relative lack of O_2 supply to muscle loci, further suggesting that an important percentage of the energy

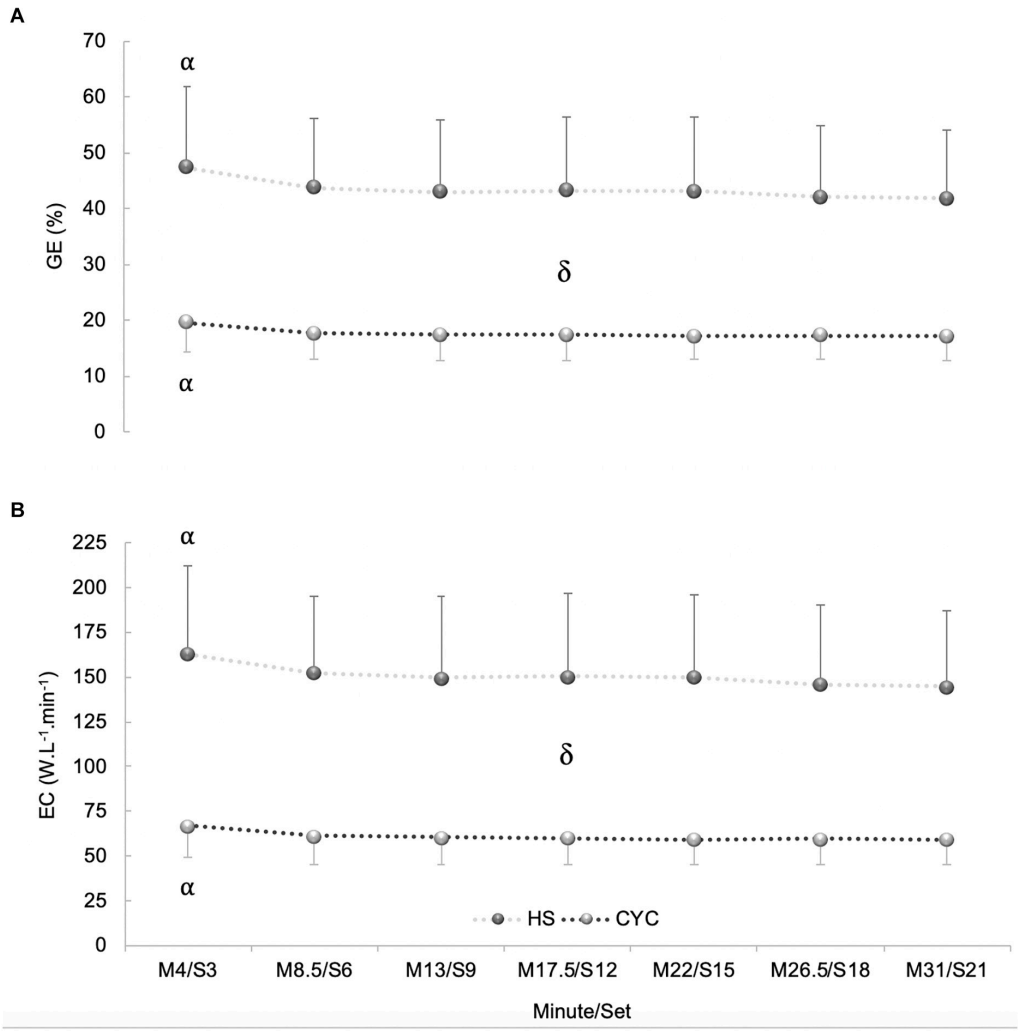

FIGURE 5 | Differences between half-squat (HS) exercise and cycle ergometer (CYC) during constant-load test at each checkpoint: **(A)** Gross efficiency (GE). **(B)** Economy (EC). αSignificantly different from M8.5/S6, M13/S9, M17.5/S12, M22/S15, M26.5/S18, and M31/S21 ($p < 0.05$). δSignificant differences between cycle ergometer and HS exercise at each checkpoint ($p < 0.001$).

derived from anaerobic metabolism might not be quantified by measuring metabolic gas exchange (Garnacho-Castaño et al., 2018a). The HS test probably stimulated a release of anaerobic sources to EE (Tesch et al., 1986) that make it impossible to use steady-state VO_2 to exactly estimate the EE (Scott et al., 2011).

Another feasible mechanism that would help to better understand the etiology of the VO_2sc in resistance exercises links the slight increase in pulmonary VO_2 with the VO_2 rise into the muscle. It has been suggested that increased leg VO_2 could explain for ∼85% of the rise in pulmonary VO_2 (Poole et al., 1991). Probably, VO_2sc discovered in HS exercise increased leg VO_2 within the active muscle to a greater magnitude than in the cycle ergometer test and, consequently, EE was augmented only during HS test. The VO_2sc and EE increase would presumably be associated with an increased ATP cost of force production and or increased O_2 cost of ATP resynthesis (Cannon et al., 2014; Korzeniewski and Zoladz, 2015). This energy mechanism would force a delayed recruitment of larger and less efficient motor units from the oxidative point of view to compensate the production of attenuated force in those already active motor units. So, a preferential glycogen depletion of the type I fibers (Vøllestad and Blom, 1985) and the recruitment of type II fibers (Whipp, 1994; Barstow et al., 1996) has been postulated as the most acceptable explanation for the VO_2sc (Gaesser and Poole, 1996).

Glycogen depletion patterns have been detected in type I/II fibers, confirming that both fast-twitch glycolytic muscle fibers and slow-twitch oxidative muscle fibers were activated during high intensity cycling exercise at 80% of VO_{2max}. When cycling exercise was performed at moderate intensity (50% of VO_{2max}), only type I fibers were recruited and no VO_2sc was observed (Krustrup et al., 2004). These findings suggest the recruitment of type I fibers by this mechanism probably occurred during constant-load cycling test. For this reason, VO_2 was not increased and lower limbs fatigue was not induced at the end of

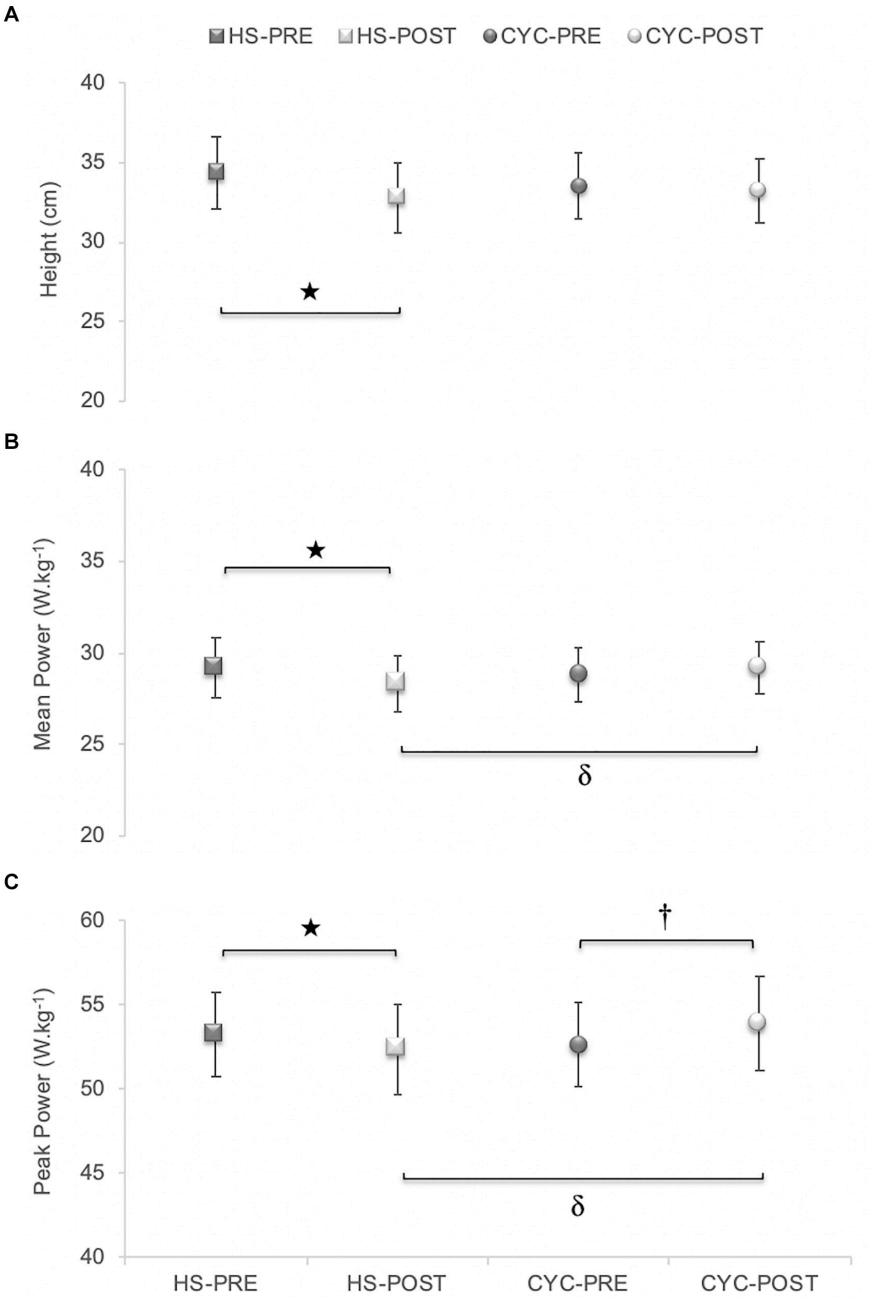

FIGURE 6 | Mechanical fatigue evaluated before and after constant-load exercises using a counter-movement jump test. ★Significant differences between pre- and post-test in HS exercise ($p < 0.01$). †Significant differences between pre- and post-test in cycling exercise ($p = 0.011$). δSignificant differences between cycling and HS tests ($p < 0.05$).

constant-power output cycling. The goal of this study was not to evaluate the gradual fibers-type recruitment associated with the energetic cost; therefore, our arguments are based on findings of others. Nevertheless, it can be assumed that VO_2sc and the corresponding increase in EE could be related to progressive fatigue in HS exercise (Garnacho-Castaño et al., 2018a).

In theory, the recruitment forced of type II fibers should induce higher blood lactate concentrations in HS exercise. We suspect that there was no increase in blood lactate levels because a recovery time of 1 min was established between sets. Beneke et al. (2003) demonstrated that repetitive interruptions (90 s after every 5th minute) during 30 min of constant-load testing decreased blood lactate concentrations to a greater extent than without interruptions. During the rest time between sets, the VO_2 of the whole body is still raised as a result of elevated post-exercise VO_2 while the glycolytic rate of the working muscle mass

is diminished. Therefore, the rate of lactate removal is directly linked to VO_2 under the saturation conditions of the substrate.

In addition, the RER was similar and remained stable throughout the constant-load tests despite the VO_2 was higher in the cycle ergometer than HS exercise. The RER during a constant-load test determines the percentage of carbohydrates and fats that are being used as an energy substrate. In endurance exercises have been observed that the fat oxidation is greater during running on treadmill than in the cycle ergometer at the same relative intensity (Achten et al., 2003; Cheneviere et al., 2010). This variance is partly originated by a greater degree of localized intramuscular tension during cycle exercise, which increases the recruitment of fast-twitch motor units (Carter et al., 2000) which mainly depend on carbohydrates as a fuel substrate. As the exercise intensity increases, the change in substrate metabolism toward greater carbohydrates dependence is related to a higher recruitment of the fast-twitch motor units (Coyle, 2000) and the appearance of free fatty acid entrapment (Romijn et al., 1993).

Maybe these physiological mechanisms occurred, at least in part, in the HS exercise. Probably, the HS exercise caused a higher intramuscular tension per muscular unit in the knee extensors than the cycle ergometer, intensified by the negative or eccentric work of the HS exercise. This mechanism might induce a gradual recruitment of less efficient type II muscle fibers as the initially recruited type I fibers become fatigued (Carter et al., 2000). In preliminary tests, we discovered that a recovery time between series equal to or less than 45 s produced an exponential increase in blood lactate levels and relevant muscular fatigue. The rest time of 1 min accumulated between sets throughout the constant-load HS test was a key factor to prevent a greater increase in the carbohydrates and replenish energy substrates and, therefore, for maintaining blood lactate levels in a stable aerobic metabolism.

Although the total time of the tests was the same in both exercises, the real time of execution was 10 min 30 s in HS exercise. The 20 min 30 s of recovery time during HS test could justify, at least in part, that the VO_2 and heart rate was lower in the HS test than cycle ergometer exercise. At the muscular level, probably, the HS exercise was more intense, producing a higher local muscular fatigue. Maybe for this reason, greater fatigue was found in lower limbs after the constant-load HS test. It could be deduced that the muscular fatigue produced in HS exercise stimulated the VO_2sc to a greater extent than the cycle ergometer having a higher cardioventilatory response. Despite these physiological mechanisms, the RPE was the same in both exercises.

In order to explain the VO_2sc phenomenon, GE was compared in both HS and cycling exercises. GE values in the cycle ergometer test were similar to that obtained by well-trained cyclists (∼18%) during long-term constant-load tests at moderate intensity. In HS exercise, we verify our previous findings with GE values of ∼44%. Values of ∼24–26% have been proposed in professional riders at the power outputs eliciting the LT and the respiratory compensation point during a ramp test (Lucía et al., 2002). Other studies have found lower GE values of 14–16% in world-class sprint cross-country skiers (Sandbakk et al., 2010). These values confirm the idea that GE is conditioned by the exercise modality.

According to results obtained in VO_2sc and EE during HS exercise, one could expect to discover a greater GE/economy loss throughout the constant-load HS test. Conversely, a 13% loss (non-significant) in GE was observed in both exercise modalities during constant-load tests. Previous studies have demonstrated that GE continues to diminish during prolonged constant-load tests in cycling (Hopker et al., 2017) and HS exercises (Garnacho-Castaño et al., 2018a) at moderate intensity. We suspect that the higher values and the non-loss of GE throughout the constant-load HS test in comparison with the cycle ergometer test were mainly due to the type of muscular action involved in both exercise modalities. HS execution is characterized by eccentric and concentric muscle actions; cycling prioritizes concentric muscle actions (Ericson et al., 1985). A greater increase in O_2 cost has been shown in no-rebound squats compared to eccentric-concentric squats, and rebound squats stimulate higher efficiency than only concentric squats (Villagra et al., 1993). Pre-stretch allows for storage of elastic energy in the elastic components (muscles and tendons), producing an extra energy that is released during the shortening cycle, probably decreasing O_2 cost. Furthermore, previous studies have demonstrated higher VO_2sc in cycling, compared to running (Carter et al., 2000). The authors speculated that the differences between the two exercise modalities were produced by the greater intramuscular tension induced during heavy cycling exercise and the higher eccentric muscle activity in running. This might cause a relatively lower recruitment of the less efficient type II muscle fibers in running (Carter et al., 2000). The pre-stretch could help to prevent a higher VO_2sc, decreasing O_2 cost and increasing efficiency in HS exercise to a greater extent than concentric pedaling, avoiding a higher recruitment of type II fibers. Furthermore, the eccentric phase has been demonstrated to be a key factor for improving concentric kinetic/kinematic performance during resistance exercises (Garnacho-Castaño et al., 2018b). Our results demonstrated higher power output levels and a lower VO_2 during constant-load HS exercise than in the cycling test. This increased power output contributed to improve GE in HS exercise. In consequence, variances in power output measures between a cycle ergometer and a linear position transducer should be considered.

We think that the muscle mass involved during exercise is another factor to consider. Several studies have shown a slower VO_2sc in running than in cycling (Billat et al., 1998; Carter et al., 2000), or a higher relative increase in VO_2 per unit of time during arm exercise than in a cycling test (Koppo et al., 2002) when a lower muscle mass was involved or when exercise was focused on a specific muscle group. Although the muscle groups involved in HS and cycling exercises are mainly the knee extensors, during HS exercise other muscle groups (i.e., CORE, back, etc.) are likely activated more than in the cycle ergometer exercise. The greater muscle mass involved may help to increase the whole-body efficiency, diminishing O_2 cost.

There are some limitations in this study which should be considered. Eccentric muscle action is linked to significantly higher muscle temperatures than concentric muscle action when both are performed at a comparable power output, rate of oxygen uptake or heat production (Nielsen et al., 1972;

Pahud et al., 1980). This fact may *per se* increase the metabolic rate without any other additional perturbations of the muscular milieu. This increased temperature during negative work in HS exercise could have altered the VO_2 kinetics by accelerating the rate-limiting metabolic reaction connected with oxidative phosphorylation and, moreover, accelerating a greater VO_2 delivery to the capillaries and mitochondria (Koga et al., 1997). It would have been interesting to evaluate how it affects the temperature and the positive (concentric) and negative (eccentric) work at the O_2 cost and consequently to the VO_2sc during constant-load tests.

In addition, the different methodology and protocols applied in both exercises during the incremental tests generates some controversy in the location of the LT_1. This factor could condition the cardioventilatory and metabolic responses during the constant-load tests at LT_1 intensity, producing a bias when comparing both exercises. However, the results reported during the incremental test (**Table 1**) revealed that the detection of the LT_1 in both exercises could occur in an equivalent metabolic instant and a similar exercise intensity. This idea is based on the fact that no significant differences were found in VO_2, heart rate, blood lactate concentrations and RPE between the HS and the cycle ergometer at the LT_1. Our findings are supported by the criteria established in a previous study (Binder et al., 2008). In both exercises, LT_1 occurred at a heart rate of ∼65–70% of the maximum heart rate, a rating of perceived exertion of ∼10 and a blood lactate concentrations of ∼2 $mmol.L^{-1}$, which is considered as a light intensity according to the criterion defined at the time of the LT_1.

Although it appears that the LT_1 occurred at a similar metabolic moment and intensity during both incremental tests, the cardioventilatory response during the constant-load test at LT_1 intensity was lower in HS exercise. The controversy is now focused on knowing whether both constant-load protocols occurred at the same relative intensity (% VO_{2max}). To solve this problem, both incremental protocols should have been carried out until exhaustion to determine the VO_{2max} and calculate the percentage of VO_2 in both constant-load tests. The response of blood lactate levels and RPE observed throughout the constant-load test determined, at least, a predominantly aerobic metabolic intensity.

Finally, several studies (Carter et al., 2002; Koppo et al., 2002) have compared the ventilatory responses and the VO_2sc between several exercises at the same relative intensity (at, above, below of LT_1). The behavior of VO_2 and VO_2sc is exercise- and intensity-dependent despite they are tested at the same relative or metabolic intensity. Resistance training is typically anaerobic in nature. We think that the most important contribution of this study is that resistance exercises might acquire aerobic metabolic properties selecting a suitable load and manipulating the recovery and execution time of the sets.

CONCLUSION

Although the VO_2 and heart rate responses were higher in cycling exercise, the HS constant-load test induced a greater VO_2sc and EE than the cycling test at the LT_1 intensity. GE could benefit from the eccentric phase of the HS exercise. Resistance training conducted at a load intensity equivalent to a predominantly aerobic metabolism could improve local muscular resistance and whole-body efficiency. Thus, relevant implications for both performance and health exercise programs could be considered. This would allow a faster recovery of the muscle groups from one session to another. In the fitness programs, this methodology would help complement the aerobic endurance training with resistance exercises that involve a greater muscle mass (CORE, upper limbs, stabilizers, etc.) and a higher mechanical efficiency in a metabolism that is primarily aerobic. Future research should focus on continuous protocols (without rest periods) as in endurance exercise, combining resistance exercises in the form of circuit training. This scientific knowledge could be an important advance in the assessment of resistance exercises for sports performance and health promotion.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

JM-M and MG-C conceived and designed the research. All authors performed the test protocols and edited, revised, and approved the final version of the article. MG-C and JM-M analyzed the data. LA-A, NS-P, MB, RF-R, LC, and EC contributed reagents, materials, and analysis tools. LA-A, JM-M, RF-R, and MG-C prepared the figures and drafted the article.

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Superior Effects of High-Intensity Interval vs. Moderate-Intensity Continuous Training on Endothelial Function and Cardiorespiratory Fitness in Patients With Type 1 Diabetes: A Randomized Controlled Trial

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This study aimed to compare the effect of high-intensity interval training (HIIT) with moderate-intensity continuous training (MCT) on endothelial function, oxidative stress and clinical fitness in patients with type 1 diabetes. Thirty-six type 1 diabetic patients (mean age 23.5 ± 6 years) were randomized into 3 groups: HIIT, MCT, and a non-exercising group (CON). Exercise was performed in a stationary cycle ergometers during 40 min, 3 times/week, for 8 weeks at 50–85% maximal heart rate (HR_{max}) in HIIT and 50% HR_{max} in MCT. Endothelial function was measured by flow-mediated dilation (FMD) [endothelium-dependent vasodilation (EDVD)], and smooth-muscle function by nitroglycerin-mediated dilation [endothelium-independent vasodilation (EIVD)]. Peak oxygen consumption (VO_{2peak}) and oxidative stress markers were determined before and after training. Endothelial dysfunction was defined as an increase < 8% in vascular diameter after cuff release. The trial is registered at ClinicalTrials.gov, identifier: NCT03451201. Twenty-seven patients completed the 8-week protocol, 9 in each group (3 random dropouts per group). Mean baseline EDVD was similar in all groups. After training, mean absolute EDVD response improved from baseline in HIIT: + 5.5 ± 5.4%, (P = 0.0059), but remained unchanged in MCT: 0.2 ± 4.1% (P = 0.8593) and in CON: -2.6 ± 6.4% (P = 0.2635). EDVD increase was greater in HIIT vs. MCT (P = 0.0074)

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and CON (P = 0.0042) (ANOVA with Bonferroni). Baseline VO_{2peak} was similar in all groups (P = 0.96). VO_{2peak} increased 17.6% from baseline after HIIT (P = 0.0001), but only 3% after MCT (P = 0.055); no change was detected in CON (P = 0.63). EIVD was unchanged in all groups (P = 0.18). Glycemic control was similar in all groups. In patients with type 1 diabetes without microvascular complications, 8-week HIIT produced greater improvement in endothelial function and physical fitness than MCT at a similar glycemic control.

Keywords: high-intensity interval training, endothelium, diabetes mellitus, type 1, flow-mediated dilation, microvascular complications

INTRODUCTION

Micro- and macrovascular complications are the main causes of morbidity and mortality in patients with type 1 diabetes (Nathan et al., 1993; American Diabetes Association, 1999). Endothelial dysfunction is supposed to precede atherosclerosis and microvascular disease (Bertoluci et al., 2015). The natural course of endothelial dysfunction in type 1 diabetes is unknown, but is related to chronic hyperglycemia, oxidative stress and subclinical endothelial inflammation, leading to accelerated development of atherosclerosis (Kannel and McGee, 1979; Stehouwer et al., 2002; Schram et al., 2003). We previously demonstrated that long-term poor glycemic control is associated with endothelial dysfunction development in recently diagnosed adolescents with type 1 diabetes (Ce et al., 2011). When poor glycemic control occurs in the first few years after type 1 diabetes onset, there is a greater impact of endothelial dysfunction, indicating an effect of metabolic memory (Ce et al., 2011).

Exercise training is known to improve endothelial dysfunction. In children and adolescents with type 1 diabetes, 30 min of aerobic training for 18 weeks significantly increased flow-mediated dilation (FMD) (Seeger et al., 2011). In adults with type 1 diabetes, moderate-intensity continuous training (MCT) significantly increased FMD, after 2 months of training (Fuchsjäger-Mayrl et al., 2002). In a cross-sectional study, children and adolescents with type 1 diabetes performing more than 60 min of daily moderate to vigorous exercise had greater FMD than sedentary patients (Trigona et al., 2010). Improvements in endothelium-dependent vasodilator response is also seen in type 2 diabetes without coronary artery disease, when patients are subjected to combined aerobic and resistance training (Maiorana et al., 2001).

Intensity changes during exercise seems to be an important determinant of effects on endothelial function. Studies in different populations, including type 2 diabetes, arterial hypertension, heart failure, obesity, and metabolic syndrome have demonstrated that high-intensity interval training (HIIT) (i.e., high-intensity efforts interspersed with recovery period at lower intensity) can increase endothelium-dependent dilation more effectively than traditional MCT (Wisløff et al., 2007; Schjerve et al., 2008; Tjønna et al., 2008; Molmen-Hansen et al., 2012; Mitranun et al., 2014). In addition, HIIT is associated with greater improvement in physical fitness performance ($VO_{2\,max}$) than MCT in short-term studies. A meta-analysis involving 10 studies demonstrated that HIIT exercise provided a better physical conditioning compared to MCT in subjects with established cardiovascular disease, metabolic syndrome and obesity (Weston et al., 2014). Another recent meta-analysis found that HIIT was better than MCT in increasing $VO_{2\,max}$ in type 2 diabetes (De Nardi et al., 2018).

So far, HIIT has not been tested against MCT in patients with type 1 diabetes. Our hypothesis was that if the patient is exposed to a greater exercise intensity such as in HIIT, FMD and cardiorespiratory fitness will increase more than in MCT. Therefore, the main objective of this randomized controlled trial was to compare the effects of 8-week HIIT and MCT on endothelial function, assessed by FMD, and cardiorespiratory fitness in patients with type 1 diabetes.

MATERIALS AND METHODS

Design

A randomized, parallel-group clinical trial with 3 arms and a 1:1:1 allocation ratio. We decided to include a non-exercising group in order to control the influence of blood glucose changes in FMD. The eligibility criteria is shown in **Figure 1**. No changes were made to the methods after trial commencement.

Eligibility Criteria

We searched for patients with type 1 diabetes above 18 years of age attending at the Institute for Children with Diabetes (ICD), who were included in ICD database from January 1, 2015, to January 1, 2016.

We recruited subjects of both genders, regularly attending clinic visits, who were physically inactive or not involved in exercise training programs in the previous 6 months and were interested in starting an exercise training program. We excluded smokers, pregnant women, patients with known co-morbidities not related to diabetes, patients taking drugs other than insulin and those who presented with severe diabetes-related complications, such as: loss of renal function (serum creatinine above 132.60 μmol/L), moderate to severe retinopathy or blindness, suspected or confirmed coronary artery disease, severe peripheral neuropathy, foot ulcers, or history of foot ulcers and any suspected or confirmed clinical autonomic neuropathy. Patients who met the eligibility criteria were invited to visit the research center.

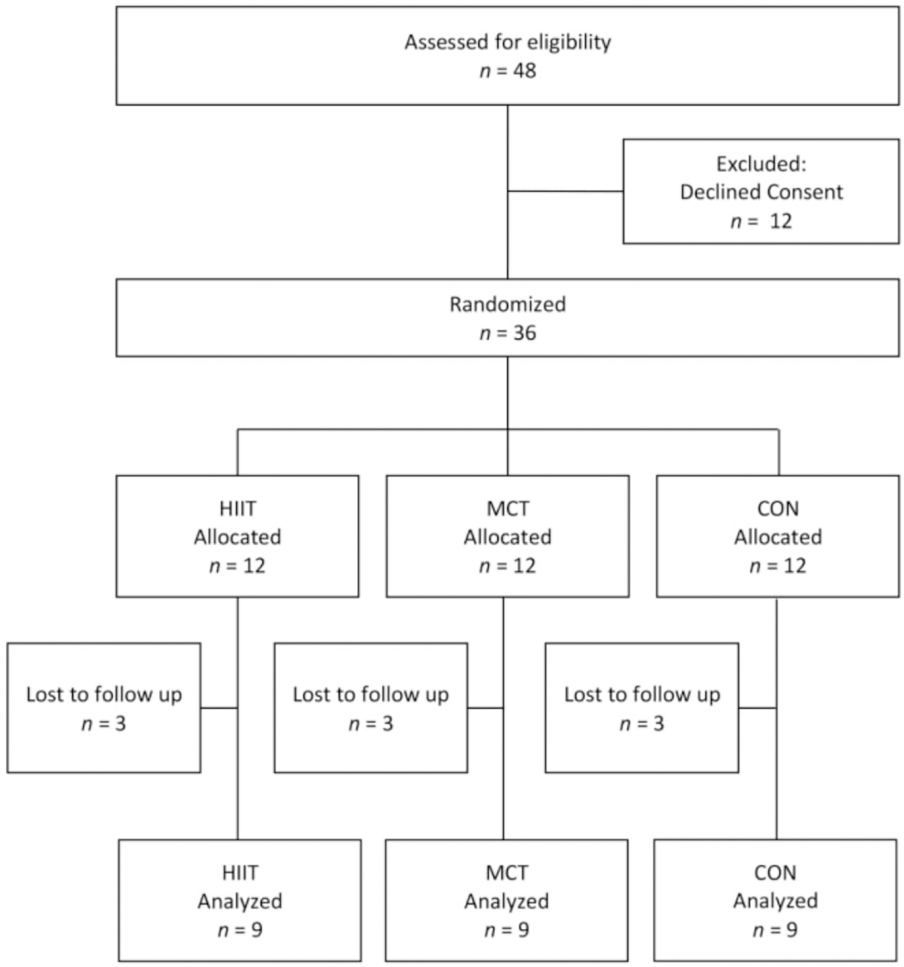

FIGURE 1 | Flow diagram of inclusion of patients in the study.

Intervention

The intervention group was submitted to the HIIT protocol. We included a moderate continuous exercise group and a non-exercising control group (CON). Training sessions were performed in the exercise training center under supervision of part of the team in the afternoon period.

As a general recommendation patients in HIIT and MCT groups exercised on cycle ergometer 3 times a week for 8 weeks. Heart rate was monitored during the whole exercise sessions using heart rate monitors (Polar® FT4, Polar Electro Oy, Kempele, Finland). All exercise sessions were supervised and adherence was monitored by group. Only individuals with more than 70% of compliance were analyzed. Capillary blood glucose was measured every 5 min during all exercise training sessions and oral 10 g glucose gels were given whenever blood glucose was $\leq$ 5.55 mmol/L and a 20% decrease in insulin basal dose was recommended to all patients in the morning of every training day to minimize the risk of hypoglycemia. In addition, patients were recommended not to exercise at the peak of insulin action.

High-Intensity Interval Training (HIIT) Protocol

High-intensity interval training protocol was divided into 3 phases according to Mitranun et al. (2014) phase 1: weeks 1–2, phase 2: weeks 3–4, phase 3: weeks 5–8. In phase 1, participants warmed up for 5 min, increasing intensity gradually to reach 50% of maximal heart rate. It was maintained for 20 min and then followed by a recovery period of 5 min. In phase 2, there was a 5-min warm up to reach 50% maximum heart rate (HR_{max}), and it was followed by 1-minute sprint at 80% HR_{max}, then slowing down intervals at 50% HR_{max} for 5 min. This procedure was repeated three more times and then followed by a recovery phase of 5 min.

In phase 3, the protocol was longer and more intense. After a 5-minute warm-up to 60% HR_{max}, patients performed six 1-minute sprints at 85% HR_{max}, followed by 4-minute slow-down intervals at 50%. The whole session lasted 40 min. At the end of each 1 min sprint, exercise intensity was also assessed through Borg RPE scale (rate of perceived exertion), which was independent from the heart rate. The participants of HIIT were

rated as 7–8 as they informed a really hard activity, as they could speak short sentences, but could not hold a conversation.

Moderate-Intensity Continuous Training (MCT) Protocol

Moderate-intensity continuous training protocol was also divided into 3 phases as described previously (12). In phase 1, training was identical as in HIIT. In phase 2, participants exercised to attain 50% HR_{max} in 5 min and then increased intensity to 60% HR_{max} for 20 min, ending with 5 min to recover, totaling 30 min. In phase 3, participants attained 50% HR_{max} in 5 min and exercised constantly at 65% HR_{max} for 30 min, recovering in 5 min and totaling 40 min.

Non-exercising Control Group (CON)

Control patients were only asked to follow general lifestyle recommendations, including to walk at least three times a week for a minimum of 30 min. This group was not supervised. No other exercise specifications were given.

Primary Outcome

Flow-Mediated Dilation (FMD)

We pre-specified the difference between post- and pre-training percentage FMD as the primary outcome. FMD was determined as follows. Within 2 weeks of the first visit, patients were assessed for pre-training endothelial function through brachial artery ultrasound in the left arm. The examination was performed by an experienced member, blinded to the results of the study, using the technique according to Corretti et al. (2002), which was previously described by our group (Ce et al., 2011). Tests were performed in the non-invasive cardiovascular methods unit of Hospital de Clínicas de Porto Alegre (HCPA). Briefly, patients were studied in the morning, after the usual dose of basal insulin and a 200-kcal low-fat standard meal for breakfast. Arterial blood pressure was measured by the auscultatory technique, using an aneroid sphygmomanometer, at room temperature (22–24°C). All measurements were performed using-high resolution ultrasound equipment (EnVisor CHD, Philips, Bothell, WA, United States) with a high-frequency transducer (3–12 MHz, L12-3 Philips) to obtain longitudinal images of the brachial artery. Post-training FMD evaluation and $VO_{2\,max}$ determination were assessed in a maximum of a week after the last training period.

The ultrasound images were obtained with two-dimensional mode, color and spectral Doppler. The simultaneous electrocardiography (ECG) was recorded. To minimize operational errors, both transducer and arm positions were maintained throughout the procedure. Images were recorded with the patients at rest for 30 min. Endothelium-dependent vasodilation (EDVD) and endothelium-independent vasodilation (EIVD) were determined, respectively, by FMD and nitroglycerin vasodilation. Measurements were done at multiple vascular sites using the measurement system of the same equipment. Arterial diameter measurements were done off-line, at the end of diastole, at the peak of the *R* wave in the ECG.

After recording of baseline images, the brachial artery was occluded for 5 min with a pressure cuff positioned on the arm and inflated to 50 mmHg above systolic blood pressure for 4 min. The EDVD response was recorded between 45 and 60 s after cuff release. After 10 min resting, baseline images were repeated and then 0.4 mg of sublingual nitroglycerin spray (Natispray Trinitrine, Procter & Gamble Pharmaceuticals, Paris-Cochin, France) was used to evaluate EIVD 4 min after the spray. EDVD and EIVD were expressed as percent change in brachial artery diameter before and after cuff release or nitroglycerin administration, respectively. Endothelial dysfunction was considered when EDVD was less than 8% in relation to baseline (Gaenzer et al., 2001; Gokce et al., 2003). Smooth muscle dysfunction was considered by the same criteria after nitrate use for EIVD (Gaenzer et al., 2001; Gokce et al., 2003).

Secondary Outcome

Maximal Oxygen Consumption (VO_{2peak})

We pre-specified the difference between post- and pre-training VO_{2peak} values during a maximum load test. Briefly, all patients were submitted to cardiorespiratory fitness assessment one day before starting the training protocols, which was repeated within 48 h after the last training session was completed. An incremental maximal cycle ergometer (Cybex, Medway, United States) test was conducted to determine peak oxygen uptake (baseline VO_{2peak}), using the breath-by-breath method in an open circuit spirometry (Quark CPET, Cosmed, Rome, Italy). After a 3-minute warm-up period, cycling at 50 W, the workload was increased by 25 W every minute until fatigue (Moser et al., 2015). VO_{2peak} was defined as the highest mean value achieved within the last 15 s prior to exhaustion.

Sample Size Calculation

The sample size, using FMD as the primary outcome, was calculated according to the study of Mitranun et al. (2014) in type 2 diabetes, in which the effect size between post- and pre-training values of FMD was 0.49. Considering a standard deviation of 3.37%, $\alpha = 0.05$ and $\beta = 0.8$, the minimal number of patients in each group was 12.

Randomization

The randomization process was in blocks, according to FMD results before training. Baseline FMD results were ranked in decreasing order in blocks of three, so that the first three patients with corresponding higher FMD results formed the first block, in a 1-2-3 sequence, respectively, Group 1 = HIIT, Group 2 = MCT, Group 3 = CON. The following block followed an inverse sequence (3-2-1) and then consecutively. This process was performed by a collaborator outside of the study and was concealed by using numbered sealed envelopes. This process ensured that baseline FMD was similar between the three groups before training intervention.

For technical reasons, the intervention was not double-blinded, since sedentary control patients knew that they would not exercise. However, the investigator responsible for FMD determinations was blinded for the rest of the study. All further

evaluations were performed before and after the exercise training interventions by the same investigators.

Biochemical Assays

Blood and urine samples were collected after 12 h fasting. Patients were asked to avoid exercise in the 48 h before blood and urine collection. Blood samples were routinely centrifuged for 15 min and serum and plasma were stored at −80°C. HbA1c was determined by immunoturbidimetry (Certified Self-Analysis of the National Glycohemoglobin Standardization Program-Cobas Integra 400, Roche, Basel, Switzerland). Plasma glucose was evaluated by the glucose-peroxidase method using enzymatic colorimetric reactions. Serum total cholesterol, high-density lipoprotein cholesterol (HDL-c) and triglyceride concentrations were also measured by the colorimetric enzyme method (Modular, Roche, Mannheim, Germany). LDLc was estimated by the Friedewald equation. Creatinine was measured by the method of Jaffe (Modular; Roche) and high-sensitivity C-reactive protein (hs-CRP) by nephelometry (BN II; Dade-Behring, Deerfield, IL, United States). Albuminuria was determined in a single urine sample obtained in morning using the immunoturbidimetric method: Url-Pack Bayer® MAlb Kit, Cobas Mira® Roche (AlbUCobas) (Sacks et al., 2011).

Oxidative Stress Parameters

Total thiol group concentrations (T-SH) were assessed by reaction with [5,5′-dithiobis (2-nitrobenzoic acid); DTNB] (Ellman, 1959), and reading at 412 nm. Levels of plasma thiobarbituric acid-reactive substances (TBARS) were evaluated as previously described (Ohkawa et al., 1979), determined spectrophotometrically at 532 nm.

TABLE 1 | Baseline characteristics of patients.

	HIIT (n = 9)	MCT (n = 9)	CON (n = 9)
Age (year)	26.1 ± 7.8	23.7 ± 5.8	20.8 ± 2.6
Male/Female	3/6	5/4	4/5
Duration of type 1 diabetes (years)	9.1 ± 2.9	10.4 ± 2.8	9.7 ± 2.7
Total daily insulin dose (UI/kg)	0.48 ± 0.09	0.56 ± 0.22	0.47 ± 0.11
BMI (kg/m^2)	23.2 ± 2.4	24.1 ± 2.0	22.7 ± 2.6
Systolic BP (mmHg)	108.3 ± 7.9^{ab}	120.5 ± 8.8	116.5 ± 7.5
Diastolic BP (mmHg)	71.1 ± 8.2^{c}	78.8 ± 7.8	79.6 ± 6.5
Fasting plasma glucose (mmol/L)	11.49 ± 4.05	8.66 ± 2.94	11.32 ± 6.16
HbA1c (%)	8.2 ± 1.3	8.4 ± 0.9	8.8 ± 2.3
Total cholesterol (mmol/L)	4.77 ± 0.77	4.57 ± 0.84	5.33 ± 1.73
LDL cholesterol (mmol/L)	2.87 ± 0.70	2.31 ± 0.57	3.16 ± 1.37
HDL-c (mmol/L)	1.53 ± 0.31	1.47 ± 0.63	1.57 ± 0.38
Triglycerides (mmol/L)	0.78 ± 0.34	1.70 ± 0.21	1.29 ± 0.87
Serum creatinine (μmol/L)	51.85 ± 9.91	59.48 ± 9.15	50.33 ± 11.44
Mean UAC (mg/L)	12.6 (3.0–41.0)	30.4 (3.3–184)	30.5 (3.0–142)
Microalbuminuria (%)	1/9 (11.1)	2/9 (22.2)	2/9 (22.2)
Endothelial dysfunction (%)	5/9 (55.5)	7/9 (77.7)	6/9 (66.6)

BMI, body mass index; BP, blood pressure; HbAlc, glycated hemoglobin; HDL-c, high-density lipoprotein cholesterol; UAC, urinary albumin concentration. Microalbuminuria was defined as UAC > 30 mg/g. Data are expressed as mean ± standard deviation, except for UAC.

Statistics

Data distribution was evaluated by the Shapiro-Wilk test. ANOVA with Bonferroni/Dunn post-test was used to study FMD, NTG, and VO_{2peak}. The differences between pre and post values for EDVD, EIVD, and VO_{2peak} were referred to as DELTA. ANOVA with Bonferroni was used to make comparisons of DELTA between groups. The chi-square test was used for qualitative variables. Pearson's correlation coefficient was used to study the association between VO_{2peak} and FMD. Statistical tests were performed with the standard software package Statistical Analysis System (SAS) version QC (GraphPad, United States) and StatView (Abacus, United States).

Ethics Statement

This clinical trial was registered at ClinicalTrials.gov Identifier: NCT03451201. This study protocol was approved by the HCPA ethics board, and the reported investigations were carried out in accordance with the principles of the Declaration of Helsinki. All participants provided oral and written consent prior to inclusion in the study. Those who agreed to participate were registered for further evaluation at HCPA and School of Physical Education, Physiotherapy and Dance (ESEFID).

RESULTS

The study randomized 36 patients with type 1 diabetes. Before the beginning of training, 9 individuals dropped out, 6 due to health problems not related to the study and 3 canceled consent for personal reasons. At the end of the study, 27 patients completed the study, 9 in each group (**Figure 1**). Only completers were analyzed. There were 3 random dropouts in each group that occurred immediately before the beginning of training period. All patients were analyzed in their original randomized groups. No interim analysis was performed. Patients were recruited from January 2015 to January 2016. The last follow-up visit was in May 2016. The trial ended due to the end of the protocol.

Baseline clinical and biochemical characteristics of patients are shown in **Table 1**. At baseline, the HIIT group showed slightly lower systolic and diastolic blood pressure values than the other groups. All other variables were similar between groups.

Changes in metabolic, oxidative stress, endothelial function, and cardiovascular parameters between groups before and after training are shown in **Tables 2**, **3**. Lipid profile, urinary albumin excretion, hs-CRP and oxidative stress measures did not differ between groups before and after training.

At baseline, the percentage of patients with endothelial dysfunction (% with ED) was similar in all groups (P = 0.60), as well as the baseline mean EDVD (**Table 3**). After training, % with ED was significantly lower in HIIT (22.2%) vs. MCT (88.8%) (P = 0.044) and vs. CON 88.8% (P = 0.0184) (**Table 3**). After training, EDVD increased from baseline in HIIT (P = 0.0059) and was significantly greater in relation to MCT (P = 0.0074)

TABLE 2 | Metabolic parameters and oxidative stress before (PRE) and after (POST) training and POST-PRE difference (Δ) in each group.

	HIIT (n = 9)			MCT (n = 9)			CON (n = 9)		
Variables	**PRE**	**POST**	**Δ**	**PRE**	**POST**	**Δ**	**PRE**	**POST**	**Δ**
Metabolic									
Weight (kg)	64.1 ± 7.3	61.7 ± 9.3	−2.36 ± 4.6	71.4 ± 11.6	70.7 ± 10.9	−0.7 ± 2.0	65.6 ± 9.5	63.3 ± 6.5	0.03 ± 0.9
FBG (mmol/L)	11.53 ± 4.0	11.44 ± 6.12	−0.08 ± 5.7	8.67 ± 2.96	9.70 ± 3.15	1.02 ± 4.17	11.34 ± 6.20	11.73 ± 6.95	−0.06 ± 9.20
HbA1c (%)	8.2 ± 1.3	8.0 ± 1.0	−0.2 ± 0.6	8.4 ± 0.9	8.1 ± 0.9	−0.3 ± 0.3	8.8 ± 2.3	9.2 ± 2.4	0.4 ± 0.8
TC (mmol/L)	4.77 ± 0.77	4.69 ± 0.93	−0.08 ± 0.57	4.56 ± 0.85	4.04 ± 1.37	−0.01 ± 1.45	5.34 ± 1.74	5.54 ± 2.05	0.21 ± 0.98
LDL-c (mmol/L)	2.87 ± 0.70	2.80 ± 0.80	0.09 ± 0.56	2.31 ± 0.57	1.81 ± 1.24	−0.51 ± 1.32	3.16 ± 1.37	3.16 ± 1.40	0.00 ± 0.75
HDL-c (mmol/L)	1.53 ± 0.31	1.54 ± 0.42	0.01 ± 0.18	1.47 ± 0.63	1.37 ± 0.51	−0.08 ± 0.20	1.57 ± 0.38	1.62 ± 0.55	0.05 ± 0.27
TG (mmol/L)	0.78 ± 0.34	0.80 ± 0.28	0.02 ± 0.25	1.70 ± 0.21	1.86 ± 0.16	0.16 ± 0.92	1.29 ± 0.87	1.64 ± 1.53	0.32 ± 0.71
hs-CRP (nmol/L)	18.1 ± 20.9	19.0 ± 15.2	0.9 ± 9.5	31.4 ± 23.8	37.1 ± 31.4	5.7 ± 24.7	47.6 ± 43.8	102.8 ± 147.6	55.2 ± 153.3
Oxidative Stress									
TBARS (μM MDA/L)	2.00 ± 1.41	2.31 ± 1.60	0.31 ± 0.45	2.18 ± 0.59	2.68 ± 1.54	0.49 ± 1.72	2.40 ± 0.76	2.35 ± 1.67	−0.04 ± 2.63
T-SH (nmol/mg GSH)	79.5 ± 15.2	88.3 ± 12.1	8.77 ± 17.2	95.0 ± 28.3	96.2 ± 14.0	1.2 ± 25.01	95.3 ± 29.7	91.3 ± 21.7	−4.0 ± 32.5

CON, non-exercising controls; FBG, fasting blood glucose; HbAlc, Hemoglobin Alc; HDL-c, high-density lipoprotein cholesterol; HIIT, high intensity interval training; hs-CRP, high sensitivity C-reactive protein; LDL-c, low density lipoprotein cholesterol; MCT, moderate continuous training; TBARS, plasma thiobarbituric acid-reactive substances; TC, total cholesterol; TG, triglycerides; T-SH, total thiol group concentrations; $VC_{2\,max}$, maximal oxygen consumption during exercise.

TABLE 3 | Endothelial function and cardiovascular parameters before (PRE) and after (POST) training and POST-PRE difference (Δ) in each group.

	HIIT (n = 9)			MCT (n = 9)			CON (n = 9)		
Variables	**PRE**	**POST**	**Δ**	**PRE**	**POST**	**Δ**	**PRE**	**POST**	**Δ**
Endothelial Dysfunction									
Mean FMD (%)	5.7 ± 5.0	11.2 ± 5.4^{abc}	5.5 ± 4.4^{de}	5.2 ± 3.3	5.4 ± 3.3	0.24 ± 4.0	7.6 ± 7.4	5.0 ± 3.3	−2.6 ± 6.4
% with ED	5/9 (55.5)	2/9 (22.2)^{ac}	–	7/9 (77.7)	8/9 (88.8)	–	6/9 (66.6)	8/9 (88.8)	–
Mean NTG (%)	24.1 ± 7.3	22.5 ± 5.3	−1.5 ± 5.4	18.0 ± 4.2	16.3 ± 4.7	−1.7 ± 3.9	26.3 ± 6.7	18.6 ± 7.7	−4.3 ± 6.2
% with SMD	0	0	–	0	0	–	0	0	–
Cardiovascular									
Systolic BP (mmHg)	108.3 ± 7.9	116.1 ± 9.2	7.7 ± 9.3^{d}	120.5 ± 8.8	118.2 ± 7.8	−1.6 ± 8.6	116.5 ± 7.5	120.5 ± 7.2	4.0 ± 5.5
Diastolic BP (mmHg)	71.1 ± 8.2	78.8 ± 8.9	7.7 ± 9.0	78.8 ± 7.8	81.6 ± 8.2	2.7 ± 8.7	79.6 ± 6.5	80.6 ± 7.6	1.0 ± 7.4
Resting HR (bpm)	76.5 ± 11.7	74.4 ± 8.7	−2.1 ± 12.5	73.2 ± 4.7	76.0 ± 8.4	2.1 ± 8.3	77.7 ± 10.5	84.1 ± 7.5	6.3 ± 8.5
Max HR_{peak} (bpm)	180.4 ± 14	189.0 ± 16	8.51 ± 13.9^{c}	179.2 ± 16	178.3 ± 14.9	−0.88 ± 5.32	183.2 ± 15	184.3 ± 16	0.55 ± 4.12
VO_{2peak} (ml/kg/min)	34 ± 6.3	40.1 ± 4.3	6.08 ± 2.58^{db}	33 ± 8.2	36 ± 8.8	3.04 ± 4.03^{c}	33.2 ± 10	32.7 ± 10	−0.34 ± 2.78

% with ED, percent of patients with endothelial dysfunction; % with SMD, percent of patients with smooth muscle dysfunction; BP, blood pressure; CON, non-exercising controls; HIIT, high intensity interval training; HR, heart rate; Max HR_{peak}, maximal heart rate; MCT, moderate continuous training; Mean FMD, mean flow-mediated dilation; Mean NTG, mean of nitroglycerin-mediated dilation; VO_{2peak}, peak oxygen consumption. Data are expressed as mean and standard deviation. PRE, corresponds to value obtained immediately before first training session; POST, correspond to values obtained immediately after the last exercise session; Δ corresponds to the mean of difference between post and pre values. [a]$P < 0.01$ vs. MCT; [b]$P < 0.01$ vs. baseline-HIIT; [c]$P < 0.01$ vs. CON; [d]$P < 0.05$ vs. MCT; [e]$P < 0.05$ vs. CON.

and CON (P = 0.0042) (**Figure 2**). No increase in EDVD was seen in MCT or CON. EIVD was unchanged between pre- and post-training in all groups.

Although systolic blood pressure (SBP) values were in the normal range within groups before and after training, SBP increased 7.4% in HIIT (P = 0.0378), while it was unchanged in MCT (P = 0.58) and CON (P = 0.08). Maximal heart rate was increased in HIIT in relation to CON ($P < 0.05$). There was no difference in maximal heart rate between MCT and CON (**Table 3**).

VO_{2peak} was similar at baseline between groups (P = 0.96) and increased 17.6% from baseline after HIIT training (P = 0.0001) but only 3% in MCT (P = 0.055), with no change in CON (P = 0.63). There was a trend for a greater increase in VO_{2peak} after training in HIIT compared to MCT (P = 0.055) (**Table 3**).

We found a positive correlation (r = 0.337, P = 0.007) between the delta of VO_{2peak} and the delta of FMD, indicating that a better cardiorespiratory fitness was associated with an improvement in endothelial function (**Figure 3**).

After training, HbA1c was not significantly changed compared to baseline values in any of the protocol groups. No serious hypoglycemic episodes occurred. No patient had muscular injury nor cardiovascular symptoms. All supervised exercise sessions were completed.

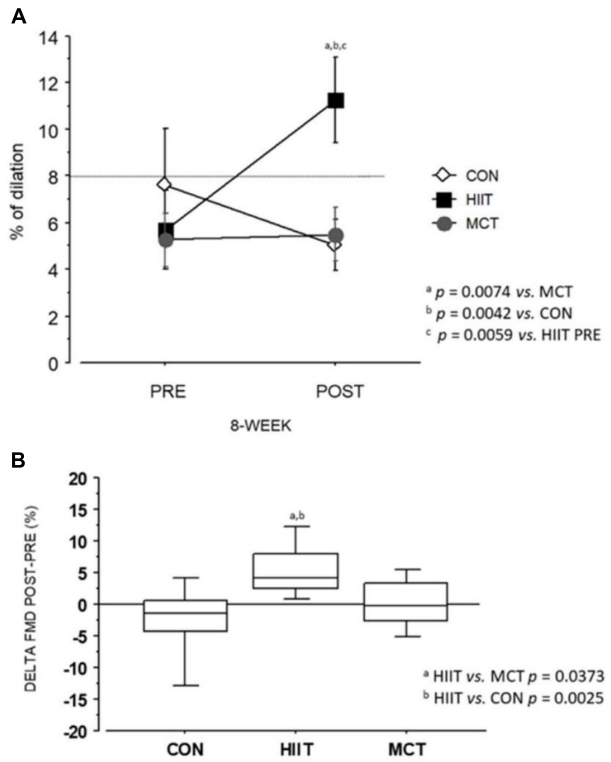

FIGURE 2 | Flow Mediated dilation (FMD): **(A)** Befor and training. **(B)** Difference between post and pre-training.

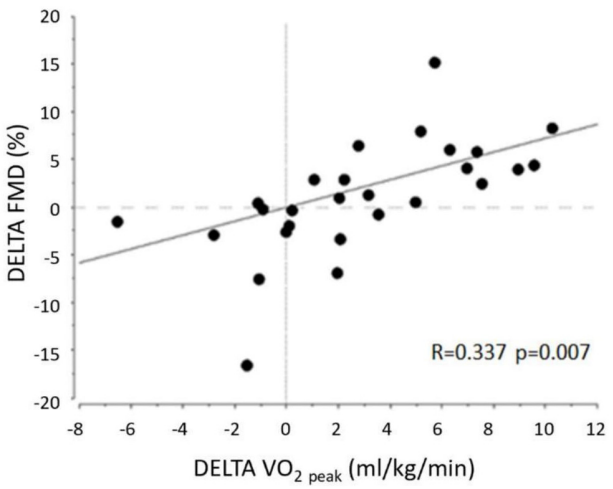

FIGURE 3 | Correlation between increase in FMD and in peak Oxygen Consumption (VO_{2Peak}) in all patients.

DISCUSSION

This randomized clinical trial examined the effects of HIIT in relation to MCT on endothelial function of young adults, with non-complicated type 1 diabetes. The study showed that 8 weeks of HIIT markedly improved vascular function, by increasing EDVD 2-fold from baseline, significantly more than MCT during a similar period of training. This was not dependent on improvements in glycemic control. Moreover, HIIT produced a robust improvement in physical fitness from baseline, while there was only a mild improvement in MCT. The strong positive correlation observed between the improvement in FMD and improvement in VO_{2peak} indicated that these variables are interdependent, and that changing intensity during exercise is an important determinant to improve physical fitness and vascular improvement in young patients with type 1 diabetes.

Exercise can improve endothelial function in both type 1 and type 2 diabetes when compared to non-exercising controls. Three studies have previously evaluated FMD in type 1 diabetes using different protocols. In a non-controlled study, Seeger et al. (2011) observed that, after performing 30-min sessions of aerobic training twice a week for 18 weeks, children and adolescents with type 1 diabetes showed a 65% increase in FMD, compared to non-exercising controls. Fuchsjäger-Mayrl et al., 2002 in a non-randomized trial tested a similar protocol of moderate continuous exercise training in older individuals with type 1 diabetes and observed improvements in FMD only after 4 months of training. They used continuous moderate workloads of 60–70% of $VO_{2\,max}$ for 40 min in a stationary cycle ergometer, 2–3 times a week and observed that FMD improved by more than 50%, in adults with non-complicated type 1 diabetes, while no change was observed in non-exercising individuals. In a cross-sectional study (Trigona et al., 2010) including children and adolescents with type 1 diabetes, there was an association between FMD and exercising, and endothelial function was enhanced in patients who engaged in more than 60 min/day of moderate-to-vigorous physical activity. These studies indicate that moderate continuous training may improve FMD in patients with type 1 diabetes. However, MCT may take too long to show benefits what is concerning because, in general, people with diabetes tend to exercise for short periods.

Although exercise training effects have been studied in type 1 diabetes, this is, to the best of our knowledge, the first study comparing HIIT and MCT in a head-to-head randomized clinical trial. The effect of short intervals during exercise sessions was studied, however, in type 2 diabetes. Interval and continuous exercise training were compared in an open label clinical trial in relation to microvascular reactivity. Mitranun et al. (2014) randomized 45 patients with type 2 diabetes to perform exercise sessions with similar energy expenditure, walking on a treadmill for 30 and 40 min per day for 12 weeks. They observed that both continuous and interval exercise training were effective in improving FMD from baseline, but there was a greater improvement in FMD in the group that performed intensity exercise intervals than in those who exercised in the continuous training group (37 vs. 27% increase, $P < 0.05$, respectively). In the present study, the differences in FMD caused by interval training were even more robust than those observed in type 2 diabetes by Mitranun et al. (2014). We found that there was an almost doubling of FMD from baseline in HIIT (97% increase) with no change in MCT at 8 weeks.

It is well known that acute exercise can enhance endothelial function compared to non-exercising controls in different clinical conditions. A meta-analysis indicated that all exercise modalities can enhance endothelial function (Ashor et al., 2015). Exercise can enhance endothelial function basically through four mechanisms: (1) by increasing nitric oxide (NO) bioavailability, which occurs secondarily to enhanced expression/stabilization of endothelial nitric oxide synthase enzyme (eNOS) and/or reduced inactivation/degradation of NO by free-radicals (Newcomer et al., 2011); (2) increasing expression of antioxidant enzymes, superoxide dismutase, glutathione peroxidase and catalases thus enhancing antioxidant capacity (Belardinelli et al., 2005) as well as reducing the expression of oxidant enzymes, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Gielen et al., 2010); (3) reducing the expression of pro-inflammatory molecules such as interleukins, adhesion molecules (Ribeiro and Oliveira, 2010); and finally (4) increasing the number of endothelial progenitor cells (EPCs), which are important determinants of vascular endothelium regeneration and angiogenesis (Ribeiro et al., 2013).

The mechanism by which interval training could exert effects on endothelial function is still speculative. It is plausible, however, that shear stress could be increased after exercise (Dopheide et al., 2017) inducing eNOS phosphorylation (Casey et al., 2017). In a pilot trial, Dopheide et al. (2017) evaluated mean tangential wall stress (TWS) in the femoral artery, a surrogate marker of shear stress, in 40 patients with known peripheral artery disease, who were compared to healthy individuals before and after supervised exercise training. Patients were asked to walk 60 min per day, at least 3–5 days a week. The intensity was limited by claudication, and they should rest for intervals of up to 5 min, repeating the same distance at lower intensity. There was a significant increase in TWS in relation to controls, indicating that intermittent exercise training could increase shear stress. Moreover, a recent study (Casey et al., 2017) demonstrated that repeated muscle contraction can induce eNOS phosphorylation in humans by increasing arterial shear stress. Casey et al. (2017) studied seven young males who performed 20 bouts of rhythmic forearm exercise at 20% maximal (3 min each) separated by 3 min of rest, over a 2 h period. Fresh endothelial cells were then obtained 2 h after exercise. They observed that protein expression and phosphorylation of eNOS was increased. This was the first evidence in humans that muscle contraction-induced increases in conduit arterial shear could lead to *in vivo* posttranslational modification of eNOS activity in endothelial cells.

Endothelial dysfunction in type 1 diabetes is known to be caused by chronic hyperglycemia and increased oxidative stress, also worsened by early vascular rigidity (Bertoluci et al., 2015). In the present study, however, exercise training did not change oxidative stress markers such as TBARS or T-SH levels, not supporting the hypothesis that a reduction in oxidative stress was critical for short-term improvements in endothelial function.

The present study had some limitations to be considered. (1) Since we studied young patients with type 1 diabetes without established diabetic complications, extrapolating these results to a group with more advanced disease is limited. (2) Our results are limited to 8 week of training. Long-term effects of exercise on endothelial function in type 1 diabetes are unknown. (3) As our study protocol was restricted to 8 weeks, it may have limited the detection of improvements of FMD in MCT group. In the study of Fuchsjäger-Mayrl et al. (2002, which was non-randomized, 18 type 1 patients were submitted to 2–4 months training at continuous (40 min) submaximal workload (60–70%$VO_{2\,max}$) for 1 h, 2–3 times a week and were assessed through FMD at the brachial artery, a very similar protocol to that used in the MCT group in the present study. They observed that the endothelium-dependent vasodilatory response to reactive hyperemia increased significantly only after 4 months of training. This indicates that MCT may also be effective in improving FMD in type 1 diabetes, but may take a longer time. By this way, our study might be underpowered to detect such FMD changes in MCT group.

On the other hand, there were some important strengths to be considered. (1) This is the first randomized clinical trial study to compare HIIT protocol head-to-head with MCT in young adults with type 1 diabetes. We used the in-block randomization process, considering FMD, which favored to obtain very similar FMD values at baseline, without selection bias. (2) We had a very high compliance level. (3) Although we may have had included a theoretically small number of patients, we had no losses during the training protocol. All losses and all included patients were studied in their original groups, as the losses occurred before training and were similar in all groups at random (4) Finally, our FMD measurements were performed by a single highly trained blinded examiner, increasing accuracy. In the future studies, however, the use of an edge detection software system can be useful to improve the validity of the FMD measurements.

CONCLUSION

In young adults with type 1 diabetes without known complications and in moderate glycemic control, HIIT proved to be superior to MCT in improving endothelial dysfunction and physical fitness during a training period of 8-weeks. The effect on endothelial function was closely related to improvement in physical fitness and did not depend on glycemic control changes. Thus, HIIT can be recommended as a useful and safe non-pharmacological alternative to improve vascular function in patients with type 1 diabetes. Long-term studies to examine the efficacy of HIIT in preventing micro- and macrovascular disease are still required.

ETHICS STATEMENT

This clinical trial was registered at ClinicalTrials.gov Identifier: NCT03451201. This study protocol was approved by the Hospital de Clínicas de Porto Alegre (HCPA) ethics board (CAEE 54928116.0.0000.5327) and the reported investigations were carried out in accordance with the principles of the Declaration of

Helsinki. All participants provided oral and written consent prior to inclusion in the study.

AUTHOR CONTRIBUTIONS

WB was the mentor of the study, designed, and organized the logistics at Hospital de Clínicas de Porto Alegre (HCPA), submitted the manuscript, conceived and executed the study, including the supervised exercise sessions. AdS performed all ultrasound examinations and obtained FMD data. JF and JR-K collected the data and organized the database at the Exercise Research Laboratory. AR-O implemented the study design in the Exercise Research Laboratory, supervised the exercise protocol, wrote and revised part of the manuscript. BT and MP organized the database and logistics at the Institute for Children with Diabetes (ICD) and revised the manuscript. WB designed the study, wrote and revised the manuscript. MB reviewed the manuscript and raised financial funds for publication.

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Evidence of Improved Vascular Function in the Arteries of Trained but Not Untrained Limbs After Isolated Knee-Extension Training

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Vascular endothelial function is a strong marker of cardiovascular health and it refers to the ability of the body to maintain the homeostasis of vascular tone. The endothelial cells react to mechanical and chemical stimuli modulating the smooth muscle cells relaxation. The extent of the induced vasodilation depends on the magnitude of the stimulus. During exercise, the peripheral circulation is mostly controlled by the endothelial cells response that increases the peripheral blood flow in body districts involved but also not involved with exercise. However, whether vascular adaptations occur also in the brachial artery as a result of isolated leg extension muscles (KE) training is still an open question. Repetitive changes in blood flow occurring during exercise may act as vascular training for vessels supplying the active muscle bed as well as for the vessels of body districts not directly involved with exercise. This study sought to evaluate whether small muscle mass (KE) training would induce improvements in endothelial function not only in the vasculature of the lower limb (measured at the femoral artery level in the limb directly involved with training), but also in the upper limb (measured at the brachial artery level in the limb not directly involved with training) as an effect of repetitive increments in the peripheral blood flow during training sessions. Ten young healthy participants (five females, and five males; age: 23 ± 3 years; stature: 1.70 ± 0.11 m; body mass: 66 ± 11 kg; BMI: 23 ± 1 kg · m^{-2}) underwent an 8-week KE training study. Maximum work rate (MWR), vascular function and peripheral blood flow were assessed pre- and post-KE training by KE ergometer, flow mediated dilatation (FMD) in the brachial artery (non-trained limb), and by passive limb movement (PLM) in femoral artery (trained limb), respectively. After 8 weeks of KE training, MWR and PLM increased by 44% ($p = 0.015$) and 153% ($p = 0.003$), respectively. Despite acute increase in brachial artery blood flow during exercise occurred (+25%; $p < 0.001$), endothelial function did not change

after training. Eight weeks of KE training improved endothelial cells response only in the lower limb (measured at the femoral artery level) directly involved with training, likely without affecting the endothelial response of the upper limb (measured at the brachial artery level) not involved with training.

Keywords: flow mediated dilation, single passive limb movement, shear rate, isolated leg extension muscle, reactive hyperaemia, vascular conditioning

INTRODUCTION

Tobacco smoking, alcohol abuse, unbalanced diet, and physical inactivity, represent the main unhealthy habits, and their prevention, with an appropriate intervention, could reduce the number of premature deaths (World Health Organization [WHO], 2016). In particular, the introduction of regular physical activity is associated with numerous benefits on the overall cardiovascular risk, such as a reduction in arterial pressure, lipids level, insulin resistance, and exercise intolerance (Green et al., 2008, 2017a,b; Riebe et al., 2015; Adamson et al., 2018; Tomschi et al., 2018). However, since the modification of these risk factors taken alone underestimate the magnitude of exercise-induced risk reduction (Mora et al., 2007), it was recently proposed the so-called "vascular conditioning" theory to explain such a gap (Green et al., 2008, 2017a). This theory is based on the possible exercise-induced effects on vasculature structure and function (i.e., vasomotor response). The mechanical effects, provided by the repetitive increases in arterial pressure, blood flow ($\dot{Q}$), and shear rate ($\dot{\Upsilon}$) occurring on inner vessels wall during each training session (Green et al., 2017a), are the main stimuli able to enhance the endothelial cells response via an increase in several vasoactive factors bioavailability, among which the most common is NO (Joyner and Green, 2009; Naylor et al., 2011).

Exercise training protocols, generally, lead to a better vascular endothelial response mainly due to a shear-dependent mechanism (Tinken et al., 2009; Birk et al., 2012a,b; Green et al., 2017a). Briefly, $\dot{\Upsilon}$ is the laminar shear force running in parallel to vessels' long axis (Niebauer and Cooke, 1996). During exercise, $\dot{Q}$ augmentation drives the increase in $\dot{\Upsilon}$, which stimulates endothelial cells to release vasoactive factors. However, the amount of skeletal muscles involved in the exercise could generate different $\dot{Q}$ and $\dot{\Upsilon}$ pattern according to the increase in muscles metabolic demands (Green et al., 2005; Thijssen et al., 2009a,b; Tinken et al., 2009; Spence et al., 2013). Indeed, it seems that repeated exercise sessions could have positive and beneficial effect on the overall endothelium health as a consequence of training-related changes in $\dot{Q}$ and $\dot{\Upsilon}$ pattern (Spence et al., 2013; Kazmi et al., 2015; Davies, 2016; Green et al., 2017a,b).

Isolated knee extension muscles (KE) training is a type of small muscle mass exercise utilized in previous investigations (Wray et al., 2006; Esposito et al., 2010, 2011, 2018). Compared to other forms of exercise, KE exercise limits muscular work to the quadriceps muscle (Andersen et al., 1985). This exercise modality has been already used to train patients with chronic heart failure (Esposito et al., 2010, 2011, 2018) or with other pathologies characterized by a central limitation to aerobic exercise performance (Richardson et al., 2004). At the end of training, improvements in muscle structure, peripheral convective and diffusive oxygen transport, and subsequently, oxygen uptake ($\dot{V}O_2$) were found (Esposito et al., 2010, 2011), thus supporting the efficacy of this small muscle mass training modality. Moreover, the $\dot{Q}$ increment in the femoral artery during a single KE session (Paterson et al., 2005) is likely happening throughout all the training period, possibly triggering the cascade of events leading to a "vascular conditioning" in the artery directly involved in the exercise.

Improvements in endothelial cells function in arteries not directly involved in exercise (e.g., brachial artery) have been observed after whole body exercise training (i.e., cycling, running) (Birk et al., 2012a; Spence et al., 2013). Interestingly, positive vascular effects were also found after small mass muscle training (handgrip muscles) in the brachial artery (Thijssen et al., 2009a), as well as in vessels remote to the body region involved in the exercises (i.e., improvement in brachial artery after a respiratory muscle training or single leg kick training) (Wray et al., 2006; Bisconti et al., 2018).

In a previous study Wray et al. (2006) investigated the effects of 6 weeks of a similar KE training on brachial, superficial and deep femoral arteries flow mediated dilation (FMD) in elderly people. KE training positively affected brachial artery FMD, with no changes in both superficial and deep femoral arteries (Wray et al., 2006). However, the effects of KE training in the lower limb vasculature (involved with training), as well as in the upper limb (not directly involved with training) in a young population still remain to be elucidated.

Small muscle mass exercise is being employed to maximize vascular adaptation due to its ability to circumvent central limitations and sympathetic restraint of limb $\dot{Q}$ (Richardson and Saltin, 1998; Richardson et al., 1998, 2004; Esposito et al., 2018). In particular, KE exercise determines a significant change in the peripheral hemodynamics (Paterson et al., 2005) without overloading the cardiopulmonary system (Richardson et al., 2004; Esposito et al., 2010, 2011, 2018).

Compared to cycle exercise (Saito and Tsukanaka, 2019), an attenuate muscle sympathetic outflow likely occurs during KE due to a lower cardiopulmonary engagement (Esposito et al., 2010). This is so not only in healthy population (Richardson and Saltin, 1998; Richardson et al., 1998) but also in patients with central (cardiac and/or pulmonary) exercise limitation

Abbreviations: AUC, area under the curve; f_H, hearth rate; FMD, flow mediated dilation; KE, isolated knee extension muscles; MAP, mean arterial pressure; MWR, maximum work rate; NO, nitric oxide; PLM, single passive limb movement; q, stroke volume; $\dot{Q}$, blood flow; $\dot{Q}_{brac}$, brachial blood flow; $\dot{Q}_{fem}$, femoral blood flow; $\dot{Q}_T$, cardiac output; $\dot{V}O_2$, oxygen uptake; $\dot{V}O_{2peak}$, peak oxygen uptake; $\dot{\Upsilon}$, shear rate; $\dot{\Upsilon}_{brac}$, brachial shear rate; $\dot{\Upsilon}_{fem}$, femoral shear rate.

(Richardson et al., 2004; Esposito et al., 2018). In addition, this exercise modality could be more easily employed in clinical populations who have significant exercise intolerance during large muscle mass exercise due to central limitation (Esposito et al., 2011). Taking all into account, KE exercise training represents a good exercise paradigm to be used in presence of exercise intolerance due to central limitation or presence of sympatho-excitation.

On these bases, this study sought to evaluate whether small muscle mass (KE) training would induce vascular conditioning not only in the vasculature of the limb directly involved with training (as results of the femoral artery measurement) but also in vasculature of a limb not directly involved with training (as results of the brachial artery measurement). The hypothesis is that the repetitive training-induced stimuli may occur also in arteries of non-trained districts via a systemic increase in $\dot{\Upsilon}$.

MATERIALS AND METHODS

Participants

Ten young, healthy participants [five females, and five males; age: 23 ± 3 years; stature: 1.70 ± 0.11 m; body mass: 66 ± 11 kg; body mass index: 23 ± 1 $kg.m^{-2}$; mean ± standard deviation (SD)] were enrolled in the study. After full explanation of the purpose and the procedures of the study, participants signed an informed consent form. Exclusion criteria were: (i) presence of neurological, vascular and musculoskeletal impairments at the lower and upper limbs level; (ii) being on pharmacological therapy related to neural and/or vascular response, including hormonal contraceptives and oral supplements; (iii) being a smoker; (iv) systolic arterial pressure higher than 140 mmHg; and (v) having an irregular menstrual cycle (26–35 days) up to 3 months before the beginning of the study. The Institutional Review Board of the Università degli Studi di Milano approved the study, which was performed in accordance with the latest Helsinki's Declaration principles.

Study Design

Before testing procedures, participants underwent a preliminary session during which they familiarized with the dynamic knee extension ergometer and with the procedure to identify the maximum isometric voluntary contraction (MVC) of the knee extensor muscles of the dominant limb. During this visit, the passive limb movement (PLM) and the FMD tests (see below for a full explanation of the procedures) were performed on each participant. At the end of the tests, the ultrasound probe position for testing was marked on a transparency sheet, together with some skin landmarks (moles, scars, angiomas, etc.) for reliability purposes. The PLM and FMD outcomes obtained during the familiarization and the pre-training experimental session were used to calculate intersession reliability.

Pre- and post-8 weeks of KE training, participants were tested at the same time of the day in a climate-controlled laboratory (constant temperature of 20 ± 1°C and relative humidity of 50 ± 5%) to minimize any possible confounds due to circadian rhythm. For females, the tests were assessed on the same day of the menstrual cycle (early follicular phase days 3 ± 3). Female participants recorded their menstrual cycle in a personal diary throughout the study, which was used to assess the early follicular phase and allowed the subjects to be tested on the same menstrual day pre- and -post KE training. On the test days, participants came to the laboratory after fasting overnight, abstaining from caffeine and other similar substances for at least 12 h, and not participating in heavy exercise for at least 48 h prior the tests. Post-training assessments were performed 48 h after the last KE training session. This period was observed to prevent possible biases in measurements introduced by the acute effects of the last training session. During the first session of KE training, the possible increase in brachial artery $\dot{Q}$ ($\dot{Q}_{brac}$) was also determined.

Measurements and Data Analysis

Maximum Work Rate

The maximum work rate (MWR) was determined by an incremental square wave test on a dynamic knee extension ergometer (Andersen et al., 1985). As shown in **Figure 1**, the test was performed while sitting on an adjustable chair in order to fit body sizes of different dimensions. Both knees were flexed at 90°, with the ankle of the dominant limb connected to a bicycle ergometer pedal arm by a rigid bar. The concentric phase occurred actively from 90° of the knee to full extension, while the eccentric phase was completely passive, driven by the flywheel momentum. The mechanical brake applied to the ergometer and the pedal frequency were measured to determine the mechanical power output. The mechanical friction, i.e., the force applied to each revolution, was measured by a force transducer (mod. SM-100 N, Interface, Crowthorne, United Kingdom), while the pedal frequency was determined by a magnetic transducer integrated in the cycle ergometer (mod. 839E, Monark, Vansbro, Sweden).

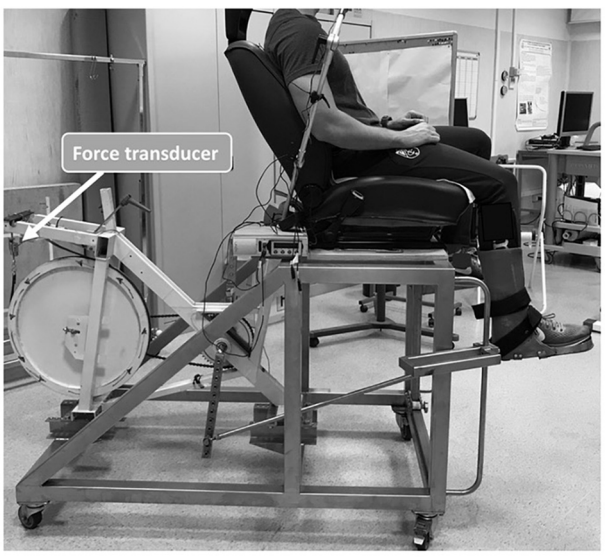

FIGURE 1 | Photographic representation of participant's positioning on the knee extension ergometer. The load-cell to calculate mechanical power is indicated by the arrow.

Both the force and the pedal frequency signals were amplified (gain × 100) and acquired by a personal computer after an analog-to-digital conversion (model UM150, Biopac System, CA, United States) at a sampling rate of 1 kHz.

The square wave test started with an initial work load of 20 W for males and 10 W for females. Loads increased by similar amplitude steps (+20 W for male and +10 W for female) until exhaustion. Each load was maintained for 3 min, with 5 min recovery in between. The load of the last completed step was considered as MWR. The amplitude of the load increments during the square wave test was chosen as the best compromise between the identification of the maximum workload and the necessity to minimize the onset of muscle fatigue. A non-invasive impedance cardiograph device (Physio Flow®, Manatec Biomedical, Paris, France) was used to assess the cardiac output ($\dot{Q}_T$), stroke volume (q) and heart rate (f_H). At rest and during test, pulmonary $\dot{V}O_2$ was detected on a breath-by-breath modality by gas analysers (mod. Quark b^2, Cosmed, Rome, Italy). The system was calibrated before each test with gas mixtures of known concentrations (16% O_2; 5% CO_2; balance N_2). Data were averaged on the last 60 s of baseline and the last 30 s of the last work load. The average $\dot{V}O_2$ matched to MWR was considered as peak $\dot{V}O_2$ ($\dot{V}O_{2peak}$).

Maximum Isometric Voluntary Contraction

Knee extensors MVC was measured with the participants sitting on an ergometer with the hip and the knee flexed at 90° and firmly secured at the ankle level by a Velcro® strap (Velcro Industries Inc., Willemstad, Netherlands Antilles) to a load cell (mod. SM-2000N operating linearly between 0 and 2000 N; Interface, Crowthorne, United Kingdom) for the force signal detection. After a standardized warm-up (10 × 2-s contractions at 50% MVC previously determined during familiarization), three MVC attempts were performed. The participants were instructed to push as fast and hard as possible for 3 s. Each MVC attempt was interspersed by 3 min of recovery. The force signal was driven to an A/D converter (mod. UM 150, Biopac, Biopac System Inc., Santa Barbara, CA, United States), sampled at 1000 Hz, and stored on a personal computer. The maximum force value recorded during tests was considered as MVC and was inserted into the data analysis.

Knee Extensor Muscles Volume

With thigh length, circumferences, and skinfold measurements, knee extensor muscles volume was calculated to allow an estimate of quadriceps muscle mass, as previously published in other studies (Jones and Pearson, 1969; Andersen et al., 1985; Esposito et al., 2011, 2018).

Single Passive Limb Movement

Single passive limb movement was performed in accordance to the recommended procedures (Gifford and Richardson, 2017; Venturelli et al., 2017b). Sitting on a chair, subjects rested in the upright-seated position for 10 min before starting the data collection, and remained in this position until the end of the test. The PLM protocol consisted of 60 s of baseline peripheral hemodynamic data collection, followed by a single passive knee flexion and extension of 1 s, after which the leg was maintained fully extended for the remaining 59 s for the post-movement data collection. PLM was performed by a member of the research team, who moved the subject's lower leg through a 90° range of motion at 1 Hz. Throughout the test, measurements of arterial blood velocity and vessel diameter were performed in the common femoral artery of the passively moved leg, distal to the inguinal ligament and proximal to the deep and superficial femoral bifurcation by Doppler ultrasound (mod. Logiq-7, General Electric Medical Systems, Milwaukee, WI, United States). After being positioned to an insonation angle of 60° or less, a 9-MHz linear array transducer was used to measure the mean blood velocity. The sample volume was centered and size-maximized according to vessel's diameter (Trinity et al., 2012). Femoral artery $\dot{Q}$ ($\dot{Q}_{fem}$) was calculated by using data of arterial diameter and mean blood velocity as:

$$\dot{Q}(\text{ml} \cdot \text{min}^{-1}) = \text{mean blood velocity} \cdot \pi \cdot (\text{vessel diameter}/2)^2 \cdot 60$$

Flow Mediated Dilation

Flow mediated dilation measurements were performed according to recommended procedures (Harris et al., 2010; Bisconti et al., 2018). Before FMD, the subjects laid supine for approximately 20 min to restore baseline values of cardiovascular parameters. An arterial pressure cuff was placed on the forearm immediately distal to the olecranon process in order to provide an ischemic stimulus on the forearm when inflated. Following baseline assessments, the forearm blood pressure cuff was inflated to 250 mmHg for 5 min. Brachial diameter and flow velocity recordings resumed at baseline, 30 s prior to cuff deflation and continued for 2 min post-deflation, in accordance with previous literature (Corretti et al., 2002; Harris et al., 2010; Wray et al., 2013). A 9-MHz linear array transducer attached to a high-resolution ultrasound machine was used to image the brachial artery in the distal third of the upper arm above the cuff placement. When an optimal image was obtained, the probe was held stable and longitudinal in B-mode, acquiring images of the lumen-arterial wall interface. Continuous Doppler velocity assessments were also obtained and collected using the lowest possible insonation angle (<60°). The Doppler ultrasound settings were maintained consistent pre vs. post-KE assessment among subjects. Additionally, anatomical marks were considered to ensure the same ultrasound probe, as well as pressure cuff position between the two visits. The FMD data were exported in AVI format and analyzed using commercially available software (Brachial Artery Analyzer for Research, Medical Imaging Applications, LLC, Coralville, IA, United States), in which the lumen diameter, distance between upper intima-media to lower intima media, was measured from within the same region-of-interest (Faita et al., 2011; Ratcliffe et al., 2017). This method is largely independent of investigator bias (Faita et al., 2011; Ratcliffe et al., 2017). FMD was quantified as the maximal change in brachial artery diameter after cuff release, expressed as a percentage increase (%Δ) above baseline:

$$(\text{Maximum} - \text{rest diameter})/\text{rest diameter} \times 100$$

Brachial artery $\dot{Q}$ ($\dot{Q}_{brac}$) was calculated as previously described for PLM assessments.

$\dot{\Upsilon}$ was calculated post-cuff release using the following equation:

$$\dot{\Upsilon}\ (s^{-1}) \ = \ 8\ v_{mean}/\text{vessel diameter}$$

The cumulative $\dot{\Upsilon}$, corresponding to the reactive hyperaemia post-cuff release (total $\dot{\Upsilon}$ from cuff release to time-to-peak), was integrated (AUC) by using the trapezoidal rule, and calculated as:

$$\sum\left[y_i \cdot (x_{i+1} - x_i) + \frac{1}{2}(y_{i+1} - y_i) \cdot (x_{i+1} - x_i)\right] \quad (1)$$

The $\dot{\Upsilon}$ AUC reflects the amount of mechanical stimulus applied on the endothelium during the cuff release hyperaemic response until time-to-peak. Considering that FMD is primarily dependent on the endothelium response to mechanical stimuli, the FMD was therefore divided by cumulative $\dot{\Upsilon}$ (FMD/$\dot{\Upsilon}$) (Pyke and Tschakovsky, 2005).

Isolated Knee Extension Muscles Training

A schematic drawn of the KE training design is given in **Figure 2**.

Similar to previous studies (Esposito et al., 2011, 2018), participants underwent an 8-week KE training (3 sessions.w^{-1}) involving both legs, one at a time. The KE training was performed on the same ergometer used for MWR assessment, which allows participants to train only a single limb leg extensor muscles. The KE training was similar to that proposed in a previous study (Esposito et al., 2010). Briefly, workloads ranged mainly from 50% to 95% MWR, with an average session duration of 40 min for each leg (a report of the characteristics of each KE training session is provided as **Supplementary Table S1**). The session's workloads were readjusted every 2 weeks reassessing a new MWR. Each training session was supervised by an expert operator, who monitored the attendance, the correct exercise execution, and the maintenance of the workload. The participants not attending at least the 80% of the total training sessions were excluded from the study. In this case, a new participant was recruited to replace the drop out.

To assess the acute effects of exercise on $\dot{Q}_{fem}$ and $\dot{Q}_{brac}$, the related $\dot{Q}$, together with $\dot{Q}_T$, q, f_H, and mean arterial pressure (MAP) were measured during the first 6 min of the first session of KE training (performed at 50% of MWR, representing the minimum workload intensity within the training). Femoral and brachial artery mean blood velocity and diameter were assessed by ultrasound. As previously described (Venturelli et al., 2017a), a modelflow method (Finapres Medical System) automatically assessed the q, with $\dot{Q}_T$ calculated as the product of q and f_H. f_H and the MAP were detected by electrocardiography and finger photoplethysmography positioned at the heart level (Finometer PRO, Finapres Medical System, Amsterdam, Netherlands). Before starting the bout of exercise training, a baseline measurement of 30 s was recorded. From mean blood velocity and diameter assessments during the last minute of exercise, the $\dot{Q}_{fem}$, $\dot{Q}_{brac}$ and the $\dot{\Upsilon}$ at femoral ($\dot{\Upsilon}_{fem}$) and brachial artery ($\dot{\Upsilon}_{brac}$) level were calculated, together with the central hemodynamic parameters.

Statistical Analysis

Statistical analysis was performed using a statistical software package (IBM SPSS Statistics v. 22, Armonk, NY, United States). Shapiro–Wilk test was used to check the normal distribution of the sampling. Based on a previous work in which percentage changes of about 45% were detected in brachial artery FMD/$\dot{\Upsilon}$ (main outcome) after a training protocol not directly involving the upper limbs (Bisconti et al., 2018), a sample size of ten participants was selected to ensure a statistical power > 0.80 and a type-1 error < 0.05, To determine intersession reliability in the endothelial function parameters, the intraclass correlation coefficient (ICC) and percentage change of the standard error of the measurement (SEM%) were calculated. The ICC was interpreted as follows: >0.90: very high; 0.89–0.70: high; 0.69–0.50: moderate (Munro, 2004). The minimal detectable change at 95% confidence interval ($MDC_{95\%}$) was used to detect sensitivity of the effects on endothelial function parameters between pre- and post-isolated knee extension muscles training (Donoghue et al., 2009). To assess significant effects of KE training on MWR, PLM and FMD parameters, a paired Student's t-test was applied pre and post data. Statistical significance was positioned at $p < 0.05$. Effect sizes measure expressed as Cohen's d was calculated for each parameter to quantify within-group magnitude

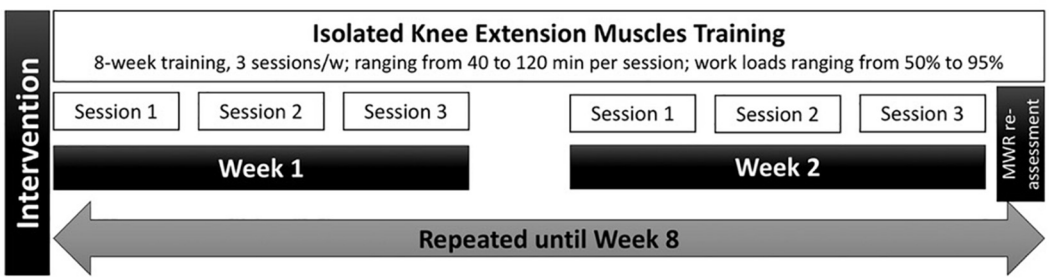

FIGURE 2 | Schematic chart of the experimental design for isolated knee extension muscles training.

TABLE 1 | Intersession reliability (intraclass correlation coefficient, ICC; standard error of measurement as a percentage, SEM%), and sensitivity (minimum detectable change at 95% confidence interval, $MDC_{95\%}$) values in the main endothelial function parameters calculated during the single passive limb movement (PLM) and flow mediated dilation (FMD) tests.

		Trial 1	Trial 2	ICC	SEM%	$MDC_{95\%}$
PLM	Femoral artery diameter (cm)	0.78 ± 0.09	0.78 ± 0.09	0.999	0.37	1.01
	Rest $\dot{Q}_{fem}$ ($ml \cdot min^{-1}$)	224 ± 116	228 ± 113	0.998	2.27	6.28
	Max $\dot{Q}_{fem}$ ($ml \cdot min^{-1}$)	552 ± 305	538 ± 282	0.997	2.95	8.18
	AUC (ml)	3042 ± 2392	3078 ± 2370	0.999	2.46	6.82
FMD	Rest brachial artery diameter (cm)	0.29 ± 0.07	0.28 ± 0.08	0.997	1.47	4.07
	Peak brachial artery diameter (cm)	0.36 ± 0.08	0.38 ± 0.08	0.936	5.17	14.32
	$\dot{\Upsilon}$ AUC (s^{-1}; × 1000)	292 ± 39	294 ± 42	0.701	8.98	25.42

Femoral blood flow at rest and at maximum value after PLM maneuvre, $\dot{Q}_{fem}$; shear rate, $\dot{\Upsilon}$; area under the curve of the $\dot{Q}$ and $\dot{\Upsilon}$ as a function of time in PLM and FMD, respectively, AUC. Data are presented as mean ± SD.

changes (Cohen, 1992). Cohen's *d* value was classified as trivial for values < 0.19, small between 0.2 and 0.6, moderate between 0.6 and 1.2, large between 1.2 and 2.0, and very large > 2.0. If not otherwise stated, data are presented as mean ± standard deviation.

RESULTS

Participants' attendance was about 95% (228/240 training sessions). Two participants dropped out throughout the study because of injury (not related to the training protocol) and lack of time. They were immediately replaced in order to maintain the required sample size.

Reliability

Intersession reliability for endothelial function parameters is reported in **Table 1**. ICC and SEM% in PLM parameters ranged from 0.997 and 0.37 to 0.999 and 2.95, respectively. ICC and SEM% in FMD parameters spanned from 0.701 and 1.47 to 0.997 and 8.98, respectively. In both PLM and FMD, $MDC_{95\%}$ was comprised between 1 and 25%.

Acute 50% MWR Exercise

Central hemodynamic and peripheral blood parameters assessed during the first 6 min of the first session of KE training (50% MWR) are presented in **Table 2**. All the parameters were significantly higher at the end of the sixth minute of exercise compared to baseline. In details, $\dot{Q}_T$, q, f_H and MAP increased by 114, 16, 85, and 18%, respectively. Both $\dot{Q}_{fem}$ and $\dot{\Upsilon}_{fem}$ increased by about fivefolds, whereas $\dot{Q}_{brac}$ and $\dot{\Upsilon}_{brac}$ increased by 26 and 60%, respectively.

Isolated Knee Extension Muscles Training

As shown in **Table 3**, MWR, MVC, KE muscle volume, and $\dot{V}O_{2peak}$ increased significantly by 44, 21, 7 and 11%, respectively, after KE training. No significant changes were retrieved in the other central hemodynamic parameters, both at rest and at MWR.

Single Passive Limb Movement and Flow Mediated Dilation

$\dot{Q}_{fem}$ kinetics response to PLM is illustrated in **Figure 3A**. $\dot{Q}_{fem}$ AUC, maximum $\dot{Q}_{fem}$, (max $\dot{Q}_{fem}$), and $\Delta\dot{Q}_{fem}$ (maximum − resting $\dot{Q}_{fem}$) are reported in **Table 4**. The pre-post changes in $\Delta\dot{Q}_{fem}$ and $\dot{Q}_{fem}$ AUC for each participant are presented in **Figure 4**. After training, $\dot{Q}_{fem}$ AUC, max $\dot{Q}_{fem}$ and $\Delta\dot{Q}_{fem}$ increased significantly by 161, 104, and 153 compared to pre-training, respectively (**Table 4** and **Figure 3A**). The pre-post changes in FMD and $\dot{\Upsilon}$ AUC for each participant are provided in **Figure 5**. No significant differences were detected after 8 weeks of KE training in any FMD parameter (**Table 4** and **Figure 3B**).

DISCUSSION

This study sought to investigate possible positive effects of KE training on the endothelial function in artery of a limb not directly involved with training, such as the brachial artery. The main finding was that KE training increased endothelial function only in the lower limb, assessed by the femoral artery measurements, without any significant change in the upper limb, assessed by brachial artery measurements. Despite the increase during every exercise session in peripheral $\dot{Q}$ and, in turn, in $\dot{\Upsilon}$ in the brachial artery (limb not directly involved with KE training), the mechanical stimulus in that area was not strong enough to trigger the chain of events turning to an endothelial function enhancement in the brachial artery.

Cardiac, Metabolic, and Skeletal Muscle Response to KE Training

The KE training protocol led to marked improvements at the peripheral level with no changes in heart hemodynamics due to the minimum taxing of this small muscle mass exercise paradigm on central factors. These findings are in line with previous reports in health and disease (Andersen et al., 1985; Richardson et al., 2004; Esposito et al., 2010, 2011) and indicate that this specific training paradigm can induce improvements in peripheral convective and diffusive oxygen transport without detectable changes in central hemodynamic. The 44% increase in MWR is only partially supported by the enhancement in $\dot{V}O_{2peak}$ (+11%). Indeed, the larger improvement in MVC (+21%) than in $\dot{V}O_{2peak}$, accompanied by the increase in estimated leg extensor muscle volume (+7%) suggested that the higher post KE training MWR was also induced by other metabolic pathways beside the aerobic power. These findings are somewhat in agreement with a previous investigation in chronic heart

TABLE 2 | Central hemodynamic parameters, femoral, and brachial artery blood flow detected at baseline and at the end of the sixth minute during the first session of isolated knee extension muscles training performed at 50% of the maximum work rate.

	Baseline	Sixth minute	Paired Student's *t*-test	Cohen's *d*
$\dot{Q}_T$ (l · min^{-1})	6.43 ± 0.48	13.75 ± 1.02	$t_{(9)}$: −20.53; $p < 0.001$	−8.79
q (ml)	95 ± 9	110 ± 10	$t_{(9)}$: −3.48; $p = 0.003$	−1.49
f_H (bpm)	68 ± 7	125 ± 13	$t_{(9)}$: −12.21; $p < 0.001$	−5.23
MAP (mmHg)	99 ± 9	117 ± 11	$t_{(9)}$: −4.17; $p < 0.001$	−1.78
$\dot{Q}_{fem}$ (ml · min^{-1})	284 ± 150	1759 ± 646	$t_{(9)}$: −7.04; $p < 0.001$	−2.87
$\dot{\Upsilon}_{fem}$ (s^{-1})	11.61 ± 6.13	72.04 ± 26.45	$t_{(9)}$: −7.35; $p < 0.001$	−3.01
$\dot{Q}_{brac}$ (ml · min^{-1})	34 ± 3	43 ± 5	$t_{(9)}$: −4.31; $p < 0.001$	−1.85
$\dot{\Upsilon}_{brac}$ (s^{-1})	14.80 ± 3.92	23.70 ± 8.14	$t_{(9)}$: −3.13; $p = 0.006$	−1.34

Cardiac output, $\dot{Q}_T$; stroke volume, q; heart rate, f_H; mean arterial pressure, MAP; femoral artery blood flow, $\dot{Q}_{fem}$; femoral artery shear rate, $\dot{\Upsilon}_{fem}$; brachial artery blood flow, $\dot{Q}_{brac}$; brachial artery shear rate, Υ_{brac}. Data are presented as mean ± SD.

TABLE 3 | Maximum knee-extension muscles (KE), isometric voluntary contraction (MVC), work rate (MWR), KE volume, and central hemodynamic parameters before (Pre) and after (Post) KE training.

	Pre	Post	Paired Student's *t*-test	Cohen's *d*
MWR (W)	48 ± 13	69 ± 21	$t_{(9)}$: −2.69; $p = 0.015$	−1.15
MVC (N)	501 ± 17	604 ± 23	$t_{(9)}$: −11.39; $p < 0.001$	−4.88
KE volume (cm^3)	3919 ± 355	4199 ± 452	$t_{(9)}$: −2.17; $p = 0.003$	−1.69
Rest $\dot{V}O_2$ (ml · kg^{-1} · min^{-1})	4.0 ± 0.8	4.1 ± 0.7	$t_{(9)}$: 0.20; $p = 0.844$	−0.09
Peak $\dot{V}O_2$ (ml · kg^{-1} · min^{-1})	22.7 ± 3.8	25.1 ± 4.2	$t_{(9)}$: −1.98; $p = 0.042$	−0.94
Rest $\dot{Q}_T$ (l · min^{-1})	5.47 ± 2.26	6.19 ± 1.35	$t_{(9)}$: −0.87; $p = 0.398$	−0.37
Peak $\dot{Q}_T$ (l · min^{-1})	15.11 ± 7.24	15.68 ± 4.04	$t_{(9)}$: −0.22; $p = 0.830$	−0.09
Rest *q* (ml)	77.05 ± 37.00	83.57 ± 22.18	$t_{(9)}$: −0.48; $p = 0.638$	−0.20
Peak *q* (ml)	100.39 ± 43.47	106.29 ± 27.63	$t_{(9)}$: −0.37; $p = 0.360$	−0.16
Rest f_H (bpm)	72 ± 8	72 ± 8	$t_{(9)}$: 0.00; $p = 1.000$	0.00
Peak f_H (bpm)	148 ± 22	147 ± 19	$t_{(9)}$: 0.11; $p = 0.915$	0.05

Pulmonary oxygen uptake, $\dot{V}O_2$; cardiac output, $\dot{Q}_T$; stroke volume, q; heart rate: f_H. Data are presented as mean ± SD.

failure participant engaged in an 8-week KE training at 65/75% MWR (Magnusson et al., 1996). After training, the authors demonstrated an increment of the MWR not accompanied by increases in $\dot{V}O_{2peak}$, whereas the knee extensor muscles MVC and mass were increased.

Effect of KE Training on Vascular Endothelial Function

It was previously demonstrated that KE training is an effective strategy to highly challenge the peripheral skeletal muscles, involved with exercise, without taxing central heart and respiratory function (Andersen et al., 1985). This training modality is particularly useful to counteract sarcopenia in the elderly and to decrease exercise intolerance in patients with central hemodynamic limitation, such as heart failure or chronic obstructive pulmonary disease (Richardson et al., 2004; Esposito et al., 2010, 2011, 2018). After 8 weeks of KE training, the endothelial function in the femoral artery, which was directly involved with KE training, increased as shown in **Table 4**. Contrary to the experimental hypothesis, this was not the case in the brachial artery.

As a result of KE training, all the PLM-related parameters, such as PLM AUC, max $\dot{Q}_{fem}$ and $\Delta\dot{Q}$, increased together with MWR, highlighting the positive effect of this type of training not only on the mechanical power (MWR) but also on the functionality of the lower limb vasculature directly exposed to exercise. Remarkably, despite PLM is a relative new research tool, its response provides an important insight into the function of the vascular system with clinical relevance. The most representative and common factors reported to describe the PLM response are: (i) the peak flow; (ii) the change from baseline to peak flow (Δ peak flow); and (iii) AUC (Gifford and Richardson, 2017). As previously reported, all these parameters are strictly related to NO bioavailability (Gifford and Richardson, 2017). Considering vascular tube length and blood viscosity relatively constant, and applying the Poiseuille's law, PLM-induced hyperemia might be driven by two main factors: an increased perfusion pressure and an increased peripheral vasodilation (i.e., decreased vascular tone). In detail, the vascular endothelium seems to play an important role in the PLM-induced change in vascular tone (Mortensen et al., 2012; Trinity et al., 2012, 2015; Groot et al., 2015). Indeed, the passive movement of the leg causes the mechanical deformation of vessels determining also NO release (Cheng et al., 2009; Jufri et al., 2015), that, in turn, results in the dilatation of the vascular bed (Mortensen et al., 2012; Trinity et al., 2012). Therefore, considering the nature of this hyperemic response, we can ascribe the present improvement in PLM's data

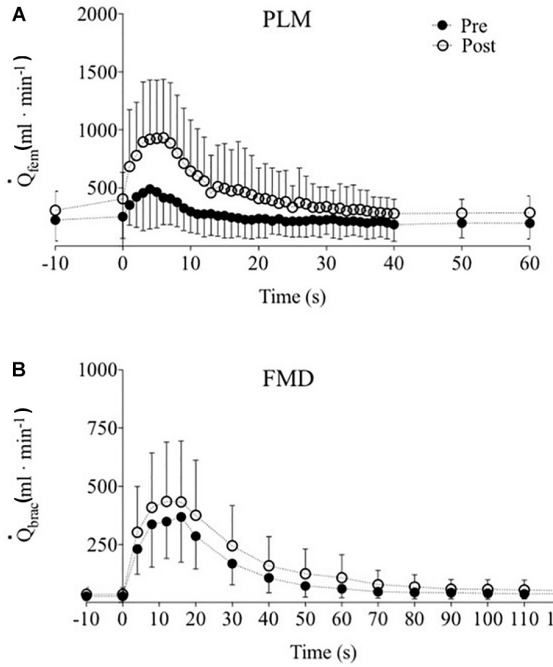

FIGURE 3 | Femoral and brachial blood flow ($\dot{Q}_{fem}$ and $\dot{Q}_{brac}$, respectively) hyperaemic response to single passive limb movement (PLM, **A**) and flow mediated dilatation (FMD, **B**) tests pre- (closed circles) and post- (open circles) training. Data are presented as mean ± SD.

to an enhanced NO-bioavailability (Trinity et al., 2015; Venturelli et al., 2017b). Oscillations in $\dot{Q}$ during training sessions, indeed, may be an important stimulus to the endothelial cell membrane deformation that trigger a series of signaling events favoring NO production, as previously mentioned in the introduction (Green et al., 2005).

However, it is important to underline that although PLM and FMD refer to two different part of the circulation (micro- and macro-circulation, respectively) (Eskurza et al., 2001; Mortensen et al., 2012), a relationship exists between these two different type of vascular assessments (Rossman et al., 2016), possibly due to the influence of reactive hyperemia/shear rate on FMD (Padilla et al., 2008), which is itself largely determined by the microcirculation (Pyke and Tschakovsky, 2005).

To date a consistent body of literature reported the positive effect of a small muscle training (i.e., handgrip training) on endothelial function (Tinken et al., 2010; Credeur et al., 2012; Badrov et al., 2016). Even in these studies, the positive effects of training on the vasculature were primarily ascribed to the $\dot{\Upsilon}$ stimulus acting on the inner vessel lay and leading to an improvement in endothelial cells response (Tinken et al., 2010; Credeur et al., 2012; Badrov et al., 2016), likely due to an increase in the NO-bioavailability (Corretti et al., 2002; Harris et al., 2010; Tinken et al., 2010; Thijssen et al., 2011; Green et al., 2017a).

Our data in acute condition (**Table 2**) indicate that during acute KE exercise an increase in peripheral circulation occurred also at the brachial artery level. This observation confirmed our hypothesis that the $\dot{\Upsilon}$ stimulus increased in limbs not directly involved with training, and possibly could have led to positive effects also in the endothelial cells response. As a matter of fact, chronic $\dot{\Upsilon}$ stimulus occurring in the brachial artery during different training modalities not directly involving upper limb muscles (such as cycling or respiratory muscle exercise) led to enhancements in brachial artery endothelial function (Birk et al., 2012a; Bisconti et al., 2018). More in details, acute cycle exercise increased $\dot{Q}_{brac}$ by about 30% with respect to baseline (Green et al., 2005) and $\dot{\Upsilon}_{brac}$ by about +280% (Birk et al., 2012a). Respiratory muscles exercise increased $\dot{Q}_{brac}$ up to +241% from baseline, with a $\dot{\Upsilon}_{brac}$ increase by about +115%. In the present study, KE exercise increased $\dot{Q}_{fem}$ by about +519% from baseline, while $\dot{Q}_{brac}$ increased only by +26%, generating a different level of mechanical stress ($\dot{\Upsilon}_{fem}$ and $\dot{\Upsilon}_{brac}$ +520 and +60%, respectively). Given that the positive effects of $\dot{\Upsilon}$ on the endothelial function are likely dependent on the magnitude with which the $\dot{\Upsilon}$ stimulus acts on the vessel (Green et al., 2011), the difference in $\dot{\Upsilon}$ in the two districts during exercise may explain the lack of training effects on brachial artery endothelial properties. A major strength of this

TABLE 4 | Changes in femoral and brachial arteries endothelial function parameters before (Pre) and after (Post) isolated knee extensor muscles training.

		Pre	Post	Paired Student's *t*-test	Cohen's *d*
PLM	AUC (ml)	53 ± 28	138 ± 112	$t_{(9)}$: −2.33; p = 0.032	−1.00
	Femoral artery diameter (cm)	0.77 ± 0.09	0.78 ± 0.09	$t_{(9)}$: −0.25; p = 0.807	−0.11
	Max $\dot{Q}_{fem}$(l · min^{-1})	528 ± 318	1078 ± 505	$t_{(9)}$: −2.91; p < 0.001	−1.25
	$\Delta\dot{Q}_{fem}$ (l · min^{-1})	304 ± 158	769 ± 399	$t_{(9)}$: −3.43; p = 0.003	−1.47
FMD	FMD (%)	19 ± 9	18 ± 9	$t_{(9)}$: 0.25; p = 0.807	0.11
	Rest brachial artery diameter (cm)	0.29 ± 0.08	0.31 ± 0.07	$t_{(9)}$: −0.60; p = 0.559	−0.25
	Peak diameter (cm)	0.35 ± 0.08	0.37 ± 0.08	$t_{(9)}$: −0.59; p = 0.583	−0.24
	Time-to-peak (s)	27 ± 11	26 ± 8	$t_{(9)}$: 0.23; p = 0.819	0.10
	$\dot{\Upsilon}$ AUC (s^{-1}; × 1000)	294 ± 42	399 ± 192	$t_{(9)}$: −1.69; p = 0.108	−0.72
	FMD/$\dot{\Upsilon}$ (%/s^{-1})	0.06 ± 0.03	0.05 ± 0.03	$t_{(9)}$: 0.75; p = 0.466	0.32
	AUC (ml)	30.62 ± 14.76	40.81 ± 25.72	$t_{(9)}$: −1.09; p = 0.292	−0.47

Single passive limb movement, PLM; area under the curve of the blood flow and $\dot{\Upsilon}$ as a function of time in PLM and FMD, respectively, AUC; femoral artery maximum blood flow, max $\dot{Q}_{fem}$; difference between rest and maximum femoral artery blood flow, $\Delta\dot{Q}_{fem}$; flow mediated dilation, FMD; shear rate, $\dot{\Upsilon}$. Data are presented as mean ± SD.

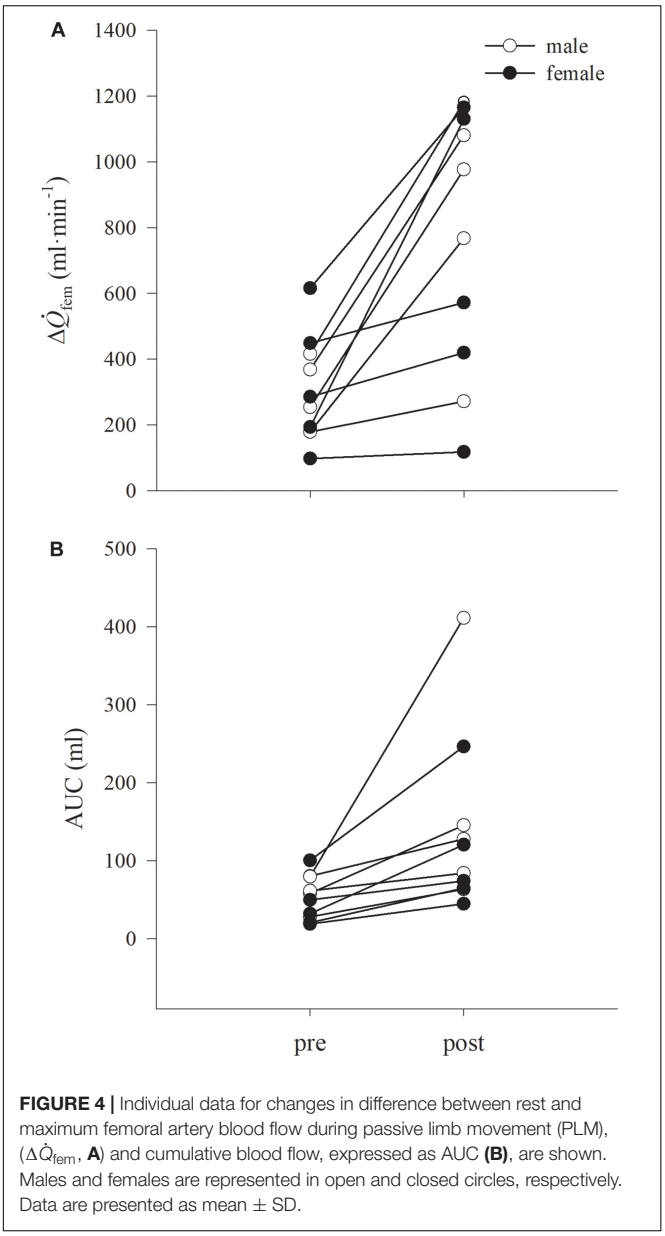

FIGURE 4 | Individual data for changes in difference between rest and maximum femoral artery blood flow during passive limb movement (PLM), ($\Delta\dot{Q}_{fem}$, **A**) and cumulative blood flow, expressed as AUC **(B)**, are shown. Males and females are represented in open and closed circles, respectively. Data are presented as mean ± SD.

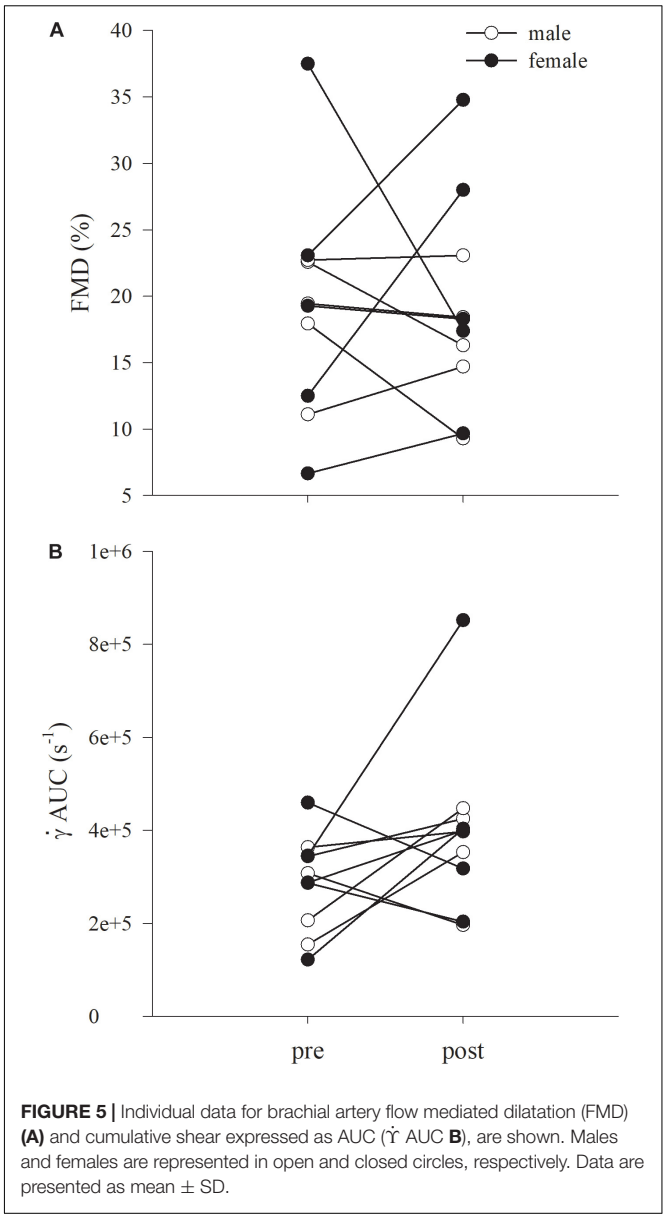

FIGURE 5 | Individual data for brachial artery flow mediated dilatation (FMD) **(A)** and cumulative shear expressed as AUC ($\dot{\Upsilon}$ AUC **B**), are shown. Males and females are represented in open and closed circles, respectively. Data are presented as mean ± SD.

study is the PLM data following KE training. As mentioned before, this significant finding of improved vascular function following 8 weeks of training contradicts what is reported with the FMD model and may implicate PLM as a more robust measure of vascular adaptation/health in response to increased exercise/physical activity patterns, thus explaining some discrepancies with previous reports (Wray et al., 2006).

However, it should be also taken into account that at the end of training the increase in the PLM response due to $\dot{\Upsilon}$ repeated stimuli does not involve the vasodilator capacity of the common femoral artery (Gifford and Richardson, 2017; Venturelli et al., 2017b), which is the largest conduit artery in the thigh with the main role of $\dot{Q}$ delivery rather than regulation. Therefore, another possible concomitant explanation, for the lack of effects on the brachial artery, could be that an exercise-induced $\dot{Q}$ increase in the common femoral artery (with minimum vasodilator capacity) leads to a much higher $\dot{\Upsilon}$ than in the brachial artery, where increases in $\dot{Q}$ are accompanied by vasodilation.

Interestingly Wray et al. (2006) investigated the effects of 6 weeks of a similar KE training on brachial, deep and superficial femoral arteries with age. KE training positively affected brachial artery FMD, with no changes in both deep and femoral arteries (Wray et al., 2006). Such results suggest that the pre-training vascular functionality level could play an important role in determining or not some positive results. It is therefore likely that, in the face of a "normal vascular functionality", arteries not directly involved with exercise possibly require a greater stimulus to achieve a significant improvement. An alternative mechanism to explain the lack of increase in FMD in the brachial artery after KE training could be the occurrence of a structural

arterial remodeling. Studies in humans reported that during a training protocol, changes in FMD occur during the first few weeks of training before returning to pre-training values, often not accompanied by modifications of the baseline diameter (Tinken et al., 2008; Weber et al., 2013), suggesting that structural remodeling of the vessels may have likely occurred. Based on an animal model, it appears that structural arterial remodeling may occur to counteract the endothelial cells response to reactive hyperemia (i.e., %FMD) (Green et al., 2017a). Despite the model utilized (animal vs. human), adaptations in terms of vascular remodeling after a training protocol are shear-stress dependent (Tinken et al., 2008; Green et al., 2017a). Indeed, exercise training may affect vascular tissue not only by modifying the function (e.g., %FMD), but also by inducing structural modifications in baseline and peak diameter (Green et al., 2017a). In the present study, neither progressive measurements during training (e.g., after 4/6 weeks of training), nor specific tests to observed these possible structural changes [e.g., ischemic handgrip exercise (Naylor, 2005)] were performed, thus a possible vascular remodeling could have not been disclosed.

Study Limitations

This study comes with some known limitations. First, although a sample size of ten participants was higher enough to reach a statistical power > 0.80, the enrolment of a higher number of sex-balanced participants may allow to highlight possible gender differences. Nevertheless, the individual changes in FMD% and $\dot{\Upsilon}$ AUC did not demonstrate sex differences. Further studies are therefore needed to evaluate specific sex-related difference in brachial artery vascular response after training. Second, this study was not matched against a group of elderly and/or people presenting cardiovascular dysfunction. As mentioned in the discussion, a similar KE training model was previously demonstrated to have a positive effect on brachial artery FMD in a group of elderly without any changes in young people (Wray et al., 2006), leading to the hypothesis that the vascular health level pre-exercise training might be a pivotal factor to consider when changes in arteries functionality are expected.

Third, as previously stated, the lack of progressive FMD measurements throughout the 8-week training period could have disguised a possible brachial artery structural remodeling. However, no changes in baseline and peak brachial diameter were found after training. Moreover, in the light of the high reliability level obtained here, the lack of changes in FMD data after KE training could likely not be ascribed to the operator's skills level. Future studies investigating the effects of KE training are necessary to reveal a possible arterial remodeling in a distal artery from exercise.

CONCLUSION

The hypothesis that KE exercise could represents a paradigm able to increase peripheral $\dot{Q}$ was confirmed by the increase in $\dot{Q}_{brac}$ and $\dot{\Upsilon}_{brac}$ assessed during an acute KE training session, suggesting that possible positive results could have been found also in the upper limb as results of brachial artery measurement. In the present study, though, the magnitude of this stimulus was not sufficient to promote a significant vascular conditioning in the upper limb (i.e., brachial artery FMD), as in the lower limb (i.e., femoral artery PLM). Future studies are needed to assess possible effects of KE training on arteries in districts not directly involved with training in populations with reduced endothelial function, such as patients with heart failure of chronic obstructive pulmonary disease.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the "Institutional Review Board of the Università degli Studi di Milano with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Università degli Studi di Milano".

AUTHOR CONTRIBUTIONS

AVB, EC, SL, MV, RG, GC, SS, SR, EL, and FE conceived and designed the study. AVB, EC, SL, MV, RG, and SS performed the experiments. AVB, EC, and SL analyzed the data. AVB, EC, SL, and FE interpreted the results. AVB and EC prepared the figures. AVB, EC, SL, and FE drafted the manuscript. AVB, EC, SL, MV, GC, SS, SR, EL, RG, and FE edited and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00727/full#supplementary-material

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Influence of Hyperoxic-Supplemented High-Intensity Interval Training on Hemotological and Muscle Mitochondrial Adaptations in Trained Cyclists

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Background: Hyperoxia (HYPER) increases O_2 carrying capacity resulting in a higher O_2 delivery to the working muscles during exercise. Several lines of evidence indicate that lactate metabolism, power output, and endurance are improved by HYPER compared to normoxia (NORM). Since HYPER enables a higher exercise power output compared to NORM and considering the O_2 delivery limitation at exercise intensities near to maximum, we hypothesized that hyperoxic-supplemented high-intensity interval training (HIIT) would upregulate muscle mitochondrial oxidative capacity and enhance endurance cycling performance compared to training in normoxia.

Methods: 23 trained cyclists, age 35.3 ± 6.4 years, body mass 75.2 ± 9.6 kg, height 179.8 ± 7.9 m, and VO_2max 4.5 ± 0.7 L min^{-1} performed 6 weeks polarized and periodized endurance training on a cycle ergometer consisting of supervised HIIT sessions 3 days/week and additional low-intensity training 2 days/week. Participants were randomly assigned to either HYPER (F_IO_2 0.30; n = 12) or NORM (F_IO_2 0.21; n = 11) breathing condition during HIIT. Mitochondrial respiration in permeabilized fibers and isolated mitochondria together with maximal and submaximal VO_2, hematological parameters, and self-paced endurance cycling performance were tested pre- and posttraining intervention.

Results: Hyperoxic training led to a small, non-significant change in performance compared to normoxic training (HYPER 6.0 ± 3.7%, NORM 2.4 ± 5.0%; p = 0.073, ES = 0.32). This small, beneficial effect on the self-paced endurance cycling performance was not explained by the change in VO_2max (HYPER 1.1 ± 3.8%, NORM 0.0 ± 3.7%; p = 0.55, ES = 0.08), blood volume and hemoglobin mass, mitochondrial oxidative phosphorylation capacity (permeabilized fibers: HYPER 27.3 ± 46.0%, NORM 16.5 ± 49.1%; p = 0.37, ES = 3.24 and in isolated mitochondria: HYPER 26.1 ± 80.1%, NORM 15.9 ± 73.3%; p = 0.66, ES = 0.51), or markers of mitochondrial content which were similar between groups post intervention.

Conclusions: This study showed that 6 weeks hyperoxic-supplemented HIIT led to marginal gain in cycle performance in already trained cyclists without change in VO_2max, blood volume, hemoglobin mass, mitochondrial oxidative phosphorylation capacity, or exercise efficiency. The underlying mechanisms for the potentially meaningful performance effects of hyperoxia training remain unexplained and may raise ethical questions for elite sport.

Keywords: cycling performance, hyperoxia, high-intensity interval training, mitochondria, OXPHOS, VO_2max

INTRODUCTION

It is still under the debate whether strategies such as hyperoxia supplementation enabling acute improvement of exercise performance leads to superior training adaptations and therefore greater performance enhancement (Ploutz-Snyder et al., 1996; Hamalainen et al., 2000; Morris et al., 2000; Perry et al., 2005, 2007; Kilding et al., 2012; Przyklenk et al., 2017). However, despite the fact that previous studies have employed different study designs, training intervention lengths (3–6 weeks), exercise mode (continuous or high-intensity interval training), fractions of O_2 inspired (F_IO_2 range: 0.26–0.70), training status of the participants (i.e., untrained or trained cyclists), and different physical performance tests (all-out effort or time to exhaustion), an overall "likely positive" effect on performance has been found following hyperoxic-supplemented training compared to normoxic training (Mallette et al., 2017; Cardinale and Ekblom, 2018).

As recently reviewed (Cardinale and Ekblom, 2018), hyperoxia acutely improves lactate metabolism (Ekblom et al., 1975), reduces muscle glycogen utilization (Stellingwerff et al., 2006), and enables a higher exercise work rate compared to exercise in normoxia (Powers et al., 1989; Nielsen et al., 1999) while reducing submaximal exercise efficiency (Manselin et al., 2017). Acutely, hyperoxia increases oxygen (O_2) delivery to the working muscles (Ekblom et al., 1975) and completely prevents exercise-induced arterial hypoxemia (EIAH), i.e., "oxyhemoglobin SaO_2 below 95%" (Powers et al., 1989), a condition often found in individuals exercising at intensities approaching maximal oxygen uptake (VO_2max) (Dempsey and Wagner, 1999; Dempsey et al., 2008). Considering the O_2 delivery limitation at near maximal exercise intensities (Saltin and Calbet, 2006) and that the mitochondria possess an excess capacity above the O_2 delivery (Boushel et al., 2011), we postulated that hyperoxic-supplemented exercise training allowing a higher training load leads to a greater training stimulus at the muscle level and therefore greater performance enhancement compared to the same training regimen breathing normoxia. This hypothesis is supported by the finding that exercise training with recombinant human erythropoietin treatment enhanced skeletal muscle mitochondrial capacity compared to controls (Plenge et al., 2012).

Only a few studies have assessed markers of skeletal muscle metabolic adaptions following hyperoxic-supplemented endurance training (Ploutz-Snyder et al., 1996; Perry et al., 2007; Przyklenk et al., 2017). The greater enzyme activity involved in β-oxidation following hyperoxic training indicated by the higher β-hydroxyacyl-coenzyme A dehydrogenase shown by Ploutz-Snyder et al. (1996), was not later found by Perry et al. (2007). In both studies, glycolytic enzymes activities (phosphofructokinase, creatine kinase, and glyceraldehyde phosphate dehydrogenase) and components of oxidative phosphorylation (cytochrome c-oxidase and citrate synthase) were not enhanced with hyperoxic training. Furthermore, previous studies have been conducted on untrained or recreationally trained individuals, and the mechanism of action of hyperoxic-supplemented endurance training may differ in trained individuals. Only two studies have been conducted on trained individuals (Hamalainen et al., 2000; Kilding et al., 2012) with opposite results on performance, and without detail on potential physiological mechanisms. For these reasons, we designed a 6-week randomized controlled training study with parallel groups where both participants and training supervisors were blinded to the type of gas inhaled with the aim to assess the effect of hyperoxic-supplemented high-intensity interval training on physiological and performance outcomes in trained cyclists.

We hypothesized that hyperoxic-supplemented high-intensity interval training in trained cyclists would enhance the training stimulus on skeletal muscle and thereby improve cycle performance to a greater extent than a normoxic breathing condition due to an upregulated skeletal muscle mitochondrial oxidative phosphorylation capacity independent of cardiorespiratory or hematological adaptations.

MATERIALS AND METHODS

General Design

This study used a 6-week double-blind randomized controlled training study design with parallel groups stratified for participants' baseline VO_2max. Participants were randomly assigned to either an experimental group that trained breathing hyperoxia (F_IO_2 0.30; HYPER) or a sham-hyperoxia control group that trained in normoxia (F_IO_2 0.21; NORM). Within a week prior to and posttraining intervention, participants reported to the laboratory three times for baseline and post-tests, respectively. The training intervention consisted of 15 supervised high-intensity interval training (HIIT) bouts breathing either HYPER or NORM and 10 non-supervised low-intensity exercise sessions distributed within a 6-week period (explained in detail in "the training intervention" section). Inclusion criteria in the final analysis were an attendance of at least 85% of

the HIIT and low-intensity training sessions. Participants were informed of the possible risks and discomfort involved before giving their written consent to participate. The study was undertaken according to the Declaration of Helsinki and was approved by the Swedish Regional Ethics Committee (2014/1764-31/2 and 2017/630-32).

Participants

A group of 32 trained cyclists (24 men and 7 women), age 34.8 ± 7.3 years [mean ± standard deviation (SD)], body mass 72.9 ± 10.8 kg, height 177.7 ± 9.6 m, and VO_2max 4.4 ± 0.8 L min^{-1} participated in this study. Prior to inclusion, a larger group of cyclists completed a health screening survey and exercise tests for assessment of their cardiorespiratory fitness. Only the healthy subjects who regularly conducted endurance and ultra-endurance races and had been competing at a national or amateur level in the last 5–10 years prior to this study were selected for this study. The aim of including experienced cyclists is that this group of athletes shows a small magnitude of change in performance, cardiorespiratory fitness as well as metabolic adaptation even when intensifying their training. On average, each subject was accustomed to about 10–15 h exercise training per week. High-intensity interval training and resistance training was consistently implemented in their normal training regimen on average once per week; however 80–90% of the whole training time was spent at low and average exercise intensity.

Training Intervention

The training intervention consisted of a 6-week polarized and periodized endurance training on a cycle ergometer. The term *polarized* refers to the combination of both HIIT and low-intensity exercise sessions within the same mesocycle (i.e., 6 weeks) (Stöggl and Sperlich, 2014), whereas the term *periodized* refers to the variation of training load and intensity within the mesocycle. The 6-week intervention protocol used a periodization model with a relationship of 2:1 between hard weeks and easy weeks. This approach was taken to reduce the risk of overtraining syndrome and included a tapering period of a week before the post-tests assessments. Participants were scheduled to perform 15 supervised HIIT sessions breathing either HYPER or NORM and 10 low-intensity exercise sessions.

The HIIT sessions, scheduled on Monday, Wednesday and Friday each week, consisted of either three times 8-min intervals or four times 4-min intervals performed at maximal sustainable effort [equivalent to a rating perceived exertion, RPE, of 18–20 (Borg, 1970)], with 3-min active relief in between the exercise bouts on a cycle ergometer. The longer intervals were performed three times per week during weeks 1, 2, 4, and 5 of the training intervention, whereas the 4-by-4-min intervals were performed twice in week 3 and once in week 6. To progressively increase the participants' training load during the training intervention, a fourth interval of 4 min in length in week 4 and a fourth interval of 8 min in length in week 5 was added, in addition to the three times 8-min intervals as described above.

Each HIIT session was preceded by a standard 20-min warm up protocol on a cycle ergometer which included two bouts of 1 min at moderate/high-intensity exercise. This type of warm up has been shown to be superior to continuous warm up either at an intensity below or above threshold (McGowan et al., 2015). Overall, the 20-min warm up was performed as follows: 10 min cycling at an exercise intensity equivalent to 10–12 RPE, 1 min at 14–16 RPE, 3 min at 11–14 RPE, 1 min at 15–17 RPE, and 5 min at 10–12 RPE. During the last 30 s of the warm-up protocol, the participants wore a full-face mask with headgear used for gas administration (refer to section "Gas Administration" for the complete setup description). The participants removed the mask during the relief periods. At the completion of the HIIT session, a 10-min cool-down was performed at an exercise intensity equivalent to 10–12 RPE.

During the HIIT sessions, the only data shown to the participants were the cycling cadence and the elapsed exercise time, whereas the participants were blinded to breathing condition, power output, and heart rate. Since the HIIT sessions were self-paced by the participants who were blinded to power output, one or two training supervisors guided and encouraged the participants to assure that each interval of each session was performed at the highest sustainable intensity possible by the participants. For motivational and time-efficiency purposes, participants completed HIIT sessions in the company of 2–4 other participants. The training supervisors were also blinded to the breathing condition that the participants were assigned to during the whole duration of the training intervention.

Participants were encouraged to perform two low-intensity exercise training sessions consisting of about 2 h (LOW1) and 4 h (LOW2) long continuous exercise at ~75% of the individual maximal heart rate (equivalent to an RPE of 14–15). The participants conducted the LOW1 cycling either outdoors or on a stationary bike indoors on Tuesday or Thursday of weeks 1, 2, 4, 5, and 6 of the training intervention. A LOW2 session was scheduled each weekend during the 6-week training intervention.

Participants were requested to maintain supplementary training involving flexibility exercise and exercise specifically recruiting upper body muscles. No other strenuous exercise training was allowed during the 6-week intervention except for the one above described.

Gas Administration

The gas was delivered to the participants through a face mask which was connected, by tubing, to a dosage unit (Oxelerate, Tumba, Sweden) which in turn was connected to a gas tank filled with either pure medical oxygen or medical air gas (i.e., 21% O_2). For subjects assigned to the hyperoxia group, the dosage unit intermittently delivered a gas bolus at the beginning of each participant's inhalation that was mixed with room air inside the mask cavity resulting in a final F_IO_2 of ~0.30, as established by previous work (Lindholm et al., 2017). The participants and the training supervisors were blinded to the type of gas inhaled.

Procedures and Measures

Participants reported to the laboratory on three separate occasions with 2–3 days in between within the week prior to the start

of the exercise intervention and a week after the last training session. Participants abstained from strenuous physical activity 24–48 h prior to each occasion.

The first occasion consisted of skeletal muscle biopsy collection and total hemoglobin mass assessment which was scheduled in the morning between 07:00 and 10:00 h to limit the circadian influence. At the second occasion, participants performed a submaximal and maximal incremental test on cycle ergometer in the afternoon between 15:00 and 19:00 h. The third occasion consisted of a self-paced cycling performance test scheduled between 09:00 and 17:00 h.

Muscle Biopsy Sampling

A skeletal muscle sample was obtained from the middle portion of the *vastus lateralis* muscle at a depth of 2–3 cm, about one-third of the distance from the upper margin of the patella to the anterior superior iliac spine with the participants resting in a semi recumbent position lying on a bench. After local anesthesia (2–4 ml carbocaine 20 mg ml^{-1}; AstraZeneca, Södertälje, Sweden), an incision (0.5–1 cm) was made through the skin and fascia and a muscle sample (50–100 mg) was obtained with the Weil-Blakesley chonchotome technique. A portion of the sample was snap frozen in liquid nitrogen, while second and third portions were rapidly placed in ice-cold mitochondrial isolation medium and relaxing medium (see section below), followed by mitochondria isolation and muscle fiber permeabilization, respectively, as later described.

Hematological Parameters

A subgroup of 12 participants (n = 6 per group) was tested for total hemoglobin mass (Hb_{mass}) assessment performed as described elsewhere (Burge and Skinner, 1995) with some minor modification of the rebreathing technique. Briefly, with the participants still lying on the bench in a semi recumbent position following the biopsy collection, 15 ml of blood was sampled from an antecubital vein *via* a 20-gauge venflon and analyzed immediately for Hb concentration (Hb) using HemoCue® Hb 201+ System (HemoCue AB, Ängelholm, Sweden); and hematocrit in quadruplets with micro-method (3 min at 13,500 rpm). About 1.5 ml of the blood sample was then quickly transferred to a 2-ml Eppendorf tube and stored at −80°C until percent carboxyhemoglobin (%HbCO) analysis using an hemoximeter (ABL800, Radiometer, Copenhagen, Denmark). After baseline collection, the participants breathed from a Douglas bag previously filled with pure oxygen for 4 min to flush nitrogen from the airways. During this time, the operator flushed the re-breathing circuit with the pure oxygen which was then closed. After 4 min, the operator switched the participant to the rebreathing circuit and a precisely measured bolus of 1.2 ml kg^{-1} body mass of 99.997% chemically pure CO (CO N47, Air Liquide, Paris, France) was injected into the circuit. The participants then breathed the gas mixture for 10 min. Thereafter, an additional venous blood sample was collected from an antecubital vein for assessment of the change in (%HbCO) accounting for the CO remaining in the re-breathing circuit which was determined (Monoxor III, Bacharach Inc., New Kensington, USA). Hb_{mass} was calculated from the change in %HbCO and total red blood cell volume (RCV), blood volume (BV), and plasma volume (PV) were derived (Burge and Skinner, 1995).

Submaximal Exercise Test

To determine the power output at lactate inflection point using a modified Dmax method, a sub-group of 12 participants (n = 6 per group) cycled at 4–6 intervals (i.e., 30, 150, 185, 220, 255, 290, and 325 W) each of 4 min in length on a cycle ergometer (Monark LC6, Monark Exercise AB, Vansbro, Sweden). Participants cycled with a constant, freely chosen cadence until blood lactate concentration (Biosen C-Line Clinic; EKF-diagnostic GmbH, Barleben, Germany) measured at the end of each interval from fingertips, reached a concentration higher than 4 mMol L^{-1}. Participants cycled wearing a Hans Rudolf mask (Hans Rudolph Inc., Kansas, USA) which covered mouth and nose for assessment of pulmonary oxygen consumption (Jaeger Oxycon Pro; CareFusion GmbH, Hoechberg, Germany). The metabolic cart was calibrated prior to each test according to the manufacturer's instructions, with high-grade calibration gases (Air Liquide, Paris, France). Respiratory variables were measured and averaged every 10 s. The averaged VO_2 recorded during the last minute of each interval was taken as the representative oxygen consumption for that specific power output. After the last interval, participants were allowed 10 min cycling at an intensity equal to 10–12 RPE to recover and get ready for subsequent graded incremental exercise test.

The blood lactate concentrations representative for each exercise intensity were used to calculate the power output at lactate inflection point using a modified Dmax method (Cheng et al., 1992), i.e., defined as the derivate to the exponential curve created from exponential lactate increase, including maximal lactate concentration obtained from the successive maximal increment test. The increase in lactate relative to power output was defined as the increase in blood lactate from the point where the exponential curve crossed the lactate baseline.

The mean oxygen consumption and the respiratory exchange ratio between minute 3 and 4 at each cycled stage was used to calculate energy expenditure with the equation developed by Brouwer (1957). Cycling efficiency when cycling at 150 W was expressed as gross efficiency (GE) and work efficiency (WE) as described elsewhere (Mogensen et al., 2006).

Maximal Incremental Test

All participants performed a graded incremental exercise test until volitional exhaustion on a cycle ergometer to determine VO_2max and time to exhaustion. Participants pedaled at a fixed cadence and the load was increased by 25 and 20 W min^{-1} for men and women, respectively. Participants cycled wearing a Hans Rudolf mask (Hans Rudolph Inc., Kansas, USA) which covered mouth and nose for assessment of pulmonary oxygen consumption (Jaeger Oxycon Pro; CareFusion GmbH, Hoechberg, Germany). VO_2max leveling-off criteria were applied (i.e., a VO_2 plateau, followed by exercise cessation or decrease of VO_2 at higher work rates, with an RER >1.10).

The power output that the participants pedaled at the time of the volitional exhaustion was taken as the maximal power output (Winc.). O_2 consumption was measured with a metabolic cart (OxyconPro, Jaeger GmbH, Germany), calibrated prior to each test according to the manufacturer's instructions, with high-grade calibration gases (Air Liquide, Paris, France). Respiratory variables were measured and averaged every 10 s. The highest 60 s averaged VO_2 recorded was taken as the VO_2max.

Cycle Performance Test

The self-paced endurance cycling performance test consisted of pedaling for 20 min at the maximal sustainable effort with the intent to obtain the highest mean power output on a cycle ergometer (Monark LC2, Monark Exercise AB, Vansbro, Sweden) equipped with a power meter (SRM power meter science road; SRM International, Jülich, Germany) for power output assessment. The test was preceded by a standard 20-min warmup as described in the "Training intervention" section. Briefly, the participants cycled for 10 min at an exercise intensity equivalent to 10–12 RPE, 1 min at 14–16 RPE, 3 min at 11–14 RPE, 1 min at 15–17 RPE, and 5 min at 10–12 RPE. During the last 5 min of the warm up, the participants were equipped with a Hans Rudolf mask (Hans Rudolph Inc., Kansas, USA) which covered mouth and nose for assessment of pulmonary oxygen consumption (Jaeger Oxycon Pro; CareFusion GmbH, Hoechberg, Germany) and a pulse oximetry sensor (Rad-97 Masimo Corporation; Neuchatel, Switzerland) was positioned on the participant's forehead for assessment of the peripheral capillary oxygen saturation (SpO_2).

During the test, the only data shown to participants were the cycling cadence and the elapsed exercise time, whereas the participants were blinded to power output and heart rate. The test leader verbally encouraged the participants during the whole test length. For motivational purposes, music chosen by the participant was played during test. The participants completed the test with a 10 min cool-down performed at an exercise intensity equivalent to 10–12 RPE. Participants were all familiar to this test and performed one or two familiarization tests prior the baseline test, which showed a typical error of measurement of about 2%.

Permeabilized Fiber Preparation

Muscle fiber bundle permeabilization was performed as previously described (Pesta and Gnaiger, 2012; Cardinale et al., 2018a). Briefly, a portion of the muscle biopsy (~5 mg wet weight) was immediately transferred into ice-cold relaxing medium (BIOPS) containing 10 mM/L Ca^{2+}/EGTA buffer, 20 mM/L imidazole, 50 mM/L K^+-4-morpholinoethanesulfonic acid (Mes), 0.5 mMol/L dithiothreitol, 6.56 mM/L $MgCl_2$, 5.77 mM/L ATP, and 15 mMol/L phosphocreatine at pH 7.1. A portion of the 5 mg wet weight sample (~1–3 mg) was transferred into BIOPS in a small petri dish on an ice-cold metal plate where the fiber bundles were mechanically separated using forceps and needles. Thereafter, approximately 10–15 fibers were slowly agitated for 30 min on a platform shaker in BIOPS containing saponin (5 mg/ml saponin) solution at 4°C. Fibers were then washed for 10 min at 4°C in ice-cold mitochondrial respiration medium (MiR06; 0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 g/L BSA essentially fatty acid free, adjusted to pH 7.1, 2.8 U/mg solid catalase lypophilized powder). The fibers were weighed on a microbalance after having been blotted on filter paper and transferred into the respirometry chamber.

Isolation of Mitochondria

A portion of the muscle biopsy (40–80 mg wet weight) designated for mitochondrial isolation was first weighed and then cut in ice-cold isolation medium (sucrose 100 mM, KCl 100 mM, Tris-HCl 50 mM, KH_2PO_4 1 mM, EGTA 100 µM, BSA 0.1%; final pH was set to 7.4). The homogenate was washed in 1 ml isolation medium and the supernatant was removed. One ml of isolation medium containing 0.2 mg ml^{-1} bacterial protease was added to the homogenate. The homogenate was gently agitated every 30 s for ~2-min time and then transferred in a pre-cooled glass jacket connected to an ice-cold bath pump and further homogenized with a hand held electrically driven drill (80 rpm). The final homogenate was then transferred to a falcon tube containing 3 ml isolation medium and then subsequently centrifuged at 700 *g* at 4°C for 10 min. After removing the pellet, the suspension was again centrifuged at 10,000 *g* at 4°C. The resultant mitochondrial pellet was re-suspended in the same medium. The Eppendorf was then centrifuged at 7,000 *g* for 5 min and the pellet was dissolved in 0.6 µl preservation medium (EGTA 0.5 mM, $MgCl_2 \cdot 6H_2O$ 3 mM, K-lactobionate 60 mM, Taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, sucrose 110 mM, BSA 1 g L^{-1} histidine 20 mM, vitamin E succinate 20 µM, glutathione 3 mM, leupeptine 1 µM, glutamate 2 mM, malate 2 mM, and Mg-ATP 2 mM) per mg wet weight.

Mitochondrial Respiration

Mitochondrial respiration was performed in a two-channel high-resolution respirometer (Oroboros Oxygraph, Paar, Graz, Austria). The glass chamber volume (2 ml capacity) was sealed with rubber-ringed stoppers to minimize the O_2 back diffusion into the chamber (Steinlechner-Maran et al., 1996). Polyvinylidene difluoride magnetic stirrers set to 750 rpm were used to stir the sample in to the medium. Data were collected at 1-s intervals and averaged over 40 s. All experiments were run in duplicate and the respiration data for each of the two chambers were then averaged. The medium in the respiration chamber was MiR05 containing EGTA 0.5 mM, $MgCl_2 \cdot 6H_2O$ 3 mM, K-lactobionate 60 mM, taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, sucrose 110 mM, and BSA 1 g L^{-1}. All experiments were performed at 37°C. Time constants for complete mixing in the chamber were calculated by briefly stopping and starting the stirrers. O_2 consumption and zero-drift of the O_2 electrode were calculated using DatLab 5.2 software (Oroboros, Paar, Graz, Austria). At least five different O_2 tensions (400–0 nMol/ml) were used during the background calibration to calculate and account for the diffusion of O_2 into the chamber.

Mitochondrial Respiration in Permeabilized Fibers

Mitochondrial respiration was measured by adding the following substrates into the chambers (final concentrations): octanoylcarnitine (0.2 mM), malate (2 mM) for assessment of leak respiration (EFT_L), ADP (2.5 mM) to support electron entry from fatty acid β-oxidation through electron-transferring flavoprotein and complex I (EFT_P), followed by pyruvate (5 mM) and glutamate (10 mM) to stimulate complex I (CI_P), and succinate (10 mM) to stimulate complex I and II linked respiration ($CI + II_P$). Following inhibition of complex III with antimycin A (mM), N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (0.5 mM) and ascorbate (2 mM) were added followed by sodium azide (100 mM) for cytochrome C oxidase activity assessment. O_2 flux from the permeabilized fiber preparation was normalized per initial fiber wet weight.

Mitochondrial Respiration in Isolated Mitochondria

The same titration protocol described above was used to measure O_2 flux in isolated mitochondria with the exception that ADP was left to be completely phosphorylated to ATP following β-oxidation respiration allowing respiration proceed to state 4 (S4). Furthermore, carbonyl cyanide m-chloro phenyl hydrazine (0.05 μM steps) was used to measure maximal uncoupled oxidative phosphorylation (Unc). O_2 flux from isolated mitochondria was normalized by protein concentrations determined in aliquots of supernatant diluted 1:10 in distilled water using the Pierce 660 nm protein assay (Thermo Scientific).

Citrate Synthase Activity

A portion of the freeze-dried muscle samples was first cleansed of visible blood, fat and connective tissue and subsequently homogenized in ice-cold buffer (100 μl/mg dry weight) consisting of 50 mM KH_2PO_4, 1 mM EDTA and 0.05% Triton X-100 using a Bullet Blender (NextAdvance, Averill Park, NY) with 0.5 mm ZrO beads. The Eppendorf tubes containing the homogenates were rotated for 30 min at 4°C before being centrifuged at 1,400 *g* for 1 min at 4°C. CS activity was measured on a 96-well plate in a reagent solution (in mM): 50 Tris-HCl, 0.2 DTNB, and 30 acetyl-CoA. The reaction was initiated by adding oxaloacetate (10 mM) and the change in absorbance at 412 nm was measured spectrophotometrically at 25°C.

Protein Extraction and Immunoblot Analysis

A portion of the snap frozen biopsy sample was (1) freeze-dried, (2) cleansed of visible blood, fat, and connective tissue and subsequently, and (3) homogenized in ice-cold buffer (100 μl/mg dry weight) consisting of 2 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 1% Triton X-100, 2 mM dithiothreitol, and 1.5% phosphatase and protease inhibitor cocktail (Halt™, Thermo Scientific, Rockford, MD) using a Bullet Blender (NextAdvance, Averill Park, NY) with 0.5 mm ZrO beads. The Eppendorf tubes containing the homogenates were 360° rotated for 30 min at 4°C before being centrifuged at 10,000 *g* for 10 min at 4°C. The obtained supernatant was stored at −80°C. Protein concentrations of the homogenates were determined using the Pierce 660 nm protein assay (Thermo Scientific). Muscle homogenates were diluted with 4× Laemmli sample buffer (Bio-Rad, Richmond, CA) and homogenizing buffer to obtain a final protein concentration that was similar between all samples. Subsequently, all samples were heated at 95°C for 5 min to denature proteins, and then stored at −20°C until further analysis.

Samples were separated by SDS polyacrylamide gel electrophoresis (PAGE) on 26-well Criterion TGX gradient gels (4–20% acrylamide; Bio-Rad). Samples from all three groups were loaded on the same gel. The blots were quantified using Quantity One software version 4.6.3 (BIORAD). To control for appropriate loading and transfer, target proteins were expressed relative to total protein stained at ~95 kDa obtained by staining the membranes with MemCode Reversible Protein Stain Kit (Thermo Scientific) (Antharavally et al., 2004).

The monoclonal primary cytochrome c oxidase antibody (#4850; 1:1,000; Cell Signaling Technology, Danvers, USA) conjugated with a secondary anti-rabbit antibody (#7074; 1:10,000; Cell Signaling Technology, Danvers, USA) was used for the detection of target total protein.

Statistical Analysis

Normal distribution of the data was checked by assessing skewness. Baseline characteristics of each group were summarized using descriptive statistics. Exact χ^2 tests were used to evaluate if differences existed between groups for categorical variables at baseline. For between-groups analyses, one-way analysis of variance (one-way ANOVA) was conducted using the variable changes pre to post intervention. A two-tailed $p < 0.05$ was considered significant. Analysis of covariance (ANCOVA) with baseline-test results as a covariate and the post-test as the dependent variable was also performed on the same dataset to ensure that baseline measurements did not affect the statistical results. The use of one-way ANOVA or ANCOVA did not influence the interpretation of the study results. Effect sizes and associated confidence intervals were interpreted according to Cohen's guidelines (Cohen, 1988), effect sizes with scores of 0.2–0.5, 0.5–0.8, and >0.8 were considered small, medium, and large effects, respectively. Paired sample *t*-test was used to assess within-group differences pre- to post measurement. Statistical analyses were carried out using SPSS statistical software version 21 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Training Adherence, Exercise Intensity, and Training Load

Of the initial 32 trained cyclists, 23 of those (19 men and 4 women), with age 35.3 ± 6.4 years [mean ± standard deviation (SD)], body mass 75.2 ± 9.6 kg, height 179.8 ± 7.9 m, and VO_2max of 4.5 ± 0.7 $L \cdot min^{-1}$, successfully adhered to the training regimen (NORM attended 95.8 ± 5.4% of the HIIT sessions and 90.1 ± 8.6% of the LOW1/LOW2 sessions; HYPER attended 94.4 ± 5.6% of the HIIT sessions and 95.5 ± 4.7% of the LOW1/LOW2 sessions) and were included for further analysis. The two groups NORM ($n = 11$) and HYPER ($n = 12$)

did not differ at baseline for any measured variables. Participants' characteristics are shown in **Table 1**. During the HIIT sessions, HYPER consistently trained at 3.3 ± 1.2% higher relative intensity than NORM despite a similar rating of perceived exertion (i.e., NORM RPE 8.3 ± 1.0 and HYPER RPE 8.2 ± 0.7). However, the higher training intensity did not lead to a significantly higher training load over the intervention compared to NORM (p = 0.37) (**Figure 1**).

TABLE 1 | Characteristics and baseline measures of the hyperoxia (HYPER) and normoxia (NORM) group.

	NORM	HYPER
n	11	12
Women	1	3
Men	10	9
Age (years)	37.3 ± 4.5	33.4 ± 7.6
Height (cm)	182.8 ± 5.0	177.2 ± 8.6
Body mass (kg)	77.2 ± 7.5	73.2 ± 11.8
Time to exhaustion during maximal incremental test (s)	395.8 ± 55.3	406.3 ± 47.1
Winc. (W)	404.1 ± 45.0	387.9 ± 55.2
VO_2max (L min^{-1})	4.7 ± 0.7	4.4 ± 0.7
VO_2max (ml min^{-1} kg^{-1})	60.3 ± 5.1	60.4 ± 5.3
Mean power output during 20 min trial (W)	300.9 ± 41.5	281.0 ± 41.0
Mean power output during 20 min trial (W kg^{-1})	3.9 ± 0.4	3.9 ± 0.4
EIAH during 20 min trial (*n*)	7 (64%)	6 (50%)

Data are mean ± SD. Winc., maximum watt pedaled during the maximal incremental test; EIAH, exercise induced arterial hypoxemia (defined as SaO_2 lower than 95%).

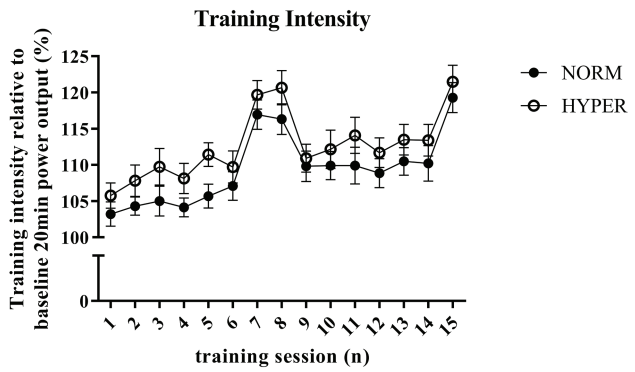

FIGURE 1 | Overview of the training intensity and progression of the normoxic (NORM) and hyperoxic (HYPER) group during the 6-week training intervention. Training intensity of each high-intensity interval session is expressed in percentage of the mean power output obtained during the baseline self-paced 20-min cycling trial. Note that the higher training intensity during training session number 7, 8, and 15 is attributed to the higher exercise intensity obtained when performing 4 × 4 min interval training session instead of the otherwise performed 3 × 8 min intervals. This figure shows (1) the numerically but not significantly higher training intensity performed by HYPER compared to NORM throughout the training intervention which was independent of the interval duration, (2) the increasingly higher training intensity performed by both groups during the intervention period which indicated that cycle performance was enhanced in both groups independent of hyperoxia.

Skeletal Muscle Mitochondrial Adaptations

The permeabilized fibers assay revealed that the training intervention had no effect on mass-specific (normalized to tissue wet weight) leak respiration, fatty acid oxidation, maximal oxidative phosphorylation respiratory, and electron transfer system capacity of the skeletal muscle with no difference between groups (**Figure 2A**). Maximal oxidative phosphorylation numerically increased (22.6 ± 46.1%) but did not reach the level of significance over the intervention (p = 0.20), between (p = 0.37, ES = 3.24) or within groups (NORM 16.5 ± 49.1% p = 0.86; HYPER 27.3 ± 46.0%, p = 0.15) and showed a great individual variability in response to exercise training (**Figure 3**). Although no differences over the intervention were detected between groups, fatty acid oxidation decreased over the intervention only in NORM (p = 0.03).

Similarly, the isolated mitochondria assay showed only a small relative increase (21.0 ± 75.1% p = 0.90) in intrinsic maximal mitochondrial respiration over the intervention

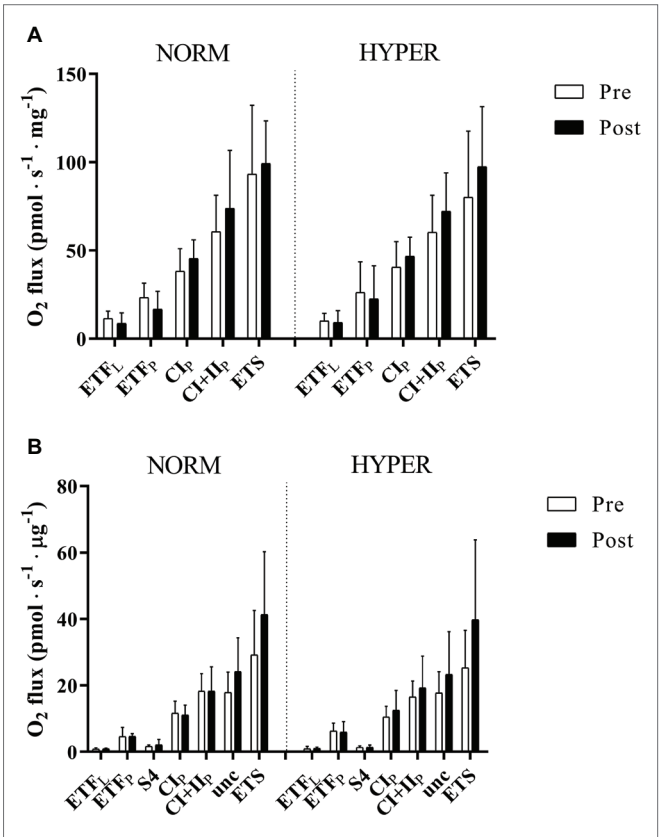

FIGURE 2 | Mitochondrial respiration pre and post 6 weeks polarized and periodized high-intensity interval training either in normoxia (NORM) or hyperoxic-supplemented (HYPER). All values are expressed as means ± SD. In panel **(A)**, O_2 flux rates obtained from measurement of mitochondrial respiration using permeabilized fibers technique are expressed in pmol s^{-1} mg^{-1} fibers initial wet weight, whereas in panel **(B)**, O_2 flux obtained from measurement of isolated mitochondria the O_2 flux rates are presented in pmol s^{-1} µg^{-1} protein. Electron-transferring flavoprotein complex (ETF), state 4 (S4), complex I (CI), complex I + II-linked substrate state (CI + II), and uncoupling state (Unc) electron transport system capacity (ETS). Leak respiration and oxidative phosphorylation are indicated by subscripts L and P, respectively.

(**Figure 2B**) with no change between (p = 0.66, ES = 0.51) or within groups (NORM 15.9 ± 73.3% p = 0.99; HYPER 26.1 ± 80.1%, p = 0.54).

The unchanged *ex vivo* mitochondrial oxidative phosphorylation over the intervention paralleled the unchanged biomarkers of mitochondrial content, i.e., citrate synthase (CS) activity (p = 0.42 and between groups p = 0.41). CS activity in NORM went from 238.7 ± 53.2 to 239.3 ± 39.5 nM min^{-1} mg^{-1} (p = 0.97) and HYPER from 239.4 ± 37.3 to 221.9 ± 48.6 nM min^{-1} mg^{-1} (p = 0.28). Similarly, cytochrome C oxidase protein levels did not change over the intervention (p = 0.93) and between groups (p = 0.95). Cytochrome C oxidase protein levels in NORM went from 11.6 ± 6.4 to 11.6 ± 4.2 a.u. (p = 0.99) and HYPER from 7.7 ± 3.6 to 7.6 ± 3.6 a.u. (p = 0.90).

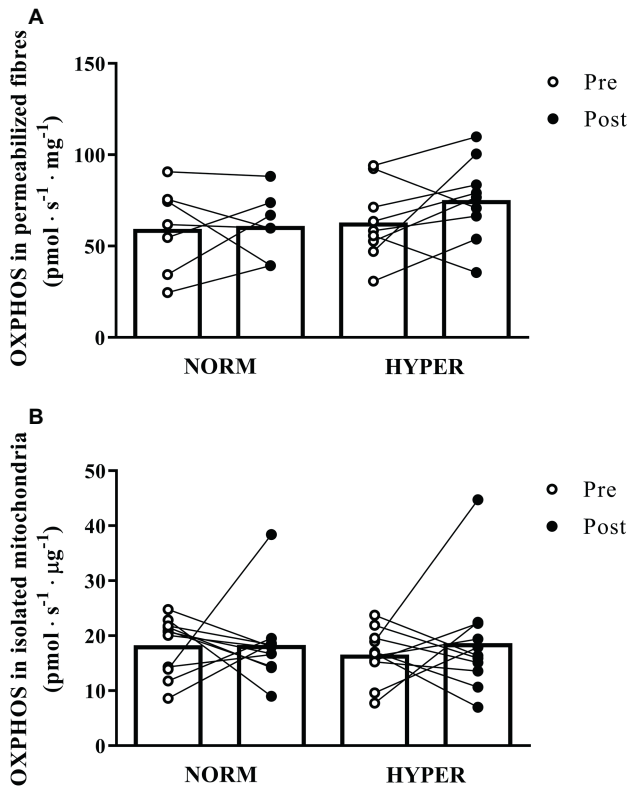

FIGURE 3 | Maximal mitochondrial respiration (OXPHOS) pre and post 6 weeks polarized and periodized high-intensity interval training either in normoxia (NORM) or hyperoxic-supplemented (HYPER). All values are shown as mean and individual response following the intervention. In panel **(A)**, O_2 flux rates obtained from measurement of mitochondrial respiration using permeabilized fibers technique are expressed in pmol s^{-1} mg^{-1} fibers initial wet weight, whereas in panel **(B)**, O_2 flux obtained from measurement of isolated mitochondria are presented in pmol s^{-1} μg^{-1} protein.

Hematology

Hematology variables are presented in **Table 2**. All parameters were unaltered by the training intervention and no differences were detected between groups.

Submaximal Measurements

Power outputs at lactate inflection point did not change over the intervention (p = 0.73). NORM went from a power output at lactate inflection point of 281.8 ± 42.1 to 282.9 ± 44.9 W (p = 0.78) and HYPER from 288.2 ± 37.2 to 289.3 ± 29.2 W (p = 0.84). No change in gross efficiency (GE) and work efficiency (WE) when cycling at 150 W were observed pre to post intervention with no difference between groups (NORM GE from 19.0 ± 0.9 to 19.3 ± 1.1% and HYPER GE 19.7 ± 0.1 to 20.0 ± 1.3%; NORM WE from 24.2 ± 1.0 to 24.9 ± 0.5% and HYPER WE from 25.0 ± 1.3 to 25.7 ± 1.7%).

VO_2max and time to exhaustion

Overall, VO_2max was maintained over the course of the intervention (p = 0.58) with no difference between groups (p = 0.55, ES = 0.08). NORM went from a VO_2max of 4.7 ± 0.7 to 4.6 ± 0.7 L min^{-1} (0.0 ± 3.7%, p = 0.84) and HYPER from 4.4 ± 0.7 to 4.5 ± 0.8 L min^{-1} (1.1 ± 3.8%, p = 0.33). Time to exhaustion significantly improved pre to post intervention from 401.3 ± 42.1 to 426.8 ± 49.7 s (p = 0.001) with no difference between groups (p = 0.31, ES = −0.18).

Endurance cycling performance

Overall, the 6-week training intervention enhanced the absolute (3.7 ± 4.6%; p = 0.001) and relative to body mass

TABLE 2 | Body mass and intravascular volumes before and after training intervention.

	NORM		HYPER		E.S.	*p*
	Pre	Post	Pre	Post		
[Hb] (g L^{-1})	14.8 ± 0.7	14.8 ± 042	14.9 ± 1.1	14.4 ± 0.7	−1.06	0.56
Hct (%)	44.4 ± 2.2	45.3 ± 1.8	45.9 ± 3.0	44.7 ± 1.7	−1.87	0.31
*n*Hb (g)	962.6 ± 131.2	964.8 ± 160.7	986.4 ± 255.2	978.5 ± 292.8	−0.36	0.82
Hb_{mass} (g kg^{-1})	12.3 ± 0.5	12.3 ± 0.9	13.3 ± 1.7	13.0 ± 1.9	−0.16	0.92
RCV (ml)	2,889.9 ± 423.1	2,958.1 ± 516.6	3,038.6 ± 748.9	3,026.5 ± 891.8	−0.12	0.94
PV (ml)	3,650.0 ± 690.8	3,562.3 ± 520.1	3,547.7 ± 679.2	3,732.3 ± 1,060.7	1.11	0.22
BV (ml)	6,539.8 ± 1,097.5	6,520.4 ± 1,016.1	6,586.7 ± 1,385.0	6,758.7 ± 1,941.9	0.88	0.37

Values are means ± SD. [Hb], hemoglobin concentration; Hct, hematocrit; Hb_{mass}, total hemoglobin mass; RCV, total red blood cell volume; PV, plasma volume; BV, blood volume. Measurements were performed before (Pre) and after Post training intervention in the group breathing normoxia (NORM: n = 6) and in the hyperoxia group (HYPER: n = 6). The level of significance and the effect size (E.S.) is indicated.

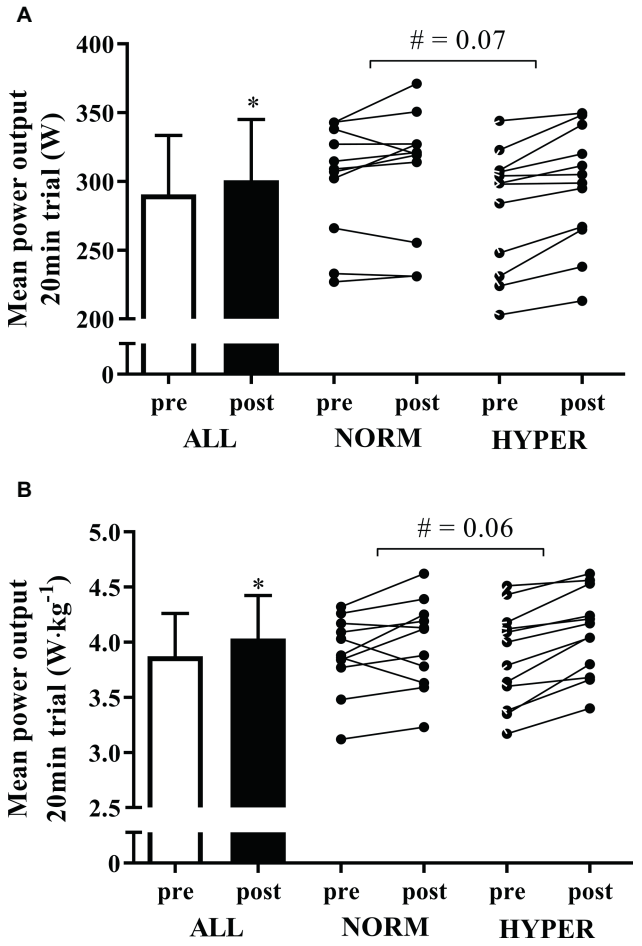

FIGURE 4 | Mean ± SD of the mean power output during the self-paced 20-min cycling trial pre and post intervention expressed in absolute Watts **(A)** and Watts relative to body mass **(B)** for all the participants as well as the individual response of the participants in normoxia (NORM) and hyperoxia (HYPER) groups. Note: $^{*}p < 0.05$, $^{\#}p$ between groups.

(4.3 ± 4.7%; $p = 0.0002$) mean power output during the self-paced 20-min cycling trial (**Figure 4**). The magnitude of improvement in mean power output during the 20-min cycling trial was larger in HYPER compared to NORM (for mean power output, $p = 0.07$, ES = 0.22; for mean power output relative to body mass, $p = 0.06$, ES = 0.32). NORM numerically increased absolute mean power output during the 20-min cycling trial by 1.6 ± 4.3% ($p = 0.23$), whereas HYPER significantly enhanced power output by 5.6 ± 4.2% ($p = 0.0006$) (**Figure 4**). The power output during the 20-min test normalized per body mass numerically increased by 2.4 ± 6.0% in NORM ($p = 0.15$) and significantly in HYPER by 6.0 ± 3.7% ($p = 0.0001$).

DISCUSSION

This study presents novel findings on performance effects and physiological responses to high-intensity interval training with hyperoxia in already trained cyclists. We showed that 6-week high-intensity interval training induced non-significant, but potentially meaningful performance gains without affecting VO_2max, hematological parameters, mitochondrial oxidative phosphorylation capacity, and biomarkers of mitochondrial content in already trained cyclists. Our results are in line with the study of Kilding et al. (2012) who, using a similar polarized training (Seiler, 2010) intervention with a F_IO_2 of 0.60, showed that training with hyperoxia had no significant physiological benefit in trained cyclists of a similar performance level to those tested in our study. This study shows that training with hyperoxia induces no change in hematological or muscle oxidative metabolic capacity as underlying mechanisms for endurance performance.

Hyperoxia acutely enables a higher exercise intensity and therefore larger mechanical work produced over the intervention compared to when breathing normoxia (Perry et al., 2005, 2007; Kilding et al., 2012). Despite a numerically greater exercise intensity in HYPER compared to NORM, contrary to our hypothesis, the higher relative intensity and greater mechanical work led to a small positive effect in performance compared to normoxia but did not induce superior physiological training adaptations. Of note, cyclists could only see cadence data during the HIIT sessions and were blind to breathing assignment, power output and heart rate while pedaling at the maximum effort during the 4- and 8-min intervals. Our findings indicate that a further increase in exercise intensity of an already high-intense exercise regimen does not necessarily lead to additional gains in skeletal muscle training adaptations. However, individual performance change over the intervention (**Figure 4**) revealed that breathing hyperoxia allowed a positive training response compared to normoxia in some individuals (2 of 11 cyclists in NORM slightly decreased power output during the 20-min cycling trial pre- to postintervention). We speculate that hyperoxic-supplemented training may be advantageous in individuals who show a lower magnitude of performance change compared to the group response. With this consideration, hyperoxic-supplemented endurance high-intensity training may have an effect in erroneously categorized "non-responders" prior to increasing training frequency as previously suggested (Montero and Lundby, 2017).

Improved arterial oxygen saturation with hyperoxia has been reported at exercise intensities near to VO_2max thus preventing EIAH (Nielsen, 2003). EIAH is linked to the alveolar-capillary diffusion limitation due to decreased Hb mean-transit time in the lung (Dempsey and Wagner, 1999) caused by mechanical ventilatory constraint during exercise (Dominelli et al., 2013). EIAH has been reported to occur in ~50% of highly trained endurance athletes at sea level (Powers et al., 1988) and is more pronounced in both active and well-trained females (St Croix et al., 1998; Guenette and Sheel, 2007) than in male athletes (Anselme et al., 1994). In line with previous findings, 57% of our participants showed EIAH at intensities near to maximum; 7 of the 11 cyclists in the NORM, and 6 of the 12 cyclists in HYPER. The lower the SaO_2 during exercise intensity near to maximum the larger is the effect of acutely breathing hyperoxia on exercise tolerance (Ohya et al., 2016). Therefore, it can be expected that maintaining Hb fully saturated

at an exercise intensity near to VO_2max in cyclists who otherwise exhibit EIAH in normoxia would lead to improved skeletal muscle training adaptations resulting from the increased O_2 delivery. Contrary to what we hypothesized, increasing O_2 delivery, partially preventing EIAH to occur, did not improve exercise adaptations when breathing hyperoxia. Furthermore, in HYPER, the change in performance did not correlate with the SaO_2 levels during the cycle performance test (**Figure 5**). By contrast, there was a trend for an opposite relationship whereby cyclists manifesting high SaO_2 during the cycle performance test in normoxia demonstrated a larger magnitude of change in performance over the hyperoxic intervention.

Fundamental papers have shown that the convective component of the oxygen cascade limits VO_2max (Ekblom et al., 1968; Saltin and Calbet, 2006) and that the maximal mitochondrial oxidative phosphorylation is in excess over the O_2 delivery (Boushel et al., 2011) in healthy individuals. We hypothesized that increasing microvasculature PO_2 by hyperoxia would increase *in vivo* mitochondrial relative activation (Cardinale et al., 2018b), such that each mitochondrion would respire at a higher rate *in vivo* and in turn be exposed to a higher adaptive training stimulus. This hypothesis is supported by the greatly enhanced peripheral adaptations of skeletal muscle following one-legged cycling where higher O_2 delivery per active muscle mass occurs compared to cycling (Abbiss et al., 2011). By contrast, we did not find support for this notion. It is likely that mitochondrial respiratory capacity may be still in excess of the O_2 delivery despite the increased O_2 carrying capacity due to hyperoxia while cycling. Our findings indicate that the greater mitochondrial activation induced when breathing hyperoxia compared to normoxia during cycling did not induce greater mitochondrial biogenesis and/or intrinsic function. It has been shown that hyperoxia does not increase *in vivo* mitochondrial respiration during exercise in obese untrained but only in patients with type II diabetes with impaired *ex vivo* mitochondrial respiration (Cree-Green et al., 2018). The unchanged *in vivo* mitochondrial respiration in obese untrained but overall healthy individuals when breathing hyperoxia can be explained by a lower mitochondrial O_2 affinity ($p50_{mito}$), a novel mechanism regulating oxygen diffusion from microvessels to muscle mitochondria with direct effects on oxygen consumption (Cardinale et al., 2018b). In the present study, the lack of change in the hematological variables that could have altered the O_2 carry capacity and therefore O_2 delivery over the intervention indicates that training with hyperoxia does not induce changes in the capacity for oxygen delivery or utilization.

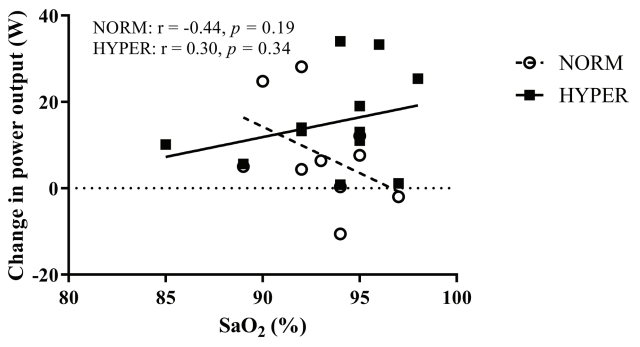

FIGURE 5 | Relationship between the change in power output during the 20-min self-paced cycling test over the training intervention and the mean arterial oxygen saturation (SaO_2) measured during the same test in participants in normoxia (NORM) and hyperoxia (HYPER) groups.

The unchanged mitochondrial content pre to post intervention suggests either that our trained cyclists had already reached a mitochondrial content plateau prior to the intervention (Montero and Lundby, 2017), that the increase in O_2 delivery with HYPER remained below mitochondrial capacity (maintained mitochondrial excess capacity) or that the training intervention did not increase the training volume to which our participants were accustomed to before the start of this study. The latter is supported by recent findings indicating that training volume significantly relates to CS activity (Granata et al., 2018) and that no plateau in CS activity should occur if training volume is constantly increased (Granata et al., 2018). However, the coefficient of variation of the change in OXPHOS measurements pre to post intervention (coefficient of variation of ~40%) was much larger than previously found in our laboratory (Cardinale et al., 2018a). The reason for this is unknown; we cannot discriminate if the variation came from the mitochondrial isolation procedure itself or if it was due to a large individual response to the hyperoxic and normoxic training stimulus. Nevertheless, the results on mitochondrial respiration should be interpreted with caution.

This study did not include direct neuromuscular measurements and it cannot be excluded that the numerically higher training intensity induced by hyperoxia increased muscle contractile properties which in turn led to small improvements in performance compared to normoxia. However, cycling efficiency was unchanged in both groups pre to post intervention. Furthermore, in Kilding et al., 2012's study, hyperoxic training did not improve peak and mean power output during a 60-s cycle sprint compared to normoxia. Therefore, it is unlikely that our results are explained by improved neuromuscular properties post intervention in the hyperoxic group.

This study attempted to recreate the real training scenario of trained cyclists including (1) fluctuations of training intensities within the micro-cycle, (2) recovery days, and (3) a tapering period prior to post testing (Meeusen et al., 2013) in a periodized and polarized training intervention program while still maintaining the rigor of controlled trials with a parallel group study design. The significant improvement in performance in our participants indicates that the overall training intervention was successful.

In conclusion, this study showed that 6-week hyperoxic-supplemented high-intensity interval training produced a small, potentially meaningful effect on cycling performance. This response was not explained by cardiorespiratory, hematological, or mitochondrial factors measured in this study. The underlying mechanisms for the performance effects of hyperoxia training remain unexplained, and may raise ethical questions for elite sport.

DATA AVAILABILITY

The datasets generated for this study can be found in figshare, https://figshare.com/s/90ea6a92bec059112c6b.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Swedish Ethics Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Regional Ethical Review Board in Stockholm.

AUTHOR CONTRIBUTIONS

DC, RB, BE, FL, and PL contributed to the conception and design of the experiment. JL, TM, and OS supervised the training. DC, RB, FL, JL, TM, OS, and SM contributed to the data collection. DC analyzed and interpreted the data and wrote the first draft of the manuscript which was reviewed by the other co-authors. All authors read and approved the final manuscript.

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frontiers in Physiology

ORIGINAL RESEARCH
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Sex Differences in Morphological and Functional Aspects of Exercise-Induced Cardiac Hypertrophy in a Rat Model

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Background: Recent evidences suggest that sex hormones may be involved in the regulation of exercise-induced left ventricular (LV) hypertrophy. However, the sex-specific functional consequences of exercise-induced myocardial hypertrophy is still not investigated in detail. We aimed at understanding the sex-specific functional and morphological alterations in the LV and the underlying molecular changes in a rat model of athlete's heart.

Methods: We divided our young, adult male and female rats into control and exercised groups. Athlete's heart was induced by a 12-week long swim training. Following the training period, we assessed LV hypertrophy with echocardiography, while pressure-volume analysis was performed to investigate *in vivo* LV function. After *in vivo* experiments, molecular biological studies and histological investigations were performed.

Results: Echocardiography and post-mortem measured heart weight data indicated LV hypertrophy in both genders, nevertheless it was more pronounced in females. Despite the more significant relative hypertrophy in females, characteristic functional parameters did not show notable differences between the genders. LV pressure-volume analysis showed increased stroke volume, improved contractility and stroke work and unaltered LV stiffness in both male and female exercised rats, while active relaxation was ameliorated solely in male animals. The induction of Akt signaling was more significant in females compared to males. There was also a characteristic difference in the mitogen-activated protein kinase pathway as suppressed phosphorylation of p44/42 MAPK (Erk) and mTOR was observed in female exercised rats, but not in male ones. Myosin heavy chain α (MHC)/β-MHC ratio did not differ in males, but increased markedly in females.

Conclusion: Our results confirm that there is a more pronounced exercise-induced LV hypertrophy in females as compared to the males, however, there are only minor differences regarding LV function. There are characteristic molecular differences between male and female animals, that can explain different degrees of LV hypertrophy.

Keywords: athlete's heart, exercise-induced hypertrophy, pressure-volume analysis, left ventricular function, sex differences

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INTRODUCTION

Physiological cardiac hypertrophy induced by repetitive intense exercise is associated with cardiomyocyte enlargement without sign of fibrotic remodeling or cell damage, that leads to a greater functional reserve to provide improved cardiac performance during the exercise session (Ellison et al., 2012). In contrast to pathological remodeling, this kind of hypertrophy is associated with benefits in myocardial energetic status and functional improvement, resulting in a protective role against cardiovascular diseases (Weiner and Baggish, 2012; Oláh et al., 2016). Although athlete's heart has been characterized by improved cardiac performance and myocardial enlargement in both genders, recent investigations suggest sex-specific regulation of the development of exercise-induced hypertrophy (Foryst-Ludwig and Kintscher, 2013; Dworatzek et al., 2014).

Most of the studies describe a more pronounced hypertrophic response, a greater relative increase of myocardial mass in female individuals compared to male ones, also in human and experimental animals (Konhilas et al., 2004; Luczak and Leinwand, 2009; Foryst-Ludwig et al., 2011; Dworatzek et al., 2014). Sex-specific molecular regulation of physiological hypertrophy has been reported in studies that were conducted in different animal models of exercise-induced hypertrophy. In previous experimental investigations that used short-term voluntary cage wheel running to train rodents, the molecular differences showed differences in the MAPK system: enhanced proportional increase in Ca^{2+}/calmodulin-dependent protein kinase (CaMK) activity and phosphorylation of p38 MAPK and ERK were observed in female animals compared to male counterparts (Konhilas et al., 2004; Dworatzek et al., 2014). The relatively greater hypertrophic response has been shown to be associated with different activation of the protein kinase B (Akt) system and these experimental reports demonstrated that estrogen receptor beta might be directly involved in the sex-specific alterations in exercise-induced hypertrophy (Mahmoodzadeh et al., 2012; Dworatzek et al., 2014). However, in these experimental studies, female sex was associated with increased running performance (distance), which complicates the interpretation.

Although there are obvious sex-specific differences in the molecular aspects of exercise-induced cardiac hypertrophy (EICH), functional consequences are still unclear. There are relatively few studies on female athletes examining cardiac size and function (Pelliccia et al., 1996; Hedman et al., 2015). Comparative non-invasive investigation of athletes could utilize only preload and afterload dependent parameters, those might not describe myocardial mechanics in detail (George et al., 1999; Yilmaz et al., 2013). According to our knowledge, no such direct comparison of exercise-induced functional consequences exists, especially using similar exercise load. Difficulties of such human study include ethical concerns (only the non-invasive possibilities to investigate myocardial mechanics) and it would also require great effort to obtain comparable training workload in male and female elite athletes. Animal models might provide an excellent tool for describing sex-specific differences in the development of exercise-induced hypertrophy, mostly due to the precisely defined exercise training conditions.

Our hypothesis was that similar exercise load results in sex-specific distinction in the degree of hypertrophy, which might be associated with different LV functional consequences in male and female rats. Therefore, we aimed at providing LV functional characterization of exercise-induced hypertrophy in male and female rats, thus providing reliable hemodynamic gender-specific comparisons in a rat model of physiological hypertrophy. Additionally, we investigated sex-specific molecular alterations in exercise-induced LV hypertrophy.

MATERIALS AND METHODS

Animals

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Guide for the Care and Use of Laboratory Animals provided by the National Institute of Health (NIH Publication No. 86-23, revised 1996.) and the EU Directive 2010/63/EU. The protocol was approved by the Ethical Committee for Animal Experimentation, Semmelweis University, Budapest (PEI/001/2374-4/2015). All animals received humane care.

Young adult, age-matched, 57–61 days old male (n = 24) and female (n = 24) Wistar rats were housed in standard rat cages at a constant room temperature (22 ± 2 °C) and humidity with a 12:12-h light-dark cycle. The animals were allowed access to a standard laboratory rat diet and water *ad libitum* during the whole experimental period.

Experimental Groups

After acclimatization, the rats were divided into four experimental groups: male control (MCo, n = 12), male exercised (MEx, n = 12), female control (FCo, n = 12), and female exercised (FEx, n = 12).

Exercise Training – Rat Model of Physiological Cardiac Hypertrophy

For long-term exercise training, both male and female exercised rats swam for a total period of 12 weeks, for 200 min/day, 5 days a week as previously described (Radovits et al., 2013). For the appropriate adaptation, the duration of swimming was increased 15 min every second training day from a basic 15 min on the first day, until achieving the maximal 200 min/day. The water temperature was maintained at 30–32°C during exercise session. Untrained control rats were placed into the water for 5 min each day during the 12-week training program.

In vivo measurements were performed at least 6 h, but not more than 24 h after last exercise session.

Echocardiography

At the completion of swimming training program, LV morphological alterations were observed by echocardiography using a 13 MHz linear transducer (12L-RS, GE Healthcare, Horten, Norway), connected to a commercially available system

(Vivid i, GE Healthcare) as described before (Olah et al., 2017). Rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.). Animals were placed on controlled heating pads, and the core temperature was maintained at 37°C. Standard two-dimensional and M-mode long- and short axis (at mid-papillary level) images were acquired. On two-dimensional recordings of the short-axis at the mid-papillary level, LV anterior (AWT) and posterior (PWT) wall thickness in diastole (index: d) and systole (index: s) as well as LV end-diastolic (LVEDD) and end-systolic diameter (LVESD) were measured. LV volume values (LVEDV and LVESV) were estimated according to the Teichholz's formula.

Fractional shortening (FS), ejection fraction (EF), and stroke volume (SV) were calculated according to standard formulas. LV mass was determined according to the following formula suggested by Devereux: LVmass = $[(\text{LVEDD}+\text{AWTd}+\text{PWTd})^3 - \text{LVEDD}^3] \times 1.04$ (Devereux et al., 1986). To calculate LV mass index, we normalized the LV mass values to the tibial length (TL) of the animal.

Hemodynamic Measurements – Left Ventricular Pressure-Volume Analysis

After completion of the 12-week long training protocols to induce myocardial hypertrophy, *in vivo* hemodynamic measurements were performed as described previously (Oláh et al., 2016). Shortly, after anesthesia using pentobarbital-sodium (60 mg/kg), using breath and temperature control, a 2-Fr pressure-conductance microcatheter (SPR-838, Millar Instruments, Houston, TX, United States) was inserted into the right carotid artery and advanced into the left ventricle through the ascending aorta.

After stabilization, such as heart rate (HR), LV end-systolic pressure (LVESP), LV end-diastolic pressure (LVEDP), the maximal slope of LV systolic pressure increment (dP/dt_{max}) and diastolic pressure decrement (dP/dt_{min}), time constant of LV pressure decay [τ; according to Glantz], LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), stroke volume (SV), ejection fraction (EF), cardiac output (CO), and stroke work (SW) were calculated and corrected according to *in vitro* and *in vivo* volume calibrations. To exclude the influence of body weight differences, CO was normalized to body weight [cardiac index (CI)].

To obtain load-independent parameters LV P-V relations were measured by transiently compressing the inferior vena cava (reducing preload). The slope of the LV end-systolic P-V relationship (ESPVR; according to the parabolic curvilinear model) and preload recruitable stroke work (PRSW) were calculated as load-independent indices of LV contractility. The slope of the LV end-diastolic PV relationship (EDPVR) was calculated as a reliable indicator of LV stiffness.

Arterial elastance (E_a) was calculated as LVESP/SV. Ventriculoarterial coupling (VAC) was described by the quotient of E_a and ESPVR.

After completing hemodynamic measurements to remove erythrocytes from myocardial tissue, an *in vivo* perfusion was performed. After opening the thoracic cavity and dissecting the inferior caval vein in the thorax, a total volume of 40 ml oxygenated Ringer solution (37°C) was infused into the LV through the apex of the heart. All animals were euthanized by exsanguination.

Thereafter, the heart was quickly removed and placed into cold (4°C) Ringer solution. Heart weight was measured and LV myocardial tissue samples were collected immediately for histology and molecular biology. Subsequently post-mortem TL measurements were done.

Histology

After organ weight measurements, the hearts were fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. Transverse, transmural, ∼5 μm thick slices of the ventricles were cut and placed on adhesive slides.

Hematoxylin and eosin staining was performed to measure cardiomyocyte diameter (CD) as a cellular marker of myocardial hypertrophy. In each sample, 100 longitudinally oriented cardiomyocytes from the LV were examined, and the diameters at the transnuclear position were defined. The mean value of 100 measurements represented one sample.

The extent of myocardial fibrosis was assessed on picrosirius-stained sections (Ruppert et al., 2018). ImageJ software (National Institutes of Health, Bethesda, MD, United States) was used to identify the picrosirius-red positive area. Three transmural images (magnification 50 ×) were randomly taken from the free LV wall on each sections. After background subtraction, eye controlled auto-thresholds have been determined to detect positive areas. The fibrosis area (picrosirius red positive area-to-total area ratio) was determined on each image, and the mean value of three images represents each animal.

Cardiac mRNA Analysis

Left ventricular myocardial tissue samples were harvested immediately after sacrifice, snap-frozen in liquid nitrogen and stored at −80°C. LV tissue of 8-8 animals from each group was homogenized in a lysis buffer (RLT buffer; Qiagen, Hilden, Germany), RNA was isolated from the ventricular samples using the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions and quantified by measuring optical density (OD) at 260 nm. RNA purity was ensured by obtaining a 260/280 nm OD ratio approximately 2.0. Reverse transcription reaction (1 μg total RNA of each sample) was completed using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was performed with the StepOnePlusTM Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) in triplicates of each sample in a volume of 10 μl in each well containing cDNA (1 μl), TaqMan® Universal PCR MasterMix (5 μl) and a TaqMan® Gene Expression Assay for the following markers (0.5 μl): atrial natriuretic factor (ANF, assay ID: Rn00561661_m1); transforming growth factor β1 (TGF-β, assay ID: Rn00572010_m1) and α and β-isoform of myosin heavy chain (α-MHC, assay ID: Rn00568304_m1; β-MHC, assay ID: Rn00568328_m1) purchased from Applied Biosystems. Gene expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; reference gene; assay ID: Rn01775763_g1) and expression levels were calculated using the

CT comparative method ($2^{-\Delta CT}$). All results are expressed as values normalized to a positive calibrator (a pool of cDNA's from all samples of the control group).

Western Blot

Western blot experiments were performed as described earlier (Koncsos et al., 2016). Freeze-clamped LV samples from six animals of each group were homogenized with RIPA buffer (Sigma Aldrich, Budapest, Hungary) containing Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, United States). Protein concentration was assessed with BCA kit (Thermo Fisher Scientific). Protein samples were resolved on precast 4–20% Criterion TGX gels (Bio-Rad, Hercules, CA, United States) and transferred to Immun-Blot PVDF membranes (Bio-Rad). Equal protein loading was verified with Ponceau staining. Membranes were blocked with bovine serum albumin (BSA; Santa Cruz Biotechnology, Dallas, TX, United States) in Tris-buffered saline with 0.05% Tween 20 (TBS-T) for 2 h. Membranes were incubated with primary antibodies in BSA in TBS-T against phospho-Akt [Ser473] (Catalog number: #4060), Akt (#9272), phospho-p44/42 MAPK (Extracellular signal-regulated kinase, ERK 1/2) [Thr202/Tyr204] (#9101), p44/42 MAPK (ERK 1/2, #9102), phospho-p38 MAPK [Thr180/Tyr182] (#4511), p38 MAPK (#9212), phospho-glycogen synthase kinase (GSK)-3β [Ser9] (#5558), GSK-3β (#12456), phospho-mammalian target of rapamycin (mTOR) [Ser2448] (#2971), mTOR (#2972), phospho-S6 [Ser235/Ser236] (#2211), and S6 (#2217) (all supplied from Cell Signaling). After three washes with TBS-T, horseradish peroxidase-conjugated secondary antibody was added for 1 h at room temperature (in BSA in TBS-T; Cell Signaling). Signals were detected with an enhanced chemiluminescence kit (Bio-Rad) by Chemidoc XRS+ (Bio-Rad) and quantitated in Image Lab 4.1 software (Bio-Rad). Antibodies bound to phospho-epitopes were removed with Pierce Stripping Buffer (Thermo Fisher Scientific) before incubation with antibodies detecting the total protein. We included all intact samples in the analysis.

Statistics

Results are expressed as mean ± SEM. After confirming normal distribution of data (Shapiro–Wilks method), two-way analysis of variance (ANOVA) with the factors "Sex" and "Exercise" was performed and *p*-values for sex and exercise interaction (p_i) were calculated. *Post hoc* pairwise comparisons were performed using the Tukey method to determine differences between groups (MCo vs. MEx; FCo vs. FEx). A p-value < 0.05 was the criterion of significance.

RESULTS

Left Ventricular Hypertrophy

Post-mortem measured heart weight and LV wall thickness values, as well as calculated LV mass data by echocardiography indicated cardiac hypertrophy in both exercised groups compared to control ones (**Figure 1**). There were notable differences between the degree of hypertrophy: female sex was associated with greater relative hypertrophy (**Figure 1**). We detected concentric hypertrophy in both male and female rats indicated by increased RWT. According to our data, no significant LV dilatation was observed after the completion of swim training program.

Histological analysis also confirmed exercise-induced hypertrophy at the microscopic level, we observed cardiomyocyte enlargement in both exercised groups (**Figure 1**). Picrosirius staining revealed no collagen deposition in the myocardium of trained rats, suggesting the physiological nature of LV hypertrophy in both genders (**Figure 1**).

Left Ventricular Function

Figure 2 shows illustrative steady-state P-V loops obtained from MCo, MEx, FCo, and FEx animals. The widening of the baseline loops can be observed both in MEx and FEx rats compared to corresponding controls and it clearly reflects increased stroke volume in case of exercise-induced hypertrophy. As the representative P-V loops depict (data shown in **Table 2**.), exercise training was associated with decreased LVESV along with unaltered LVEDV. These alterations were also confirmed by echocardiographic results (**Table 1**). Consequently, SV, EF, CI, and SW was increased in exercised rats compared to control ones suggesting increased systolic performance in the hearts, underwent exercise training. We should also mention that neither HR nor pressure relations (MAP, LV pressure values, dP/dt_{max}, and dP/dt_{min}) did differ between control and exercised groups.

To obtain load-independent, sensitive functional parameters we recorded P-V relations while cardiac preload was altered. **Figure 3** displays representative P-V loops obtained during inferior vena cava occlusions in MCo, MEx, FCo, and FEx rats. Overall results of ESPVR and PRSW have been shown on **Figure 3**. Both contractility parameters were increased in both genders at a similar degree.

τ, that is a load-independent parameter of LV relaxation, a major determinant of diastolic function, was significantly shortened (thus improved) in the case of male exercised animals, whereas there was no alteration in female rats compared to their control counterparts (**Figure 3**).

We also determined EDPVR, which characterizes LV stiffness. Although marked hypertrophy was observed in both genders, this parameter was not altered neither in male nor in female animals (**Figure 3**).

We also investigated parameters that describe the connection of ventricular and arterial system (**Figure 4**). Arterial elastance has been found to be decreased in both genders: increased stroke volume was detected along with unchanged LV pressure values in exercised rats. Additionally, VAC showed a more optimized ventriculo-arterial interaction in exercised rats compared to controls.

Molecular Differences

Left ventricular gene expression values of markers that are associated with pathological remodeling of the left ventricle (ANF, TGF-β) did not differ between the groups. There were

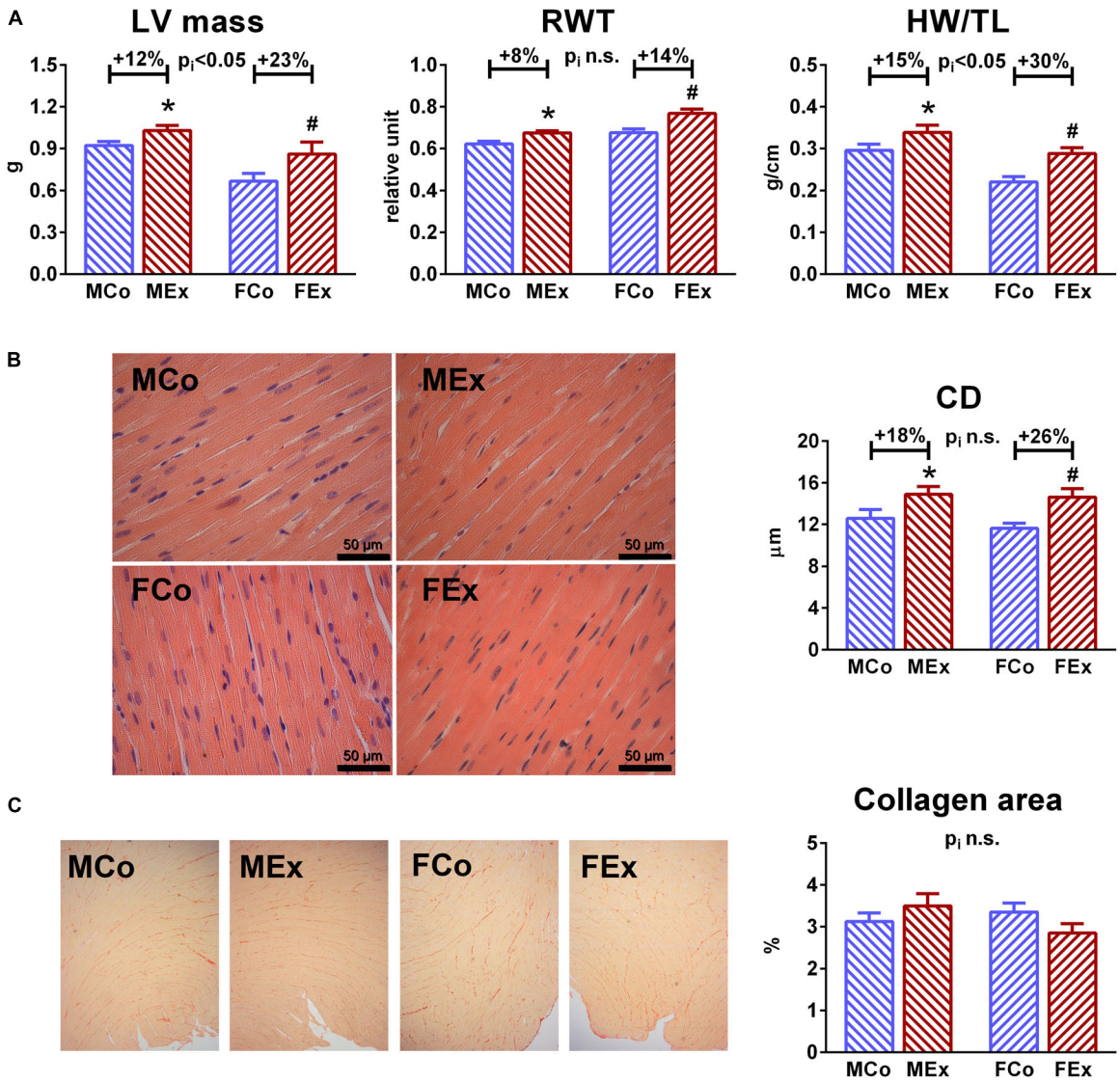

FIGURE 1 | Characterization of exercise-induced left ventricular (LV) hypertrophy. **(A)** Echocardiographic LV mass and post-mortem measured heart weight (HW, normalized to TL) showed increased values in male exercised (MEx) and female-exercised (FEx) animals compared to male control (MCo), and female control (FCo) rats, respectively. Female gender was associated with greater degree of hypertrophy. A slight, but significant increase of relative wall thickness (RWT) suggest concentric type of hypertrophy in our rat model. **(B)** Representative hematoxylin-eosin stained sections (magnification 400 ×) from all of the groups, that were used to measure transnuclear cardiomyocyte width. Mean LV cardiomyocyte diameter (CD) was increased in both genders, that confirmed hypertrophy at cellular level. **(C)** One-one representative picrosirius-stained section from each group. Red color indicates collagen fibers, magnification 50 ×. Picrosirius-staining showed unaltered collagen density in exercised rats nor in male neither in female animals. Values are means ± SEM. $^{*}p < 0.05$ vs. MCo, $^{\#}p < 0.05$ vs. FCo, p_i: interaction *p*-value of two-way analysis of variance (ANOVA).

characteristic differences between male and female rats in the mRNA expression of myosin heavy chain types. We detected a decreased expression of β-MHC in exercised female animals compared to control ones, that resulted in a marked increase of α-MHC/β-MHC ratio, while there was no difference between male exercised and control rats (**Figure 5**).

We performed additional molecular biological measurements to investigate underlying molecular mechanisms behind the differences in exercise-induced hypertrophy between sexes. Phosphorylation of Akt was increased in exercised male and female animals as compared to control counterparts, however, it was relatively more pronounced in female as in male rats. Exercise or sex had no effect on the phosphorylation of p38 MAPK and GSK-3β. Phosphorylation of Erk1/2 (p42/44 MAPK) was unaltered in exercised male rats compared to control ones, however, it was decreased by training in female animals (**Figure 6**).

The phosphorylation of mTOR was decreased in female exercised animals compared to their controls, while exercise training did not alter mTOR-phosphorylation in male rats.

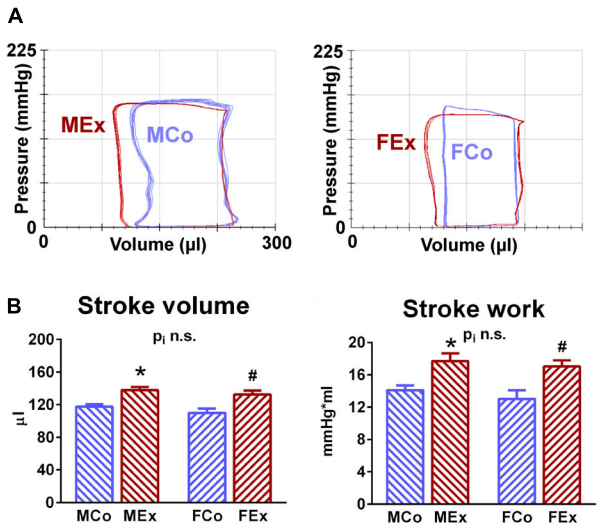

FIGURE 2 | Baseline pressure-volume relations. **(A)** Original recordings of steady-state pressure-volume (P-V) loops obtained from one representative rat from male control (MCo), male exercised (MEx), female control (FCo), and female exercised (FEx) groups. As shown by the recordings, the wider P-V loop indicates increased stroke volume along with unaltered end-diastolic volume and pressure relations, decreased end-systolic volume in exercised rats of both genders compared with untrained controls. **(B)** Exercise-induced hypertrophy was associated with increased stroke volume and stroke work in both genders. Values are means ± SEM. $^{*}p < 0.05$ vs. MCo, $^{\#}p < 0.05$ vs. FCo, p_i: interaction *p*-value of two-way analysis of variance (ANOVA).

We found sex-related distinction in the phosphorylation of S6 ribosomal protein: exercise was associated with an increment of S6 phosphorylation in male animals, while it did not differ between female control and exercised rats (**Figure 6**).

DISCUSSION

We provided the first detailed *in vivo* hemodynamic comparison of exercise-induced hypertrophy in male and female rats. Our exercised animals of both genders underwent an identical swim training program (similar workload) to enable a reliable comparison of LV functional consequences of long-term exercise training.

Left Ventricular Hypertrophy

Numerous human studies and experimental examinations investigated the sex-dependency of hypertrophic response induced by exercise training. However, it might be challenging to obtain similar workload in male and female highly elite athletes, thus human data about the sex-specific response of exercise-induced hypertrophy are controversial (Pelliccia et al., 1991; Yilmaz et al., 2013; Finocchiaro et al., 2017). Experimental studies were frequently using voluntary cage wheel running systems, that might also lead to different workload, because female mice tend to run much longer and faster voluntarily than males (Luczak and Leinwand, 2009). Our results clearly indicate a relatively more pronounced cardiac and left ventricular (LV) hypertrophy in female animals compared to male ones after completing the 12-week long training program, while we detected ∼15–20% relative increase of cardiac mass in male animals, female exercised rats were related to a ∼25–30% growth of myocardial tissue (**Figure 1**). These alterations are in line with most of the conducted studies in rodents, using voluntary or forced training systems (Konhilas et al., 2004; Foryst-Ludwig et al., 2011; Dworatzek et al., 2014). Histological analysis revealed alterations that are characteristic to physiological hypertrophy (Bernardo et al., 2010; Ellison et al., 2012; Oláh et al., 2016): cardiomyocyte enlargement without myocardial fibrosis. The unaltered ANF and TGF-β values (**Figure 5**) also confirmed the physiological nature of the observed hypertrophy (Bernardo et al., 2010; Oláh et al., 2016). Moreover, β-MHC, another pathological hypertrophy marker has not been only unaltered in female animals, but exercise training resulted in decreased myocardial expression, that also leaded to increased α/β-MHC values. Indeed, there are evidences that myocardial expression of β-MHC shows sex-specific differences also in pathological conditions (Rosenkranz-Weiss et al., 1994; Zhong et al., 2003).

We found concentric type of hypertrophy in our rat model of exercise-induced hypertrophy according to increased LV wall thickness and RWT values and unaltered end-diastolic dimensions measured by echocardiography and pressure-volume analysis (**Figure 1** and **Tables 1**, **2**). According to the Morganroth hypothesis and recent investigations in athletes, regular aerobic exercise training – such as swim training – induces rather an eccentric type of LV hypertrophy (Naylor et al., 2008). The dilatation of LV in diastole might be related to exercise-induced resting bradycardia, because of relatively prolonged LV filling and elongated diastole during the heart cycle (Pavlik et al., 2013). Hence, we should mention that during our *in vivo* measurements, animals were anesthetized, and in that condition we could not detect alterations in HR values (**Tables 1**, **2**), which can be explained by the impact of anesthesia on the autonomic nervous system. However, this discrepancy between our results and human studies might not influence parameters of pressure-volume analysis, that are independent of cardiac load and HR (Pacher et al., 2008).

We also investigated hypertrophy-associated molecular pathways (**Figure 6**). Akt, a serine-threonine kinase as the main effector of physiological hypertrophy-associated IGF-1/PI3K/Akt pathway plays a pivotal role in the development of EICH (Bernardo et al., 2010; DeBosch et al., 2006). The active, phosphorylated form of Akt was increased in both male and female animals, that is in line with other studies that investigated physiological hypertrophy (Weeks et al., 2017). In female rats, Akt activation seemed to be more pronounced, which is in line with the relatively increased hypertrophic response in female rats compared to alterations in male animals (**Figure 4**).

Glycogen synthase kinase-3β, a cellular substrate for Akt, is an important regulator with anti-hypertrophic effect and inhibition of GSK-3β has been proposed as an important mechanism for stimulating growth in hypertrophy (Antos et al., 2002; Bernardo et al., 2010). According to our data, phosphorylation of GSK-3β did not differ between the exercised

TABLE 1 | Left ventricular (LV) echocardiographic data LV echocardiographic data of male control (MCo), male exercised (MEx), female control (FCo), and female exercised (FEx) rats.

	MCo	MEx	FCo	FEx	p_i
BW, g	468 ± 10	402 ± 11*	291 ± 8	281 ± 7	0.004
HR, 1/min	355 ± 14	347 ± 11	362 ± 9	342 ± 8	0.580
LVAWTd, mm	2.15 ± 0.02	2.37 ± 0.03*	2.03 ± 0.04	2.37 ± 0.04#	0.085
LVAWTs, mm	3.13 ± 0.08	3.52 ± 0.06*	3.09 ± 0.06	3.58 ± 0.10#	0.536
LVPWTd, mm	1.96 ± 0.03	2.10 ± 0.02*	1.73 ± 0.02	1.96 ± 0.04#	0.145
LVPWTs, mm	2.97 ± 0.06	3.16 ± 0.07*	2.77 ± 0.04	3.10 ± 0.04#	0.198
LVEDD, mm	6.57 ± 0.08	6.63 ± 0.06	5.59 ± 0.12	5.72 ± 0.09	0.700
LVESD, mm	3.91 ± 0.10	3.26 ± 0.11*	3.12 ± 0.09	2.71 ± 0.09#	0.227
FS, %	40.6 ± 1.2	50.9 ± 1.3*	44.2 ± 0.8	52.6 ± 1.3#	0.430
LVEDV, µl	221.8 ± 5.8	226.3 ± 4.9	153.6 ± 7.5	161.7 ± 6.0	0.771
LVESV, µl	66.8 ± 3.8	43.6 ± 3.3*	39.0 ± 2.8	27.8 ± 2.0#	0.054
SV, µl	155.0 ± 4.6	182.8 ± 3.0*	114.6 ± 5.4	133.9 ± 5.2#	0.375
EF, %	70.0 ± 1.4	80.9 ± 1.2*	74.7 ± 0.9	82.8 ± 1.1#	0.242

*Values are means ± SEM. BW, body weight; HR, heart rate; LVAWTd and LVAWTs, left ventricular (LV) anterior wall thickness at diastole and systole, respectively; LVPWTd and LVPWTs, LV posterior wall thickness at diastole and systole, respectively; LVEDD, LV end-diastolic dimension; LVESD LV end-systolic dimension; FS, fractional shortening; LVEDV, LV end-diastolic volume; LVESV, LV end-systolic volume; SV, stroke volume; EF, ejection fraction. *$p < 0.05$ vs. MCo, #$p < 0.05$ vs. FCo. p_i: interaction value (factors: gender and exercise) of two-way analysis of variance (ANOVA).*

TABLE 2 | Left ventricular hemodynamic data LV hemodynamic data of male control (MCo), male exercised (MEx), female control (FCo), and female exercised (FEx) rats.

	MCo	MEx	FCo	FEx	p_i
HR, 1/min	414 ± 9	400 ± 8	400 ± 12	397 ± 8	0.562
MAP, mmHg	140.0 ± 5.1	138.3 ± 6.1	138.2 ± 5.0	142.2 ± 4.1	0.582
LVESP, mmHg	156.5 ± 3.9	152.3 ± 8.2	148.7 ± 6.2	155.5 ± 5.7	0.380
LVEDP, mmHg	3.3 ± 0.7	4.1 ± 1.5	3.7 ± 1.1	4.1 ± 1.1	0.861
dP/dt_{max}, mmHg/s	9241 ± 397	9821 ± 667	9331 ± 565	10231 ± 654	0.785
dP/dt_{min}, mmHg/s	−12246 ± 432	−12211 ± 670	−12579 ± 655	−13285 ± 487	0.521
LVEDV, µl	229.9 ± 2.9	239.4 ± 4.6	210.1 ± 4.3	216.1 ± 5.2	0.689
LVESV, µl	112.2 ± 1.8	101.6 ± 1.8*	100.3 ± 4.0	83.8 ± 2.7#	0.287
EF, %	51.5 ± 1.1	57.5 ± 0.7*	52.7 ± 1.9	61.1 ± 1.3#	0.370

*Values are means ± SEM. HR, heart rate; MAP, mean arterial pressure; LVESP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure; dP/dt_{max}, maximal slope of the systolic pressure increment; dP/dt_{min}, maximal slope of the diastolic pressure decrement; LVEDV, LV end-diastolic volume; LVESV, LV end-systolic volume; EF, ejection fraction. *$p < 0.05$ vs. MCo, #$p < 0.05$ vs. FCo. p_i: interaction value (factors: gender and exercise) of two-way analysis of variance (ANOVA).*

and control groups after 12 weeks of swim training, which might suggest that the active development process of hypertrophy was completed (**Figure 6**). This is in line with a study, where GSK-3β phosphorylation was altered in the early phase of training, while there was no alteration detected later (Konhilas et al., 2004). The activation of mTOR signal pathway and its downstream substrates have also been associated with the development of exercise-induced hypertrophy (Kemi et al., 2008). We found sex-related differences in mTOR phosphorylation and S6 ribosomal protein phosphorylation (**Figure 6**), that suggest a distinct regulation of mTOR pathway in male and female animals.

Although their role in the development of physiological hypertrophy is still doubtful, we also investigated proteins of MAPK system that are proposed for sex-specific exercise-induced effects and are hypothetically related to estrogen receptors (Regitz-Zagrosek et al., 2010; Dworatzek et al., 2014). p38-MAPK has been implicated in the regulation of cardiac gene expression, cardiac myocyte apoptosis, myocyte hypertrophy, contractility, remodeling and metabolism. We have not found any differences regarding activation of p38-MAPK, which is in line with an investigation of exercise-induced hypertrophy (Konhilas et al., 2004), however, it contradicts another report showing the pivotal role in sex-specific regulation (Dworatzek et al., 2014). Another important MAPK is ERK1/2 (p42-44-MAPK) that is clearly involved in the development of pathological hypertrophy, however, the over activation of ERK was not observed in the case of physiological stimuli (Clerk et al., 2006). We found a decreased phosphorylation of ERK 1/2 in hearts of female exercised rats, while the activation was unaltered in male animals (**Figure 5**).

It is indeed difficult to interpret results about activation of proteins involved in hypertrophic response, because their phosphorylation might be altered during a different phase of the development of exercise-induced hypertrophy, thus further experimental investigations are needed using standard training protocols to describe activation at different phases during the development of hypertrophy.

Left Ventricular Function

There are only a few studies in human, that compare exercise-mediated cardiac hypertrophy between male and female athletes, while most of the studies have been focused on male individuals. Although, we have significant data about gender differences in morphological aspect of exercise-induced hypertrophy, the sex-specific functional consequences are still unclear.

Systolic Function

Improved systolic performance of athlete's heart is a typical feature, however, the clinical evaluation of this improvement is yet to be resolved. Increased stroke volume is a characteristic alteration in athlete's heart in both genders (Yilmaz et al., 2013), that was also confirmed by our echocardiographic and hemodynamic data (**Table 1** and **Figure 3**). We also detected increased FS and EF in our exercised animals. Conventional parameters of systolic function, including LV FS and EF, are unreliable in athletes, while there are obvious alterations in cardiac preload and HR.

Although speckle-tracking echocardiography is a promising tool to follow-up systolic improvement (Kovacs et al., 2015), LV contractility can be assessed precisely and reliably by pressure-volume analysis. Pressure-volume recordings during a transient preload reduction maneuver (vena cava inferior occlusion) provide the opportunity to calculate load-independent indicators of ventricular contractility (Pacher et al., 2008). The most widely used sensitive contractility indices, ESPVR and PRSW, were significantly elevated in trained animals independently from sex (**Figure 4**). The exercise-mediated improvement in contractility was comparable in male and female rats.

Diastolic Function

There are two main components that describe diastolic performance of the ventricular tissue: active relaxation and myocardial stiffness.

Left ventricular relaxation has been identified as an active, energy consuming process and depends mostly on calcium reuptake by the sarcoplasmic reticulum during the early diastole. The time constant of LV pressure decay has been described as a relatively load-independent index of LV active relaxation, and the prolongation of this parameter has been widely described in pathological cardiac conditions (Zhao et al., 2008). Physiological hypertrophy was associated with enhanced relaxation in exercised male animals compared to control ones, as shown by decreased τ (**Figure 3**). In contrast, no alteration was observed between female control and exercised animals. According to our data, there is a sex-specific difference between male and female animals, that disappears after exercise training (**Figure 3**), and sexual dimorphism regarding diastolic function has indeed been reported (Luczak and Leinwand, 2009). Active relaxation is a determinative parameter and its characteristic alterations can differentiate between physiological and pathological cardiac diseases. Further investigation of active relaxation might answer the question whether lower female active relaxation time would be protective against diastolic dysfunction, and its improvement would provide additional cardiac performance improvement in male athletes.

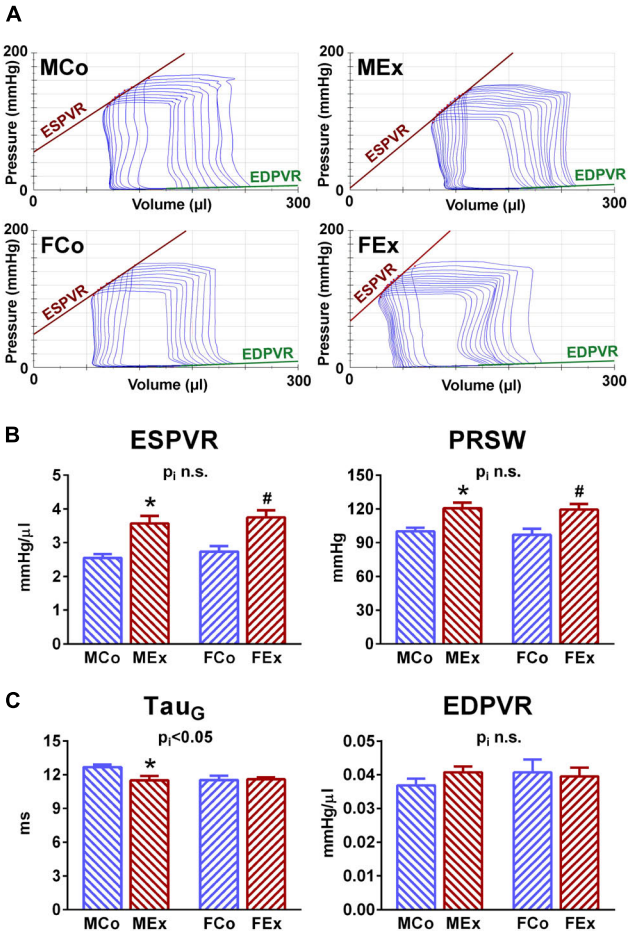

FIGURE 3 | Load-independent indices of LV systolic and diastolic function. **(A)** Characteristic indices of LV contractility and stiffness could be obtained by pressure-volume (P-V) analysis during reducing cardiac preload by the transient occlusion of vena cava inferior. Representative original recordings of P-V relations from one animal of each group during this maneuver were depicted. **(B)** Sensitive contractility indices, such as the slope of end-systolic pressure-volume relationship (ESPVR) and PRSW revealed comparable exercise-induced LV contraction improvement in both genders. **(C)** There was a notable difference regarding Tau, a load-independent parameter of LV active relaxation. In male rats a diastolic improvement was shown as indicated by decreased Tau values, whereas exercise was not associated with altered Tau in females. The slope of end-diastolic pressure volume relationship (EDPVR), that describes LV stiffness, has not been altered by exercise training. MCo, male control group; MEx, male exercised group; FCo, female control group; FEx, female exercised group. Values are means ± SEM. $^{*}p < 0.05$ vs. MCo, $^{\#}p < 0.05$ vs. FCo, p_i: interaction p-value of two-way analysis of variance (ANOVA).

Another important marker of diastolic function, describing pressure alterations during ventricular filling, is ventricular stiffness. This feature is affected predominantly by collagen metabolism in the extracellular matrix (ECM), but alterations in myocardial intracellular and other extracellular structural components (e.g., fibrosis) can also influence it. EDPVR, a sensitive marker of LV stiffness, was not altered by exercise training and showed similarity between genders (**Figure 3**). This

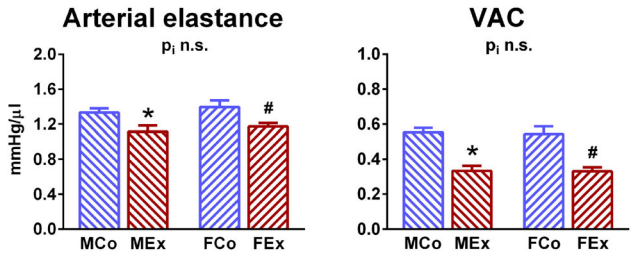

FIGURE 4 | Comparison of ventriculo-arterial connection. Arterial elastance was decreased in both genders and ventriculo-arterial coupling (VAC) was improved in both genders. MCo, male control group; MEx, male exercised group; FCo, female control group; FEx, female exercised group. Values are means ± SEM. $^{*}p < 0.05$ vs. MCo, $^{\#}p < 0.05$ vs. FCo, p_i: interaction p-value of two-way analysis of variance (ANOVA).

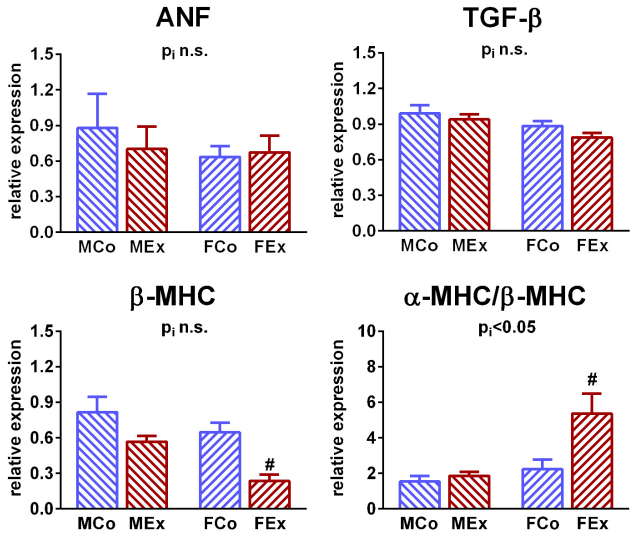

FIGURE 5 | Left ventricular gene expression analysis. LV gene expression of atrial natriuretic factor (ANF) and transforming growth factor beta (TGF-β) did not alter in exercised animals. Expression of β-myosin heavy chain (MHC) mRNA was decreased in exercised female animals, that resulted in an increase of α-MHC/β-MHC ratio, while there was no difference between male exercised and control rats. MCo, male control group; MEx, male exercised group; FCo, female control group; FEx, female exercised group. Values are means ± SEM. $^{*}p < 0.05$ vs. MCo, $^{\#}p < 0.05$ vs. FCo, p_i: interaction p-value of two-way analysis of variance (ANOVA).

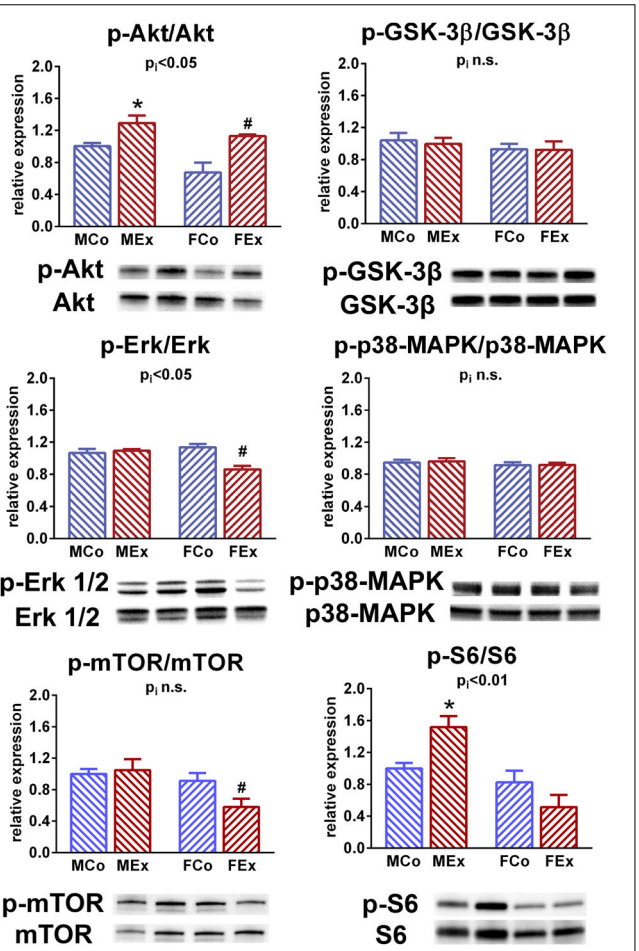

FIGURE 6 | Western blot. Protein kinase B (Akt) phosphorylation was increased in male and female trained animals in comparison with control counterparts. This alteration was more pronounced in exercised female exercised rats compared to male ones. We have not found any differences regarding the phosphorylation of p38 mitogen activated protein kinase (MAPK) and glycogen synthase kinase (GSK)-3β. Extracellular signal-regulated kinase (Erk) 1/2 (p42/44 MAPK) phosphorylation was decreased in female animals, and remained unaltered in male rats after the training program. Phosphorylation of mammalian target of rapamycin (mTOR) showed exercise-induced alteration only in female animals, while phosphorylation of S6 ribosomal protein was increased only in male exercised animals. MCo, male control group; MEx, male exercised group; FCo, female control group; FEx, female exercised group. Values are means ± SEM. $^{*}p < 0.05$ vs. MCo, $^{\#}p < 0.05$ vs. FCo, p_i: interaction p-value of two-way analysis of variance (ANOVA).

result is in good agreement with the absence of marked collagen deposition observed in the hearts of exercised animals (**Figure 1**) and with our previous findings in physiological hypertrophy (Oláh et al., 2016).

Ventricular Mechanics

Long-term exercise training was associated with increased stroke work in both sexes, reflecting improved effective external mechanical work of LV. Determining oxygen consumption in these animals would add further information about the efficiency of LV.

Ventriculo-Arterial Interaction

As a feature of the interaction between heart and arterial system, ventricular-arterial coupling expresses the interaction between left ventricle and arterial system using the ratio of arterial elastance and LV end-systolic elastance (Sunagawa et al., 1983). Arterial elastance, an integrative index that includes among others peripheral vascular resistance, arterial compliance and characteristic impedance. Decreased arterial elastance in exercised rats, independently from sex, reveals a better compliance of the arterial system in the case of

exercise-induced cardiovascular alterations (**Figure 4**). Improved VAC in exercise-trained animals reflects a more appropriate matching between the LV and the arterial system and suggests that endurance-trained individuals are able to match peripheral vascular changes with changes in the LV function following dynamic exercise. Optimized ventriculo-arterial coupling has been described only in male athletes, however, this is the first report to provide it in female individuals (Florescu et al., 2010).

CONCLUSION

In conclusion, we provided the first detailed hemodynamic comparison of physiological hypertrophy in a rodent model of athlete's heart. We confirmed physiological cardiac hypertrophy in both genders, which was more pronounced in female animals. We found that activation of Akt was increased in both genders, but even more in female rats and there were gender differences regarding ERK1/2, mTOR and S6 activation and αα/ββ-MHC proportion. Despite the differences in the degree of hypertrophy, only minor differences have been detected during functional measurements.

Both male and female hearts were associated with improved left ventricular contractility, similar "supernormal" systolic function. Differences were detected in early diastolic function: active relaxation was improved solely in male animals. LV stiffness was not affected by exercise training. LV mechanics improved by a comparable degree in the heart of male and female rats. A more optimized ventriculo-arterial interaction (VAC) is also a characteristic feature of both genders.

Limitations

The interpretation of results from the current study is limited to young male rats. The possible influence of age should be assessed in future studies. Furthermore, the present study was specifically designed to investigate sex differences in the functional consequences in a relevant model of exercise-induced hypertrophy. Also, the length of training period as well as type and intensity of training sessions might affect the observed phenotype.

In vivo investigations (echocardiography, pressure-volume analysis) could be performed under anesthesia, which might have an influence on parameters dependent on the autonomic nervous system, such as HR and pressure values. Even so, pressure-volume analysis might provide parameters that are independent of HR and loading conditions.

ETHICS STATEMENT

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Guide for the Care and Use of Laboratory Animals provided by the National Institutes of Health (NIH Publication No. 86-23, revised 1996) and the EU Directive 2010/63/EU. The protocol was approved by the Ethical Committee of Hungary for Animal Experimentation. All animals received humane care.

AUTHOR CONTRIBUTIONS

AO, BM, and TR conceived or designed the work. AO, CM, DK, MR, BB, AS, MT, GK, and TR acquired the data. AO, CM, DK, GK, ZG, and TR analyzed and interpreted the data. AO and TR were significant manuscript writers and drafted the work. ZG, PF, BM, and TR significantly revised the manuscript. All authors have read the manuscript and provided approval for its submission to Frontiers in Physiology 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.

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Effects of Acute Normobaric Hypoxia on Non-linear Dynamics of Cardiac Autonomic Activity During Constant Workload Cycling Exercise

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Aim: Measurements of Non-linear dynamics of heart rate variability (HRV) provide new possibilities to monitor cardiac autonomic activity during exercise under different environmental conditions. Using detrended fluctuation analysis (DFA) technique to assess correlation properties of heart rate (HR) dynamics, the present study examines the influence of normobaric hypoxic conditions (HC) in comparison to normoxic conditions (NC) during a constant workload exercise.

Materials and Methods: Nine well trained cyclists performed a continuous workload exercise on a cycle ergometer with an intensity corresponding to the individual anaerobic threshold until voluntary exhaustion under both NC and HC (15% O_2). The individual exercise duration was normalized to 10% sections (10–100%). During exercise HR and RR-intervals were continuously-recorded. Besides HRV time-domain measurements (meanRR, SDNN), fractal correlation properties using short-term scaling exponent alpha1 of DFA were calculated. Additionally, blood lactate (La), oxygen saturation of the blood (SpO_2), and rating of perceived exertion (RPE) were recorded in regular time intervals.

Results: We observed significant changes under NC and HC for all parameters from the beginning to the end of the exercise (10% vs. 100%) except for SpO_2 and SDNN during NC: increases for HR, La, and RPE in both conditions; decreases for SpO_2 and SDNN during HC, meanRR and DFA-alpha1 during both conditions. Under HC HR (40–70%), La (10–90%), and RPE (50–90%) were significantly-higher, SpO_2 (10–100%), meanRR (40–70%), and DFA-alpha1 (20–60%) were significantly-lower than under NC.

Conclusion: Under both conditions, prolonged exercise until voluntary exhaustion provokes a lower total variability combined with a reduction in the amplitude and correlation properties of RR fluctuations which may be attributed to increased organismic demands. Additionally, HC provoked higher demands and loss of correlation properties at an earlier stage during the exercise regime, implying an accelerated alteration of cardiac autonomic regulation.

Keywords: autonomic nervous system, heart rate variability, detrended fluctuation analysis, endurance exercise, voluntary exhaustion, hypoxia

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INTRODUCTION

Over the last 20 years, analytical data on the Non-linear dynamics of a heart rate (HR) time series have been adopted to gain further information of the complex process of cardiovascular regulation Sassi et al. (2015), both at rest and during exercise (Hottenrott and Hoos, 2017; Michael et al., 2017). Thus, measures of complexity of an HR time series, such as heart rate variability (HRV), may aid in monitoring cardiac autonomic activity and in gaining more information on the physiological status of the organismic system during exercise (Aubert et al., 2003). The present state of research suggests that cardiac dynamics is controlled by complex interactions between the two branches of autonomous nervous system, the sympathetic and parasympathetic branch, on the sinus node and other Non-neural factors (Persson, 1996; Gronwald et al., 2019a). These branches compete, resulting in parasympathetic withdrawal and sympathetic activation during exercise (Sandercock and Brodie, 2006). Looking at time- and frequency-domain HRV parameters, even low to moderate exercise intensities induce diminished variability (Gronwald et al., 2019b). During moderate to high exercise intensities, findings from such linear parameters are limited in their informative value and have led to inconsistent results (Hottenrott et al., 2006; Sandercock and Brodie, 2006). Consequently, methods for the Non-linear analysis of HRV were recently developed to detect signal properties that cannot be distinguished by linear analysis techniques (Huikuri et al., 2003; Yeh et al., 2010).

In a healthy state the HRV signal is mainly composed of quasi-periodic oscillations and also possesses fractal structures and random fluctuations (Goldberger et al., 2002). Analyses of these structures have become popular tools and have been shown to be useful diagnostic approaches in the investigation of age and disease (Voss et al., 2009). One widely applied approach to investigate the Non-linear dynamics of HRV and its scaling characteristics is detrended fluctuation analysis (DFA). Originally, this method was developed by Peng et al. (1995) to measure scale-invariant behavior; this involved the evaluation of trends of all sizes in the presence or absence of fractal correlation properties in an HR time series (Yeh et al., 2010). Thus, the DFA method allows to quantify the degree of correlation and fractal scale of an HRV signal resulting in dimensionless measures. The scaling exponents obtained by DFA are also expected to have diagnostic and prognostic abilities, especially for clinical settings. Therefore, the short-term scaling exponent of DFA, called alpha1 (DFA-alpha1), has already been applied for the prognosis of mortality, as well as cardiovascular risk assessment (Peng et al., 1995; Platisa and Gal, 2008; Huikuri et al., 2009; Sen and McGill, 2018).

The current state of research in this field shows that, regardless of the investigated disease or age group, DFA-alpha1 values that differ from the normal value (close to 1.0) (decreasing or increasing) during rest are associated with a higher morbidity or a worse prognosis, revealing a loss of the fractal dynamic toward random (disorganized randomness) or strongly-correlated (periodicity) behavior (de Godoy, 2016). In the context of homeodynamics as well as to system adaptability in response to external (environmental) stressors, that behavior could be interpreted as an effort to maintain basic stability of the control systems between order (persistence) and disorder (change) (Kauffman, 1995; Iyengar et al., 1996; Makikallio et al., 1999; Lipsitz, 2002). Physiologic systems are less adaptable and less able to cope with varied stimuli, such as exposure to different types and modes of exercise or changes in environmental conditions, when they're losing their fractal complexity (Goldberger, 1997). Hence, physiological complexity reflects the interaction of subsystems and the functioning of organismic regulation as a whole; thus, the higher the complexity, the higher the ability of the system to adapt to different conditions and situations in daily life (Goldberger et al., 2002; Gronwald et al., 2019a).

The related explanations on the Non-linear dynamics and complexity of organismic stability and self-regulation also seem to apply in endurance sports. In this respect, approaches and models from sports medicine and exercise science refer, on the one hand, to the importance of the brain in the control of fatigue processes and endurance performance and, on the other hand, to the complexity of the control and regulation processes of the subsystems limiting endurance performance (Noakes et al., 2004; Abbiss and Laursen, 2005; Marcora, 2008; Ament and Verkerke, 2009; Millet, 2011; Noakes, 2011, 2012; St Clair Gibson et al., 2018). HRV, as a marker of the integrated response of the heart to the complex, Non-linear interaction of sympatho-vagal activity and other factors could provide an adequate methodological approach, as it results from a complex central-peripheral integration of information from different cardiovascular feedback mechanisms and the central command within the central autonomic network (CAN) (Benarroch, 1993; Williamson et al., 2006).

As DFA, and its short-term scaling exponent alpha1, has a low dependence on HR and provides robustness against artifacts (Peng et al., 1995; Sandercock and Brodie, 2006; Silva et al., 2017), this method seems to be suitable for analyzing the complexity of cardiovascular regulation during endurance exercise with various exercise modalities and intensities (Gronwald et al., 2019a). Through the easy detection of HRV with a chest strap, DFA-alpha1 could be also useful as a diagnostic or monitoring metric for endurance trained athletes for assessing dose-response relationship in combination with other applicable internal and external load parameters.

Consequently, some studies have used DFA to analyze a time series during different types and modes of exercise (Tulppo et al., 2001; Hautala et al., 2003; Casties et al., 2006; Platisa et al., 2008; Hottenrott and Hoos, 2017; Gronwald et al., 2018, 2019a,b). However, further studies are necessary to analyze different modes of exercise, as well as changing environmental factors, such as hypoxic conditions (HC) or heat and cold exposure, to gain new insights for the suitability of DFA-alpha1 as a control or monitoring parameter in endurance exercise training (Gronwald et al., 2019b).

A recent systematic review by Oliveira et al. (2017) showed that acute exposure to hypoxia under resting conditions substantially-changes the HRV of healthy individuals (both time- and frequency-domain), and results in a decrease in the cardiac autonomic modulation. This is proposed to occur by

either reducing or maintaining vagal modulation, by enhancing sympathetic activation, or even by a combination of these responses. The described responses are mainly dependent on the altitude level, length of exposure, interindividual variation, and barometric pressure in the comparison of the effects of normobaric versus hypobaric hypoxia. Until now, only a few studies have analyzed the influence of hypoxia on the Non-linear parameters of HRV in general, and specifically DFA. Neither Zhang et al. (2015) or Vigo et al. (2010) could detect an effect of hypobaric hypoxia (up to 3600 and 8230 m, respectively) on DFA-alpha1 while sitting. Although there was no significant decrease detected during exposure to high hypobaric hypoxia by Vigo et al. (2010), Giles et al. (2016) found a significant increase in DFA-alpha1 during exposure to normobaric hypoxia up to 6000 m (9.8% O_2) during supine recordings. To the best of our knowledge, there are no studies to date that have analyzed the influence of hypoxia on the correlation properties of HRV (detected by DFA and its short-term scaling exponent alpha1) during exercise.

Therefore, the aim of the present study was to evaluate differences in the influence of a continuous workload exercise bout under both normoxic and normobaric HCs in terms of standard time-domain measures and Non-linear dynamics of HRV. The main objective was to determine whether characteristics of the short-term scaling exponent of DFA toward a random signal under acute prolonged exercise until voluntary exhaustion may differ in response to HCs. The ultimate goal of this study was to gain further insight into the complex organismic regulation and fatigue dynamics during exercise.

MATERIALS AND METHODS

Participants

Nine endurance trained male cyclists were recruited from local sports clubs. We included Non-smoking adults who performed cycling training sessions for at least 12 h per week (300–450 km/week) in the 6 months before the start of the study but no altitude training or visits above 1000 m in the 3 months before the start of the study. As stated in the investigation of Gronwald et al. (2019a) a preliminary medical check-up, following the S1 guidelines of the German Association for Sports Medicine and Prevention, was performed to ensure that the participants were free from cardiovascular, neurologic, pulmonary, and orthopedic problems. The check-up also included an ECG at rest and a personal anamnesis. This study protocol was approved by the ethics committee of the University Clinic of Halle (Saale), Medical Faculty of Martin Luther University of Halle-Wittenberg and was performed in accordance with the guidelines of the Helsinki World Medical Association Declaration. Following explanation of any risks and benefits associated with the study, all participants approved the study and written informed consents were obtained.

Procedure

First, aerobic fitness was assessed using spiroergometry (Metamax 3b, Cortex, Germany) during an incremental test until the point of voluntary exhaustion (start: 100 W, increment: 20 W, length: 3 min, cadence: 80–90 rpm) on a high-performance bicycle ergometer (E 2000 s, FES, Germany). On the basis of the study by Dickhuth et al. (1999), the individual anaerobic threshold (IAT) was derived from the lactate-power curves. One and two weeks after the first laboratory visit, participants completed an exercise bout of continuous workload under both normoxic (NC) and normobaric HCs, with the same exercise intensity at IAT until voluntary exhaustion (**Figure 1**). The test conditions were randomized, single-blinded, and performed in a laboratory hypoxic chamber on the same bicycle ergometer and at the same daytime. Participants were instructed and encouraged to keep a nutrition intake and training load diary in order to keep influences at a minimum. Before the exercise bout, there was a warm-up period at 100 W for 10 min, followed by 150 W for 5 min. After the exercise bout there was a cool-down period at 100 W over a period of 10 min (see **Figure 1**).

Normobaric Hypoxic Conditions

A hypoxic chamber was used (Höhenbalance AG, Germany) for the supply of oxygen deficiency conditions (hypoxia, low oxygen concentration) at constant pressure conditions (normobaric) in the breathing air. The chamber had four b-Cat high-altitude-generators with an air conditioning system. To generate oxygen deficiency conditions, the four generators exchanged oxygen proportional to nitrogen (change in the O_2-N_2-ratio). The oxygen and carbon dioxide concentration in the breathing air were constantly measured and checked using a sensor system. In addition, the carbon dioxide content was maintained under a level of 0.3% via a ventilation system. The room temperature (20°C) and humidity (50%) were controlled by air conditioning of the chamber. The condition in the hypoxic chamber corresponded to normal laboratory conditions. The exercise bout under normobaric HCs was performed at a percentage of oxygen (oxygen fraction: FiO_2) in the breathing air of 15.0%, corresponding to an increase in simulated height of approximately 2700 m. The test under normoxic (sea-level) conditions (FiO_2, 20.9%) was performed in the same chamber.

Measurements

During exercise, the HR and RR-intervals were recorded continuously (beat-to-beat-modus) using a HR-monitor with a time resolution of 1 ms (Polar s810i, Polar Electro GmbH, Germany; Weippert et al., 2010). Collected raw data were transferred to a PC via an infrared interface, and artifacts were subsequently detected with a semi-automatic approach. The RR-intervals, which were distinguished by more than a 30% difference from the previous interval, were determined as artifacts. Artifacts were replaced with the average calculated from the previous and subsequent values, and data sets with more than 5% of artifacts were completely excluded from further processing. Only the NN intervals (normal-to-normal intervals) were considered during the data analysis (Task Force, 1996). The NN intervals were stored as ASCII files for further data analysis.

Using Kubios HRV software (Version 2.1, Biosignal Analysis and Medical Imaging Group, Kuopio, Finland; Tarvainen et al., 2014), the HRV analysis was conducted on data collected from

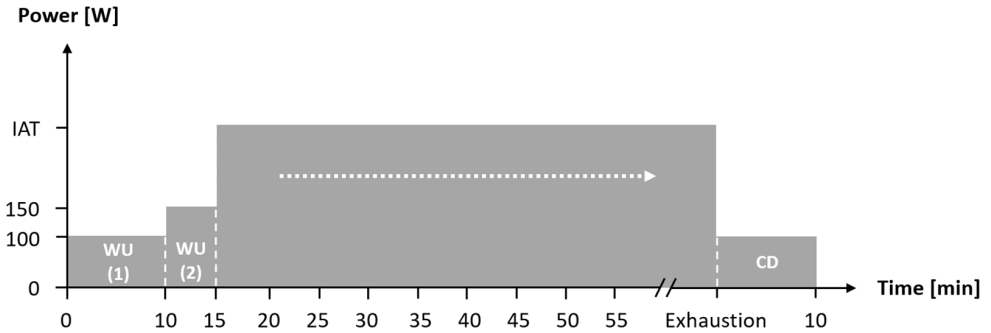

FIGURE 1 | Flow chart of the constant workload until voluntary exhaustion during normoxic and normobaric hypoxic condition. IAT: individual anaerobic threshold; WU (1): Warm-Up at 100 W; WU (2): Warm-Up at 150 W; CD: Cool-Down at 100 W.

the last 2 min of the warm-up and cool-down periods. In addition, during prolonged exercise segments of 2 min were analyzed every 5 min and before voluntary exhaustion. Besides the standard parameters obtained from time-domain analysis, including the average of the normal RR-interval length (meanRR in ms) and the total variability as the standard deviation of all normal RR-intervals (SDNN in ms), the scaling behavior was calculated using the Non-linear short-term scaling exponent DFA-alpha1. DFA has been referred to as a modification of the root mean square analysis (RMS) that is also suitable for analyzing short and Non-stationary time series data (Peng et al., 1995). Briefly, the RMS fluctuation of the integrated and detrended data is measured in observation windows of different sizes; the data are then plotted against the size of the window on a log–log scale. The scaling exponent represents the slope of the line, which relates the (log) fluctuation to the (log) window size (Mendonca et al., 2010). In this study, we only computed the short-term scaling exponent (window width: $4 \leq n \leq 16$ beats) because of the relatively short recording times for each condition (Tulppo et al., 2001; Hautala et al., 2003). The DFA-alpha1 values indicate time series fractal correlation properties, such as the type of noise (approximately 1.5 for strongly correlated Brownian noise and ≤ 0.5 for uncorrelated white noise with random signals). A value of approximately 1 signifies a mix of uncorrelated and maximally correlated signal components with $1/f$ noise; this represents a balance between the complete unpredictability (randomness) of white noise and the predictability (strong correlation) of Brownian noise (Platisa and Gal, 2008). The exponent is also an indicator of the "roughness" of the time series, with larger values of DFA-alpha1 representing a smoother time series (Peng et al., 1995; Goldberger et al., 2002).

Additionally, the blood lactate (La) and blood glucose (Glu) concentrations were assessed with Super GL ambulance (Dr. Mueller, Germany) from blood taken from an earlobe (Faude and Meyer, 2008). The oxygen saturation of the blood (SpO_2) was assessed via pulsoximetry (PM-60 OxiFlex, Mindray, Germany), and participants were asked to rate their perceived exertion (RPE: 6–20; Borg, 1982). These measures were taken every 5 min of the constant workload and after voluntary exhaustion. All parameters were also assessed during the end of the warm-up period, the end of the cool-down period, and in the resting state (with the exception of RPE).

Statistical Analysis

The statistical analysis was performed using SPSS 23.0 (IBM Statistics, United States) for Windows (Microsoft, United States). The Shapiro–Wilk test was applied to verify the Gaussian distribution of the data. The degree of variance homogeneity was verified by the Levene test. To analyze the effects of the exercise bout on dependent variables (HR, HRV parameters, La, SpO_2, and RPE) under the two conditions (NC, HC), a two-way ANOVA (factors: condition, time), with repeated measures, was applied. The main effects and interaction (condition × time) were reported and *post hoc* tests (Bonferroni) were applied to compare the differences between conditions. For the comparison of the dependent variables in the warm-up period, the cool-down period, and between the two conditions, the paired *t*-test was used. For all tests, the statistical significance was accepted as $p \leq 0.05$. η^2 was used to denote the effect sizes of main effects (small effect = 0.01, medium effect = 0.06, large effect = 0.14; Döring and Bortz, 2016) and Cohen's *d* for effect sizes in comparison of the measurement intervals (small effect = 0.2, medium effect = 0.5, large effect = 0.8; Cohen, 1988).

Because of the performance heterogeneity of the participants, the measures recorded during the constant workload were not statistically tested with the absolute test duration as an independent variable. To standardize and improve the comparability, the recorded data were normalized in relation to the individual total test duration. Therefore, before statistical processing of the data, all measures of all participants were interpolated to 10% steps using the cubic spline algorithm on MS Excel (Microsoft, United States). The preload value was defined as 0% in each case (Warm-Up at 150 W). The equidistant percentage segments calculated in this way enabled multiple statistical comparisons.

RESULTS

Participants (age: 26.4 ± 4.1 years; height: 181.7 ± 5.3 cm; body mass: 79.2 ± 9.3 kg; body fat: 13.2 ± 3.6%; VO_2peak:

53.1 ± 4.7 ml/min/kg) achieved a maximum power output of 342.2 ± 28.3W during the incremental cycling exercise test. The constant cycling bout under normoxic and normobaric HCs was performed at 266.2 ± 26.3 W (IAT) which corresponds to 80.8 ± 9.4% of peak oxygen uptake (VO_2peak) in the incremental test. Compared to the HC, participants obtained a significant longer duration during the normoxic condition (NC) until voluntary exhaustion (NC: 41:18 ± 08:21 min:sec vs. 24:42 ± 06:09min:sec; $p < 0.001$, $d = 1.553$). Maximum RPE values of 19.7 ± 0.7 (NC) vs. 19.6 ± 0.7 (HC) indicate a voluntary exhaustion during both conditions ($p = 0.738$, $d = 0.053$).

A significant main effect for condition could be found for La, SpO_2, RPE and DFA-alpha1. Despite for SpO_2, for all analyzed parameters a significant main effect of time could be determined. In addition, a significant main effect of interaction (condition × time) could be found for HR, La, RPE and meanRR, while SDNN and DFA-alpha1 showed a statistical trend. Detailed ANOVA results and descriptive values of all analyzed parameters during rest and over the time course of exercise during NC and HC are provided in **Table 1**.

In comparison of the beginning and end of the prolonged exercise bout (10% vs. 100%) during NC and HC significant changes could be found in all measures except for SpO_2 and SDNN during NC; increases for HR, La and RPE during both conditions; decreases for SpO_2 and SDNN during HC, meanRR and DFA-alpha1 during both conditions (NC – HR: $p < 0.001$, $d = 2.921$; La: $p < 0.001$, $d = 2.621$; SpO_2: $p = 0.652$, $d = 0.074$; RPE: $p < 0.001$, $d = 5.220$; meanRR: $p < 0.001$, $d = 1.766$; SDNN: $p = 0.081$, $d = 0.762$; DFA-alpha1: $p < 0.001$, $d = 3.943$; HC – HR: $p < 0.001$, $d = 3.322$; La: $p < 0.001$, $d = 2.561$; SpO_2: $p = 0.016$, $d = 0.728$; RPE: $p < 0.001$, $d = 6.388$; meanRR: $p < 0.001$, $d = 2.368$; SDNN: $p = 0.035$, $d = 0.973$; DFA-alpha1: $p < 0.001$, $d = 3.097$). In summary, we found a decrease in total variability combined with a reduction in the amplitude and correlation properties of RR fluctuations during prolonged exercise. In comparison of the first warm-up period and the cool-down period (WU (1) vs. CD) both of 10 min duration and at 100 W, significant changes could be found in all measures except for RPE; increases for HR, SpO_2 and La; decreases for meanRR, SDNN and DFA-alpha1 (NC – HR: $p < 0.001$, $d = 1.692$; La: $p = 0.001$, $d = 2.073$; SpO_2: $p = 0.013$, $d = 0.751$; RPE: $p = 0.347$, $d = 0.366$; meanRR: $p < 0.001$, $d = 1.588$; SDNN: $p < 0.001$, $d = 1.754$; DFA-alpha1: $p = 0.020$, $d = 1.337$; HC – HR: $p < 0.001$, $d = 1.374$; La: $p < 0.001$, $d = 4.446$; SpO_2: $p = 0.024$, $d = 1.393$; RPE: $p = 0.141$, $d = 0.463$; meanRR: $p = 0.001$, $d = 1.260$; SDNN: $p < 0.001$, $d = 2.544$; DFA-alpha1: $p = 0.001$, $d = 2.359$) (see **Figures 2–4**).

In comparison of the condition, we found significant higher values during prolonged exercise and HC of HR at 40–70% (40%: $p = 0.050$, $d = 0.653$; 50%: $p = 0.036$, $d = 0.659$; 60%: $p = 0.025$, $d = 0.707$; 70%: $p = 0.037$, $d = 0.676$), of La at 10–90% (10%: $p = 0.038$, $d = 0.766$; 20%: $p = 0.001$, $d = 1.403$; 30%: $p < 0.001$, $d = 1.450$; 40%: $p < 0.001$, $d = 1.331$; 50%: $p < 0.001$, $d = 1.263$; 60%: $p = 0.001$, $d = 1.371$; 70%: $p = 0.002$, $d = 1.293$; 80%: $p = 0.003$, $d = 1.098$; 90%: $p = 0.011$, $d = 1.009$) and of RPE at 50–90% (50%: $p = 0.025$, $d = 0.625$; 60%: $p = 0.013$, $d = 0.846$; 70%: $p = 0.011$, $d = 0.885$; 80%: $p = 0.019$, $d = 0.611$; 90%: $p = 0.036$, $d = 0.659$). Significant lower values during prolonged exercise and HC could be shown for SpO_2 at 10–100% (10%: $p < 0.001$, $d = 2.541$; 20%: $p < 0.001$, $d = 2.613$; 30%: $p < 0.001$, $d = 3.775$; 40%: $p < 0.001$, $d = 3.002$; 50%: $p < 0.001$, $d = 3.315$; 60%: $p < 0.001$, $d = 3.597$; 70%: $p < 0.001$, $d = 3.852$; 80%: $p < 0.001$, $d = 3.399$; 90%: $p < 0.001$, $d = 4.636$; 100%: $p < 0.001$, $d = 4.699$), for meanRR at 40–70% (40%: $p = 0.047$, $d = 0.675$; 50%: $p = 0.035$, $d = 0.659$; 60%: $p = 0.022$, $d = 0.713$; 70%: $p = 0.035$, $d = 0.687$), and for DFA-alpha1 at 20–60% (20%: $p = 0.050$, $d = 0.691$; 30%: $p = 0.006$, $d = 1.216$; 40%: $p = 0.014$, $d = 0.739$; 50%: $p = 0.050$, $d = 0.428$; 60%: $p = 0.028$, $d = 0.604$) (see **Figures 2–4**).

DISCUSSION

The presented data demonstrate that prolonged exercise until voluntary exhaustion provokes a lower total variability combined with a reduction in the amplitude and correlation properties of RR fluctuations. This may be attributed to increased organismic demands under NC and HC and could be confirmed by other parameters, such as increases in HR, La, and RPE and decreases in SpO_2. In addition, our data implies that HC provoked higher demands and loss of correlation properties at an earlier stage during the exercise regime compared to normoxia, implying an accelerated alteration of cardiac autonomic regulation. DFA-alpha1 was most sensitive to this difference by showing a large main effect for condition (η^2 of 0.765) and discriminating even at early relative exercise durations of 20%, while HR and meanRR failed to do so until 40–70% of the prolonged exercise. In this regard, DFA-alpha1 could provide added value in the interpretation of the hypoxic effects during exercise. This observation is in line with the results of previous studies supporting higher values of HR, La, and RPE and considerably lower values of SpO_2 during exercise under hypoxic compared with NCs (Bärtsch and Gibbs, 2007; Ofner et al., 2014; Moon et al., 2016; Deb et al., 2018). However, the magnitude of cardiopulmonary responses to a certain intensity of hypoxia and exercise is intra-individual (Deb et al., 2018; Wehrlin and Hallén, 2006). These differences could be interpreted as an acute compensation response to reduced aerobic exercise availability by decreased oxygen delivery and utilization capacities under HC. The higher demands result in a shorter exercise duration until voluntary exhaustion during HC. It should be noted that, through the maintenance of the required power output for as long as possible and the absence of a known endpoint, the exercise regime could be classified as "not self-paced" and "open-loop" (Smirmaul et al., 2013). Open-loop exercises require a simple behavioral decision of "continue" or "stop" by the participants. During self-regulated or self-paced (closed-loop) exercise, participants are able to compensate by voluntarily changing the power or speed at which they are performing the task, through pacing. With reference to the earlier loss of correlation properties due to DFA-alpha1 during HC (especially during the first half of the prolonged exercise), to date there is only little information on the influence of different kinds of hypoxia on DFA-alpha1 during resting conditions

TABLE 1 | Heart rate, oxygen saturation, lactate, rating of perceived exertion and HRV measures (Mean ± SD) during resting state and all cycling conditions during normoxia (NC) and normobaric hypoxia (HC).

Parameters	Condition	Rest	WU (1)	WU (2)	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	CD
HR [1/min]	NC	72.2 ± 5.7	108,4* ± 10.1	120,5* ± 13.6	147,2* ± 11.5	155,0* ± 10.9	157,3* ± 11.0	159,1 ± 10.3	160,3 ± 9.6	161,4 ± 9.3	163,9* ± 9.4	169,0* ± 8.5	174,7* ± 7.9	180,5*¥ ± 9.0	126,9*§ ± 11.0
	HC	69.3 ± 8.8	111.8* ± 10.0	127.2* ± 12.0	145.9* ± 9.9	159.3* ± 8.7	165.3* ± 7.0	167.6# ± 6.4	169.1# ± 6.3	170.6# ± 6.2	172.3*# ± 6.0	173.6* ± 6.0	175.1* ± 6.6	176.8¥ ± 7.6	127.3*§ ± 12.0
ANOVA	10–100% – Condition: F = 3.348, $p = 0.105$, $\eta^2 = 0.295$; Time: F = 181.496, $p < 0.001$, $\eta^2 = 0.958$; Interaction: F = 15.638, $p < 0.001$, $\eta^2 = 0.662$														
SpO_2 [%]	NC	98.0 ± 0.5	97.1* ± 0.8	97.1 ± 0.6	96.0 ± 1.1	95.8 ± 1.3	96.1 ± 1.0	96.0 ± 0.7	95.7 ± 0.8	95.8 ± 0.8	95.9 ± 0.8	95.9 ± 0.8	95.5 ± 1.1	95.9 ± 1.1	97.7*§ ± 0.5
	HC	92.4# ± 2.7	86.6*# ± 3.9	85.2# ± 2.7	84.5# ± 2.7	84.0# ± 2.8	83.7# ± 2.7	83.4# ± 2.3	83.3# ± 2.1	83.3# ± 2.1	83.4# ± 2.3	83.4# ± 2.3	83.1# ± 2.1	82.7#¥ ± 2.2	91.1*§ # ± 2.5
ANOVA	10–100% – Condition: $F = 591.301$, $p < 0.001$, $\eta^2 = 0.987$; Time: $F = 2.266$, $p = 0.121$, $\eta^2 = 0.221$; Interaction: $F = 1.901$, $p = 0.163$, $\eta^2 = 0.192$														
La [mmol/l]	NC	1.03 ± 0.27	0.72* ± 0.30	0.94 ± 0.62	2.19* ± 0.92	2.65 ± 1.28	2.70 ± 1.63	2.67 ± 1.90	2.69 ± 2.07	2.78 ± 2.27	3.11 ± 2.41	3.72 ± 2.53	4.75 ± 2.44	6.89¥ ± 2.09	3.30*§ ± 1.59
	HC	0.73 ± 0.21	0.65 ± 0.15	0.86* ± 0.33	2.94*# ± 0.39	4.55*# ± 0.58	5.48*# ± 0.79	5.98*# ± 0.94	6.31# ± 1.26	6.68# ± 1.73	7.13# ± 2.06	7.49# ± 2.09	7.76# ± 1.97	8.14¥ ± 1.78	3.61*§ ± 0.96
ANOVA	10–100% – Condition: $F = 31.402$, $p = 0.001$, $\eta^2 = 0.797$; Time: $F = 40.337$, $p < 0.001$, $\eta^2 = 0.834$; Interaction: $F = 9.395$, $p = 0.001$, $\eta^2 = 0.540$														
RPE [6-20]	NC	–	7.2 ± 1.6	8.8* ± 1.9	13.0* ± 1.3	14.1* ± 1.0	14.2 ± 1.2	14.5 ± 1.2	15.0 ± 1.1	15.1 ± 1.4	15.5 ± 1.5	16.5 ± 1.2	17.3 ± 1.5	19.7¥ ± 0.7	7.9* ± 2.0
	HC	–	7.2 ± 1.3	9.4* ± 1.8	12.4* ± 1.4	14.4* ± 1.2	15.2 ± 1.3	15.8 ± 1.3	16.3# ± 1.1	16.8# ± 1.2	17.5# ± 1.4	18.1# ± 1.4	18.7# ± 1.1	19.6¥ ± 0.7	7.9* ± 1.5
ANOVA	10–100% – Condition: $F = 45.320$, $p < 0.001$, $\eta^2 = 0.850$; Time: $F = 99.726$, $p < 0.001$, $\eta^2 = 0.926$; Interaction: F = 6.840, $p = 0.006$, $\eta^2 = 0.461$														
meanRR [ms]	NC	842 ± 66	558* ± 49	503* ± 52	411* ± 34	388* ± 28	383 ± 28	378 ± 25	376 ± 22	373 ± 21	367* ± 21	356* ± 18	344* ± 16	333*¥ ± 17	476*§ ± 41
	HC	884 ± 99	538* ± 53	471* ± 48	417* ± 32	379* ± 22	362* ± 15	358# ± 14	356# ± 13	352# ± 13	349*# ± 12	346* ± 12	343* ± 13	340¥ ± 15	474*§ ± 46
ANOVA	10–100% – Condition: $F = 2.931$, $p = 0.125$, $\eta^2 = 0.268$; Time: F = 110.715, $p < 0.001$, $\eta^2 = 0.933$; Interaction: F = 10.399, $p = 0.001$, $\eta^2 = 0.565$														
SDNN [ms]	NC	66.5 ± 17.5	8.7* ± 3.0	5.7* ± 1.9	2.6* ± 0.7	2.1 ± 0.4	2.3 ± 0.3	2.1 ± 0.3	2.0 ± 0.3	2.0 ± 0.3	1.9 ± 0.4	1.9 ± 0.4	2.0 ± 0.5	2.1 ± 0.5	3.6*§ ± 1.2
	HC	82.3 ± 21.9	6.8* ± 1.8	4.2* ± 1.5	2.9 ± 0.7	2.1 ± 0.5	1.9 ± 0.4	1.9 ± 0.4	2.0 ± 0.5	2.1 ± 0.5	2.1 ± 0.4	2.1 ± 0.5	2.2 ± 0.6	2.3¥ ± 0.6	3.3*§ ± 0.7
ANOVA	10–100% – Condition: F = 0.340, $p = 0.576$, $\eta^2 = 0.041$; Time: F = 8.560, $p = 0.001$, $\eta^2 = 0.517$; Interaction: F = 2.641, $p = 0.062$, $\eta^2 = 0.248$														

(Continued)

TABLE 1 | Continued

Parameters	Condition	Rest	WU (1)	WU (2)	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%	CD
DFA-alpha1 []	NC	1.35 ± 0.13	1.49* ± 0.16	1.27* ± 0.25	0.79* ± 0.14	0.66 ± 0.14	0.62 ± 0.18	0.54 ± 0.16	0.50 ± 0.13	0.51 ± 0.11	0.47 ± 0.12	0.38 ± 0.12	0.31 ± 0.14	0.31¥ ± 0.11	1.18*§ ± 0.28
	HC	1.26 ± 0.10	1.44* ± 0.18	1.11* ± 0.25	0.77* ± 0.16	0.53*# ± 0.13	0.41# ± 0.08	0.38# ± 0.08	0.39# ± 0.12	0.36# ± 0.14	0.34 ± 0.18	0.32 ± 0.16	0.32 ± 0.15	0.33¥ ± 0.12	0.99*§ ± 0.20
ANOVA	10–100% – Condition: $F = 26.098$, $p = 0.001$, $\eta^2 = 0.765$; Time: $F = 49.257$, $p < 0.001$, $\eta^2 = 0.860$; Interaction: $F = 2.897$, $p = 0.080$, $\eta^2 = 0.266$														

*WU (1): Warm-Up at 100 W; WU (2): Warm-Up at 150 W; 10–100%: Percentage of continuous workload; CD: Cool-Down at 100 W. Main effects (factors: condition, time) and interaction (condition × time) of the two-way ANOVA with repeated measures. HR: heart rate, SpO_2: oxygen saturation of the blood, La: blood lactate concentration, RPE: rate of perceived exertion, meanRR: average of normal RR-intervals, SDNN: standard deviation of all normal RR-intervals, DFA-alpha1: short-term scaling exponent of detrended fluctuation analysis; *Significant compared to preceding measurement; §Significant change WU (1) vs. CD at 100W; ¥Significant change 10% vs. 100%; #Significant change NC vs. HC ($p \leq 0.05$).*

(Vigo et al., 2010; Zhang et al., 2015; Giles et al., 2016), but not during exercise.

However, the decrease in DFA-alpha1 may verify a demand-dependent change from strongly correlated behavior in the warm-up periods, to uncorrelated/stochastic or anti-correlated behavior of the RR-intervals during prolonged exercise in both conditions (Platisa and Gal, 2008). This is consistent with previous studies reporting an almost linear reduction in complexity and correlation properties with a gradual change of the RR data structure towards an anti-correlated and merely random signal for medium-to-high exercise intensity demands (Karasik et al., 2002; Hautala et al., 2003; Casties et al., 2006; Platisa et al., 2008; Hottenrott and Hoos, 2017; Blasco-Lafarga et al., 2017; Gronwald et al., 2018, 2019a,b). Due to increased sympathetic activity and/or decreased parasympathetic activity during endurance exercise, the loss of complexity could be related to the disruption of the equilibrium and interaction between the two branches of the autonomic nervous system (Sandercock and Brodie, 2006; Lewis and Short, 2010). This particular change could be due to a protection of homeodynamic processes through an organismic system withdrawal (Casties et al., 2006; Platisa et al., 2008), which are matched by the CAN, integrating various internal and external stimuli (Benarroch, 1993, 1997). The great loss of complexity might also be a consequence of complementary neural mechanisms/circuits (Shaffer et al., 2014) which aim the maintenance of locomotor-respiratory coupling in the context of coordination between movement frequency (e.g., cadence in cycling exercise), heartbeat and breathing patterns during cycling exercise (Casties et al., 2006; Blasco-Lafarga et al., 2017; Gronwald et al., 2018, 2019a).

A possible explanatory approach for the decrease in HRV complexity during endurance exercise could be an increased reduction of the input number of different physiological systems, and/or the reduction of the interaction of various subsystems with a particular focus on either one dominant system or a few dominant systems (Nakamura et al., 1993; Casties et al., 2006). This could be interpreted in the sense of centralization or "mechanization" of a complex physiological system (von Bertalanffy, 1950). In the sense of this mechanization of the organism regulation, a dominant "performance attractor" could emerge during high physiological demand, which could be determined by sympathetic activity (Karasik et al., 2002; Hautala et al., 2003), neuro-mechanical coupling of several oscillators (Casties et al., 2006), and/or Non-neural, intrinsic HR regulation (Platisa and Gal, 2008). Thus, every fluctuation is corrected immediately in the opposite direction by the dominant attractor, which results in a random or anti-correlated signal. This organismic system withdrawal may also be interpreted as a loss of systemic integrity, in the sense of a hazardous situation for homeostasis (Seely and Macklem, 2004).

In this regard, a link between the complexity measures of HRV and their connectivity from an autonomic nervous system point of view might offer new perspectives to evaluate complex models of exercise fatigue and endurance performance. The field of research contains numerous models that emphasize the importance of the brain for the control

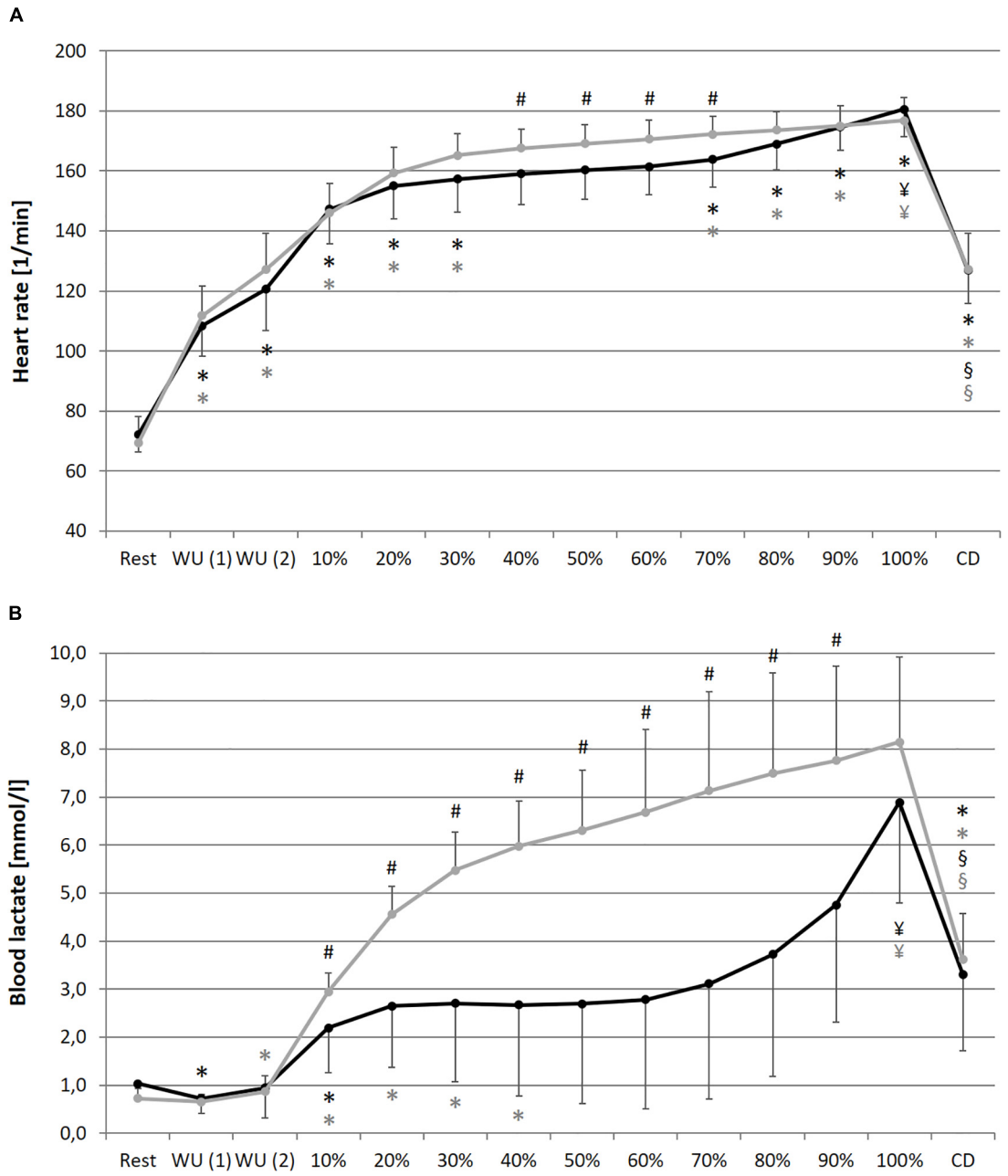

FIGURE 2 | Heart rate **(A)**, blood lactate concentration **(B)** during resting state and all cycling conditions during normoxia (NC, black color) and normobaric hypoxia (HC, gray color). WU (1): Warm-Up at 100W; WU (2): Warm-Up at 150W; 10–100%: Percentage of continuous workload; CD: Cool-Down at 100W. *Significant compared to preceding measurement; §Significant change WU (1) vs. CD at 100W; ¥Significant change 10% vs. 100%; #Significant change NC vs. HC ($p \leq 0.05$).

and regulation of organismic processes during endurance exercise (Abbiss and Laursen, 2005; Marcora, 2008; Ament and Verkerke, 2009; Millet, 2011: Noakes, 2011, 2012; St Clair Gibson et al., 2018) and other concepts of cardiovascular control during exercise that focus on central command (Boulpaep, 2009; Williamson, 2010). Some of the models have been much debated in the past (e.g., Amann and Secher, 2010; Marcora, 2010; Perrey et al., 2010). However, it should be noted that in this debate, Smirmaul et al. (2010) puts the discussion in a nutshell, so that the role of central control cannot be ignored (Noakes, 2011), the cognitive and motivational factors proposed by Marcora (2010) comprise key components of exercise tolerance and endurance performance; afferent feedback from motor muscles (Amann and Secher, 2010) is also important (but not the sole factor directly limiting endurance exercise performance) since it acts to increase the conscious awareness of organismic "discomforts." This holistic approach, with the complex interaction of all of these factors, allows for more

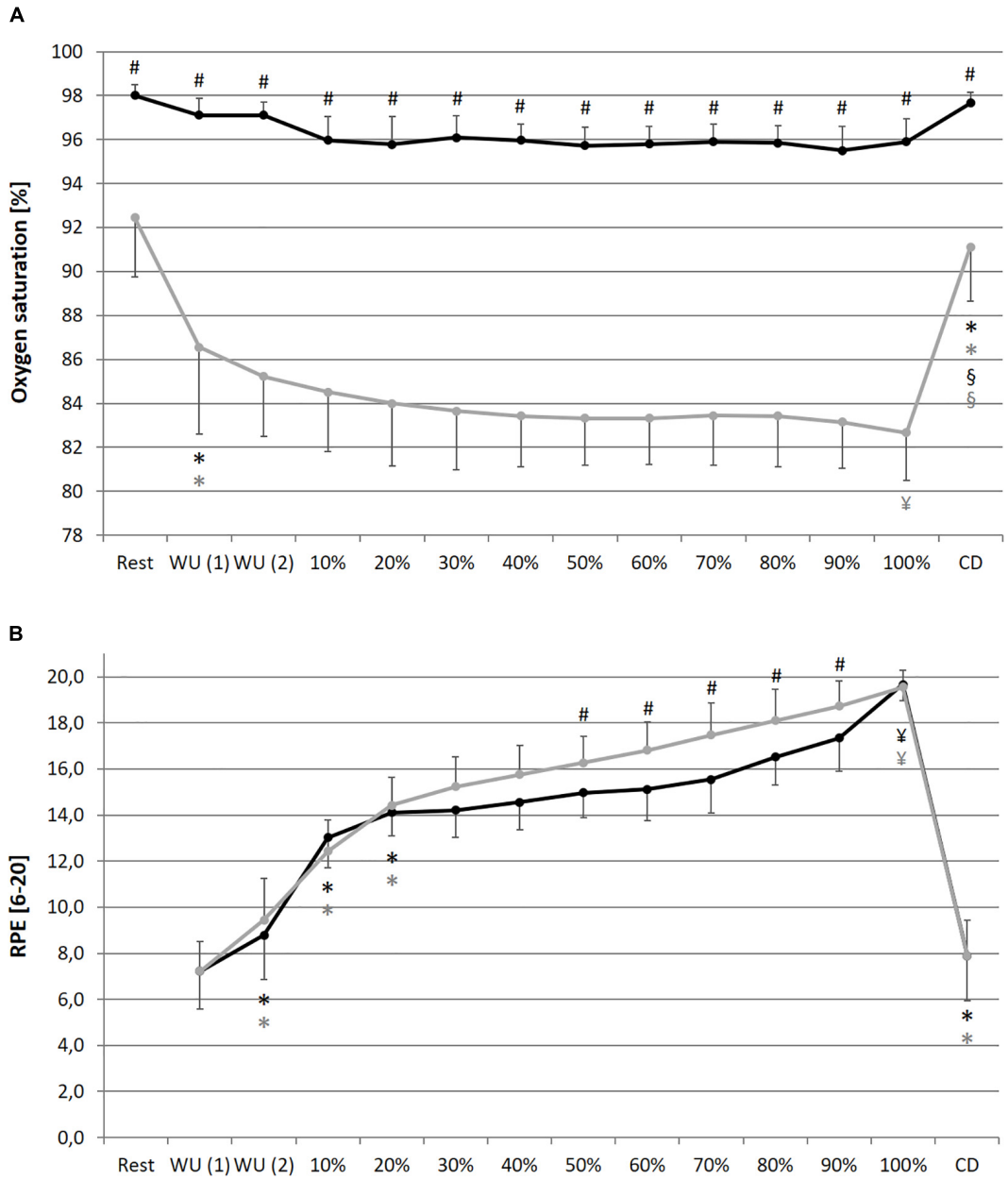

FIGURE 3 | Oxygen saturation of the blood **(A)** and rate of perceived exertion RPE, **(B)** during resting state and all cycling conditions during normoxia (NC, black color) and normobaric hypoxia (HC, gray color). WU (1): Warm-Up at 100 W; WU (2): Warm-Up at 150 W; 10–100%: Percentage of continuous workload; CD: Cool-Down at 100 W. *Significant compared to preceding measurement; §Significant change WU (1) vs. CD at 100 W; ¥Significant change 10% vs. 100%; #Significant change NC vs. HC ($p \leq 0.05$).

appropriate behavioral decisions and is crucial, especially for endurance exercise performance. Finally, fatigue processes are complex, and their analysis and understanding should not be reduced to a single isolated factor.

In this context, the Non-linear dynamics of HRV might provide a new explanatory approach. The integration of peripheral and central information for the self-controlled down regulation and limitation of muscle recruitment as a protection mechanism for organismic homeostasis, as hypothetized in the "central governor model" (Noakes, 2011, 2012), could be described in more detail as follows (Gronwald et al., 2018). On the basis of the interaction between cardiovascular feedback mechanisms and the central command within the CAN (Benarroch, 1993), this could lead to an accumulation of afferent feedback disturbances modified by many physiological systems (e.g., relevant sensory information, previous sport specific experiences, load anticipation, and current environmental conditions). This results in a multi-feedback

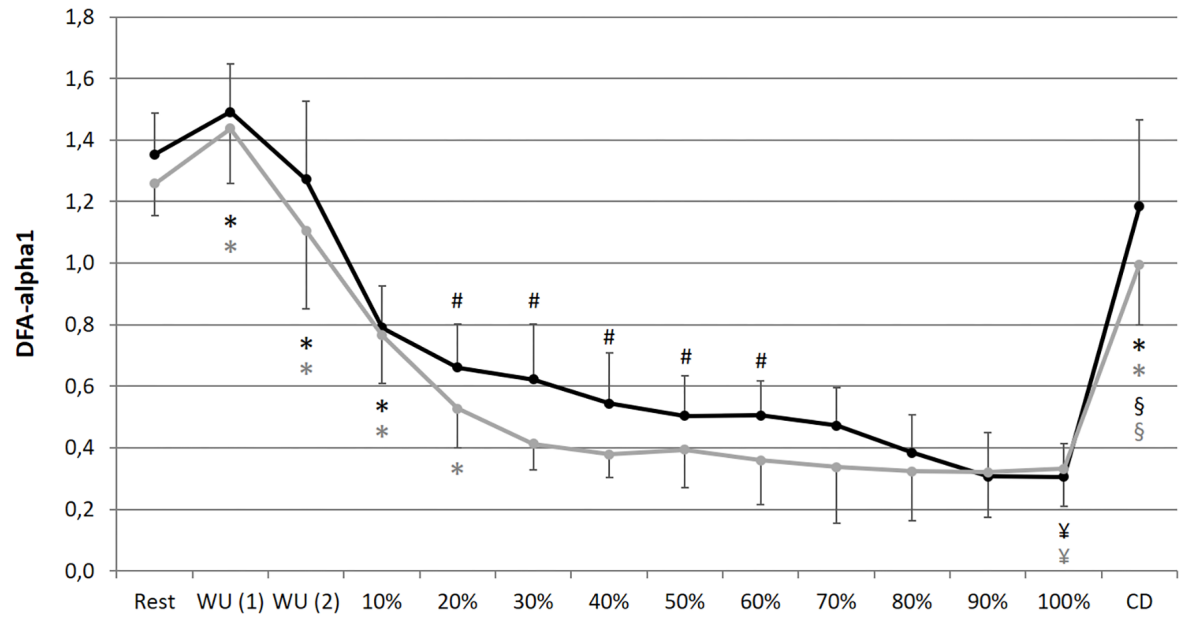

FIGURE 4 | Short-term scaling exponent (DFA-alpha1) during resting state and all cycling conditions during normoxia (NC, black color) and normobaric hypoxia (HC, gray color). WU (1): Warm-Up at 100 W; WU (2): Warm-Up at 150 W; 10–100%: Percentage of continuous workload; CD: Cool-Down at 100 W. *Significant compared to preceding measurement; §Significant change WU (1) vs. CD at 100 W; ¥Significant change 10% vs. 100%; #Significant change NC vs. HC ($p \leq 0.05$).

system which regulates physiologic processes during exercise (Gronwald et al., 2018).

The study group of Noakes et al. (2001) also provides evidence that peak cardiovascular function and peak skeletal muscle electromyographic activity is reduced during maximal exercise in hypoxia. The authors concluded that the presented data support a model in which a central neural governor constrains the cardiac output by regulating the mass of skeletal muscle that can be activated during maximal exercise. Although there is no difference between NC and HC in the presented data during voluntary exhaustion for all analyzed measures, with the exception of SpO_2, this indicates voluntary exhaustion during both conditions, with maximum values of RPE. However, if we take a closer look at the HR data, we can determine slightly (not significant) decreased maximum values during HC compared to NC. Additionally, there was only a further increase in HR data from 90 to 100% during NC, and a leveling off during HC. These observations could support the concluding remarks of Noakes et al. (2001).

In support of such a model, parameters such as blood lactate concentration (here with a substantially-accelerated increase during HC) can be considered to be indicative of functions of a signal molecule for peripheral-central information exchange (Philp et al., 2005), which, in conjunction with other psycho-physiological variables (e.g., HR, respiratory rate, overall ventilation, energy substrates, mood, and motivation; Noble and Robertson, 1996), have a decisive influence on the perceived exertion, which then, as an integrative predictor, determines exercise tolerance and fatigue during endurance exercise (Baron et al., 2008; Crewe et al., 2008, Marcora and Staiano, 2010). The fact that, so far, none of the numerous physiological, biochemical, and cognitive models used to explain peripheral and central fatigue can fully clarify the limitations and determinants of endurance performance confirms this assumption (Abbiss and Laursen, 2005; Weir et al., 2006; Enoka and Duchateau, 2008; Shephard, 2009). Here, Non-linear analysis of HRV may provide a different perspective on complex cardiovascular interaction during endurance performance.

In addition, the results of the present study, and the assumptions described above, indicate that the RPE could also be seen as a part of this feedback loop (Fontes et al., 2015; Pageaux, 2016). Indeed, RPE acts as a strong predictor of exercise tolerance and fatigue and substantially determines the duration until exercise termination with constant workload (Horstman et al., 1979; Eston et al., 2007; Crewe et al., 2008; Nakamura et al., 2008; Noakes, 2008; Marcora and Staiano, 2010). Thus, perception of effort should be considered not only as a marker of exercise intensity, but also as a factor limiting endurance performance (Pageaux and Lepers, 2016), for example as cardinal exercise stopper during high-intensity aerobic exercise rather than severe locomotor muscle fatigue or intolerably unpleasant muscle pain (Staiano et al., 2018). Furthermore, the RPE increases significantly with the onset of exercise and increases further linearly during prolonged exercise under HC until voluntary exhaustion. In the NC condition, the course could be better characterized as exponential. However, the behavior of the RPE increase corresponds to the results of other studies and shows a rather linear trend in the course of the prolonged exercise with its highest values at voluntary exhaustion (Eston et al., 2007; Crewe et al., 2008; Nakamura et al., 2008; Fontes et al.,

2010; Pires et al., 2011). Hence, the termination of endurance exercise seems to be caused by a conscious decision-making process in which the perception of effort plays an important role (Staiano et al., 2018).

As a proxy of afferent feedback, the RPE, which increased in the time course of the exercise during both conditions and with an intensified increase during HC, reflects a copy of the resulting efferent motor command, which is processed in sensory brain areas (Tucker, 2009; Williamson, 2010; Pageaux, 2016). This approach implies that the regulatory mechanism compares context-specific and teleanticipatory feedforward estimations of the internal load situation with permanent psycho-physiologically afferent feedback of the peripheral and central subsystems and organ systems; this results in a perception of effort. Thus, the function of the brain and the subjective RPE as associated markers for the protection of organismic homeostasis are considered to be of crucial importance for exercise tolerance, endurance performance, and the control of performance output during endurance exercise (e.g., pacing strategies in closed-loop exercises) (St Clair Gibson et al., 2006; Abbiss and Laursen, 2008; Tucker, 2009; Tucker and Noakes, 2009). In this context, the Non-linear analysis of HRV could provide a new perspective for the integrated and holistic consideration (as promoted by leading experts in the field) of regulatory and fatigue processes during exercise (Smirmaul et al., 2010; Marino et al., 2011; Venhorst et al., 2018), and can also describe the influence of different environmental conditions such as normobaric hypoxia. Overall, the application of DFA may provide a new possibility to analyze the relationship between different modes of exercise, environmental conditions, and the altered cardiovascular (autonomic) regulation. This could be useful in response to the concern over strongly decreased variability and weak reproducibility of amplitude dependent time- and frequency-domain HRV measures during exercise (Persson and Wagner, 1996; Tulppo et al., 2005; Millar et al., 2009; Gronwald et al., 2019a).

LIMITATIONS

The generalizability of the presented findings in this pilot study is limited due to the small number of participants. In addition, only male cyclists were included in order to exclude the confounding influence of sex. Although the DFA is a recognizably useful diagnostic method, especially because linear approaches of spectral analyses are unable to reveal changes in HRV that are related to the non-linear interaction of physiological mechanisms, their detailed physiological interpretation remains unclear (Silva et al., 2017). We are also aware that exercise physiology during exercise is too complex and too dependent on certain conditions to be broken down into a single key measure. Nevertheless, DFA analysis of HRV may represent a suitable approach with a qualitative view of physiologic exercise regulation, and it may be useful in combination with other applicable internal and external load measures for training diagnostics and monitoring (Gronwald et al., 2019a). In addition, DFA extracts intrinsic properties of HRV dynamics, where the total variability is disregarded. Although log–log plots of fluctuations vs. window size will be shifted up or down regarding total variability, the DFA-alpha1 (slope of the curve) will remain unaffected. Hence, the presented results can be assumed to be related to the cardiac autonomic modulation and not affected by different mean HRs (Peng et al., 1995; Sandercock and Brodie, 2006; Silva et al., 2017). Lastly, in order to minimize the environment's external influence and to enable coupling processes in the presented study and previous studies (Gronwald et al., 2018, 2019a,b), respiration was not prescribed and controlled. Allowing spontaneous breathing might have been a limitation, but it was necessary as we aimed to analyze individual responses during exercise as stated by Blasco-Lafarga et al. (2017).

CONCLUSION

The present data show for the first time that under both normoxic and normobaric HCs prolonged exercise until voluntary exhaustion provokes a lower total variability combined with a reduction in the amplitude and correlation properties of RR fluctuations, which may be attributed to increased organismic demands. These findings verify a demand-dependent change to anti-correlated or uncorrelated/stochastic behavior of the RR-intervals during prolonged exercise until voluntary exhaustion. This loss of complexity in the time series during exercise could be related to the disruption of the equilibrium and interaction between the two branches of the autonomic nervous system shown by DFA-alpha1. Additionally, normobaric hypoxia provoked higher demands and a more pronounced loss of correlation properties at an earlier stage during the exercise regime, implying an accelerated alteration of cardiac autonomic regulation. Hence, DFA analysis provides a complementary systemic view (Ahn et al., 2006) of cardiovascular regulation in the context of complex models of exercise and fatigue during different environmental conditions. From a practical point of view, the given approach may help to elucidate the influence of different training regimes under varied conditions and with athletes of different performance levels on complex autonomic regulation and therefore offer a new assessment and monitoring tool of the functional state of athletes (Gronwald et al., 2019a).

DATA AVAILABILITY

The datasets for this study will not be made publicly available because only raw data of HRV (rr-intervals) exist.

ETHICS STATEMENT

This study protocol was approved by the ethics committee of the University Clinic of Halle (Saale), Medical Faculty of Martin

Luther University of Halle-Wittenberg and was performed in accordance with the guidelines of the Helsinki World Medical Association Declaration. Following explanation of any risks and benefits associated with the study, all participants approved the study and written informed consents were obtained.

AUTHOR CONTRIBUTIONS

TG and KH conceived and designed the study details. TG conducted and analyzed the data collection and wrote the first draft of the manuscript. OH and KH reviewed and edited the draft critically. All authors read and accepted the final version of the manuscript.

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Cardiotonic Pills Plus Recombinant Human Prourokinase Ameliorates Atherosclerotic Lesions in LDLR$^{-/-}$ Mice

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Aim: This study was to explore the protective effects of cardiotonic pills (CP) or/and recombinant human prourokinase (proUK)on the atherosclerosis and the potential underlying mechanism.

Methods and Results: Atherosclerosis was induced in LDLR$^{-/-}$ mice by high fat diet contained 20% lard and 0.5% cholesterol. Daily oral administration of CP (130 mg/kg) or/and intravenous injection of proUK (2.5 mg/kg, twice a week) began at 8 weeks after feeding with high fat diet and continued for 4 weeks. CP alone treatment markedly decreased plasma triglyceride, but did not ameliorate atherosclerosis plaque. No effect was observed for proUK alone on any endpoints tested. CP plus proUK induced a significantly reduction in the atherosclerotic lesions, along with decreased levels of total cholesterol, triglyceride in the plasma. CP plus proUK inhibited the elevated hepatic total cholesterol and triglyceride in high fat diet-fed LDLR$^{-/-}$ mice, up-regulating the expressions of ATP-binding cassette gene 5 and 8, and adipose triglyceride lipase. In the aorta, CP plus proUK inhibited the expression of scavenger receptor A and CD36 in LDLR$^{-/-}$ mice. In addition, we observed that systemic inflammation was inhibited, manifested downregulation of plasma macrophage inflammatory protein-1α and intercellular cell adhesion molecule-1.

Conclusion: CP plus proUK effectively attenuated atherosclerosis plaque in LDLR$^{-/-}$ mice, which is associated with normalizing the lipid metabolism in the liver and aorta, reducing phagocytosis of receptor-mediated modified-LDL uptake and inhibiting systemic inflammation.

Keywords: atherosclerosis, inflammation, ATP-binding cassette gene 8, adipose triglyceride lipase, scavenger receptor A, CD36

INTRODUCTION

Atherosclerosis (AS) is a chronic disease of the arterial wall which is the leading cause of disability and death around the world (Castelli et al., 1986; Pant et al., 2014). Hyperlipidemia, system inflammation and hypertensionare thought to be the major risk factors in the formation and development of AS (Zhu et al., 2018).

Lipids play a central role in the pathogenesis of AS. Plasma low-density lipoprotein (LDL) levels have a positive association with the development of AS. AS is initiated by endothelial dysfunction predominantly due to the accumulation of plasma LDL (Alonso et al., 2014). Endothelial cell dysfunction leads to the infiltration of LDL particles and their subsequent oxidation to oxidized-LDL (ox-LDL). Macrophages differentiated from circulating monocyte take up ox-LDL via scavenger receptor A(SR-A) and CD36 to form foam cell (Lusis, 2000; Zhu et al., 2018).

Liver is the major organ that regulates plasma lipids balance especially the LDL content. Disrupted hepatic total cholesterol (TC) and triglyceride (TG) homeostasis contributes to the pathogenesis of dyslipidemia, hepatic lipid deposition and atherosclerosis (Kim et al., 2014). There are two ways to decrease hepatic cholesterol: reducing cholesterol synthesis or excreting excess cholesterol into bile by reverse cholesterol transportation (Hageman et al., 2010). It has been reported repeatedly that increasing the turnover of bile salts has a beneficial effect on LDL-C levels. Several bile salt sequestrates have been successfully used to reduce LDL-C levels in patients with hypercholesterolemia (Maxfield and Tabas, 2005). Hepatic TG deposition is manifested as increased lipogenesis, disrupted fatty acid (FA) oxidation and depressed triglyceride (TG) lipolysis. Hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) are two enzymes critical for hepatic neutral cholesterol ester hydrolase (Buers et al., 2009; Buchebner et al., 2010). ATGL$^{-/-}$ macrophages accumulate TG-rich lipid droplets resulting in altered cell morphology that resemble macrophage foam cells. These alterations and functional changes strongly argue for the involvement of ATGL in atherogenesis. Transplantation of ATGL$^{-/-}$ bone marrow into γ-irradiated LDLR$^{-/-}$ mice resulted in highly attenuated atherosclerotic lesion formation compared with WT bone marrow-transplanted LDLR$^{-/-}$ mice after feeding high fat diet for 9 weeks (Paul et al., 2008; Radovic et al., 2012).

Inflammation plays an important role in the initiation and progression of atherosclerotic plaque. Inflammatory signaling alters the behavior of the intrinsic cells of the artery wall (endothelium and smooth muscle), and recruits further inflammatory cells that interact to promote lesion formation and complications. In humans, ongoing inflammatory reactions within the coronary atherosclerotic plaques are increasingly thought to be crucial determinants of the clinical course of patients with coronary artery disease (Stoll and Bendszus, 2006).

The cardiotonic pill (CP) is a compound Chinese medicine composed of *Salvia miltiorrhiza, Panax notoginseng*, and *Borneol*, which has been widely and effectively used in cardiovascular diseases in China. The major active components of CP are dihydroxy-phenyl lactic acid, tanshinone II-A (both from *S. miltiorrhiza*), and notoginsenoside R1 (from *P. notoginseng*). Our previous studies revealed that CP attenuated myocardial I/R injury, protecting against microcirculatory disturbance, cardiac dysfunction, and myocardium infarction. CP protected against post infarction myocardial fibrosis along with a reduction in chemokine production, macrophage infiltration, and fibroblast activation (Wei et al., 2013; Yang et al., 2013). Recent reports suggested that CP normalized hyperlipimedia, improving vascular function and other pathological processes (Niu et al., 2000; Zhao et al., 2010; Guo et al., 2016; Han et al., 2017; Lim et al., 2017). Recombinant human prourokinase (proUK) (Geng et al., 2018; Hao et al., 2018; Zhao et al., 2018) is a novel type of thrombolytic drug for clinical application in thrombotic diseases, which selectively activates fibrinogen on the surface of the thrombus, and has less side effect of bleeding (Sasahara et al., 1995; Verstraete, 1999). However, no study is reported as to the effect of CP or proUK on progression of atherosclerosis. The present study is to evaluate the effect of CP or/and proUK on the atherosclerosis and the potential underlying mechanism.

MATERIALS AND METHODS

Materials

Cardiotonic pills (Batch no. 150203) and proUK (Batch no. 20170501) were supplied by Tasly Pharmaceutical Co., Ltd. (Tianjin, China). The processing of the product followed a strict quality control, and the ingredients were subjected to standardization.

Antibodies recognizing ATGL and GAPDH were from Cell Signaling Technology (Boston, MA, United States). Antibodies against CD36, SR-A, SR-BI, PPARα, ABCA1, ABCG1, ABCG5, andABCG8 were from Abcam (Cambridge, MA, United States). Oil Red O was from Sigma-Aldrich (St. Louis, MO, United States). All other reagents used in our study were of analytical grade.

Animals

LDLR$^{-/-}$ mice were purchased from Animals Center of Peking University Health Science Center. Animals were raised at a temperature of 20 ± 2°C with 12-h light/dark cycles, and fed with standard rat chow and water. All surgical procedures performed on animals were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (LA2010001). Procedures involving animals and their care were conducted in conformity with international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept).

Male 6-8-weeks-old LDLR$^{-/-}$ mice were acclimated to our animal care facilities for 5 days before experiment, with free access to normal chow diet and water. Animals were first randomly allocated to two groups: chow diet (n = 8) and high fat diet (n = 102). Animals in high fat diet group were fed with high fat diet contained 20% lard, 0.5% cholesterol, 79.5% basic diet. After 8 weeks, the high fat diet-fed LDLR−/− mice were

randomly divided into five groups: high fat diet + NS (model, n = 20), high fat diet + CP (n = 28), high fat diet + proUK (n = 20), high fat diet + CP plus proUK (n = 26),high fat diet + Atorvastatin plus Aspirin (n = 8). CP was administrated at 130 mg/kg daily by gavage, atorvastatin and aspirin also were intragastrically administered at doses of 6 and 15 mg/kg/day, respectively. ProUK was intravenously injected at 2.5 mg/kg, twice a week, for 4 weeks. CP, atorvastatin and aspirin were dissolved in purified water and used at a clinical equivalent dose. ProUK was diluted with physiologic saline and administrated at a quarter of the clinical dose. The model group mice were given the same volume of purified water and saline. Body weight and plasma lipids content was measured every 2 weeks.

En Face and Histology Analyses

$LDLR^{-/-}$ mice were sacrificed and rinsed with 20 ml phosphate buffered saline (PBS) followed by perfusion with 4% paraformaldehyde in PBS through the left ventricle. Aortas were fixed in 4% paraformaldehyde overnight, and then kept in 20% sucrose at 4°C until they sank. The aortas were then stained with Oil Red O after stripping off connective tissues and adipose tissue (Zhao et al., 2015). The en face aortic lesions were observed using a Canon EOS 80D digital camera and quantified using Image-Pro Plus 6.0 (Media Cybernetics Inc., MD, United States) analysis and expressed as percent lesion area.

Sections of the aortic tissues were harvested, embedded in optimal cutting temperature compound and then frozen. Series sections of 7-μm-thick of frozen aortic tissue were collected beginning at the aortic sinus. Every fifth section was stained with Oil Red O and counterstained with Hematoxylin and eosin (HE) as routine. Atherosclerotic lesion areas were measured using Image-Pro Plus 6.0 and expressed as average Oil Red O staining area per section in the first seven sections for each mouse.

Ultrastructure Analyses

At the end of experiment, the $LDLR^{-/-}$ mouse liver (n = 3 for each group) was perfused for 30 min with 3% glutaraldehyde (Ted Pella, Redding, CA, United States) in 0.1 mol/L PBS at a speed of 3 mL/min. For transmission electron microscopy, a slice approximate 1 mm thick was taken and stored in freshly prepared 3% glutaraldehyde overnight at 4°C. After rinsing with 0.1 mol/L PBS for 3 times, the tissue block was post-fixed in 1% osmium tetroxide in 0.1 mol/L PBS for 2 h at 4°C. The samples were dehydrated and then embedded in Epon 812 (SPI-CHEM, Westchester, PA, United States). Ultra-thin sections of liver were stained with uranium acetate and lead citrate and

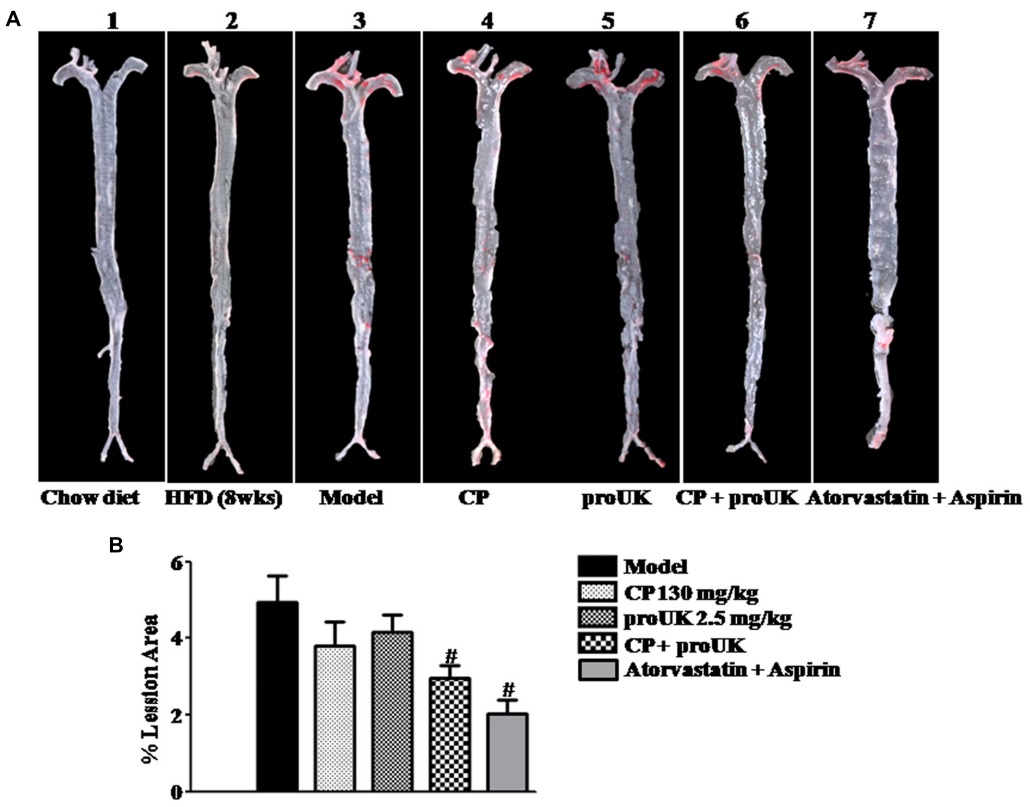

FIGURE 1 | CP plus proUK inhibits the development of atherosclerosis in $LDLR^{-/-}$ mice. High fat diet-fed $LDLR^{-/-}$ mice were randomly divided into 4 groups and received CP orally or/and proUK injection. **(A)** Representative images of en face Oil Red O staining of the full length of aorta of $LDLR^{-/-}$ mice from Chow diet group **(A-1)**, n = 3. High fat diet-fed mice for 8 weeks group **(A-2)**, n = 2. Model group **(A-3)**, n = 12. CP + HFD group **(A-4)**, n = 12. proUK + HFD group **(A-5)**, n = 12. CP plus proUK group **(A-6)**, n = 12. Atorvastatin + Aspirin group **(A-7)**, n = 8. **(B)** Percent lesion area of aorta in different groups. Data are presented as the percentages of total en face aortic area. n = 10. $^{\#}P < 0.05$ vs. Model mice.

examined in a transmission electron microscope (JEM-1400 Plus, JEOL, Tokyo, Japan).

Experiments in Cultured Macrophages

RAW264.7 macrophages were cultured in a high glucose DMEM medium containing 10% fetal calf serum, in which 50 μg/ml ox-LDL or 50 μg/ml Dil ox-LDL (Yuabio, Beijing, China) was added. The cells were incubated with or without CP, proUK, or CP plus proUK for 24 h. The final concentrations of CP and proUK were 0.5 mg/ml, 10 μg/ml, respectively. The contents of total cholesterol (TC), and triglyceride (TG) were tested by commercial kits from Applygen Technologies (Beijing, China). The cells incubated with Dil ox-LDL were observed under a Nikon Eclipse 50i microscope.

ELISA and Flow Cytometer Analyses

After LDLR$^{-/-}$ mice were fasted for 6 h, blood samples were collected from heart. The samples were centrifuged at 500 g for 20 min at 4°C to separate plasma. Plasma TC and TG contents, HDL-C and LDL-C levels were detected with kits from BioSino Bio-technology and Science Inc. (Beijing, China). Plasma AST, ALT and ICAM-1 levels were measured by enzyme-linked immunosorbent assay using an insulin ultrasensitive ELISA kit (R&D, Minneapolis, MN, United States), according the instruction of the manufacture. The color absorbance at 450 nm was measured using a Bio-Rad microplate reader.

Plasma cytokines were determined by BD Cytometric Bead Array with a flow cytometer (FACS Calibur, BD, Franklin Lakes, NJ, United States) at the end of experiments as described previously (Zhang et al., 2014; Mao et al., 2015). The following cytokines were determined in this study using their corresponding antibody: IL-1α, IL-1β, IL-6, IL-10, TNF-α, MCP-1, and MIP-1 α (R&D Systems, Minneapolis, MN, United States).

Western Blotting Analyses

Liver or aorta tissues were lysed in sample buffer containing 62 mM Tris–HCl, pH 6.8, 0.1% SDS, 0.1 mM sodium orthovanadate, and 50 mM sodium fluoride. The protein

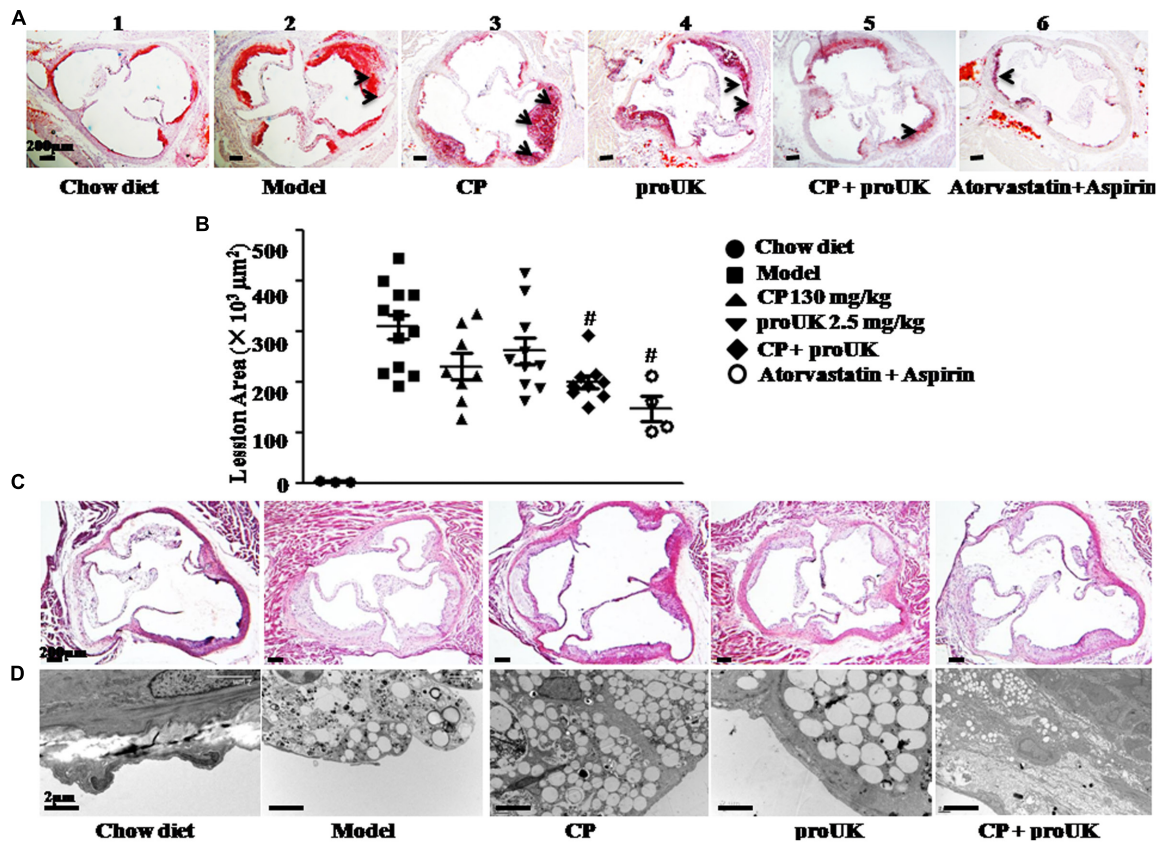

FIGURE 2 | CP plus proUK reduces atherosclerosis lesion area in aortic root of LDLR$^{-/-}$ mice. At the end of the treatment, aortic roots were prepared for transmission electron microscopy or as 7-μm frozen cross sections and stained with HE or Oil Red O solution. **(A)** Oil Red O staining of the left ventricular outflow tract in LDLR$^{-/-}$ mice from Chow diet group **(A-1)**, n = 3, Model group **(A-2)**, n = 10, CP + HFD group **(A-3)**, n = 8, proUK + HFD group **(A-4)**, n = 10, CP + proUK group **(A-5)**, n = 8. Atorvastatin + Aspirin group **(A-6)**, n = 4, Bar = 200 μm. The arrow shows the atherosclerosis plaque. **(B)** The statistic results of Oil Red O staining atherosclerosis lesions. n = 8–10. $^{\#}P < 0.05$ vs. Model mice. **(C)** HE staining of the left ventricular outflow tract in LDLR$^{-/-}$ mice from Chow diet group **(B-1)**, Model group **(B-2)**, CP + HFD group **(B-3)**, proUK + HFD group **(B-4)**, CP + proUK group **(B-5)**. Bar = 200 μm. **(D)** Transmission electron microscopic images of the aortic arch in LDLR$^{-/-}$ mice from Chow diet group **(C-1)**, Model group **(C-2)**, CP + HFD group **(C-3)**, proUK + HFD group **(C-4)**, CP + proUK group **(C-5)**. Bar = 2 μm.

content was determined by the BCA protein assay (Applygen Technologies Inc., Beijing, China). Equal amount of proteins was loaded and separated by SDS-PAGE. After electrophoresis, the proteins were transferred on membranes, after being blocked with 3% non-fat dry milk, the membrane with target proteins was recognized with primary antibodies against CD36, SR-A, SR-BI, PPARα, ABCA1, ABCG1, ABCG5, ABCG8, and GAPDH (Abcam, Cambridge, MA, United States). The bands were detected using an ECL detection kit (Applygen Technologies, Beijing, China). For quantification, band intensity was assessed by densitometry and expressed as mean area density using Quantity One image analyzer software (Bio-Rad, Richmond, CA, United States).

Statistical Analysis

All data were expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Tukey *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

CP Plus proUK Slows Down the Progression of Atherosclerosis Plaque in LDLR$^{-/-}$ Mice

The initial atherosclerotic plaque formed at the active arch after 8 weeks of high fat diet induction (**Figure 1A-2**) but not in chow diet mice (**Figure 1A-1**). To investigate the role of CP or/and proUK in atherosclerotic plaque formation, we compared aortic lesion in different groups after 4 weeks of treatment. Aortic en face Oil Red O staining showed that mice treated with CP plus proUK (**Figure 1A-6**) developed less atherosclerotic lesions than model mice (**Figure 1A-3**). The quantification of en face aortas revealed a significant reduction (36.0%) in lesion sizes in CP plus proUK-treated mice (**Figure 1B**). Treatment with CP (**Figure 1A-4**) or proUK (**Figure 1A-5**) alone did not significantly ameliorate atherosclerotic plaques compared to model mice. As a positive control, **Figure 1A-7** revealed that treatment with 6 mg/kg atorvastatin plus 15 mg/kg aspirin for 4 weeks significantly inhibited the development of high fat-diet induced atherosclerotic plaques in LDLR$^{-/-}$ mice.

The results in **Figure 1** indicate that most of the lesions are presented in the aortic arch. We further stained the frozen slices of the aortic sinus with Oil Red O (**Figure 2A**) and HE (**Figure 2C**). Compared to model mice (**Figure 2A-2**), mice treated with CP plus proUK (**Figure 2A-5**) revealed reduced lesion areas by 34.8% (**Figure 2D**), while no significant decrease in lesion area was found in CP (**Figure 2A-3**) or proUK (**Figure 2A-4**) alone treated group. As a positive control, the plaque area of the aortic sinus was less prominent in treatment with atorvastatin plus aspirin group compared with model group (**Figure 2A-6**). Consistent with the result of Oil Red O staining, HE staining (**Figure 2C**) and transmission electron microscopy (**Figure 2D**) results showed that the accumulation of foam cells was abated in the aortic sinus from CP plus proUK treated LDLR$^{-/-}$ mice compared with model mice.

Taken together, the results in **Figures 1**, **2** suggest that CP plus proUK had better protective effect on plaque formation than either of the two alone.

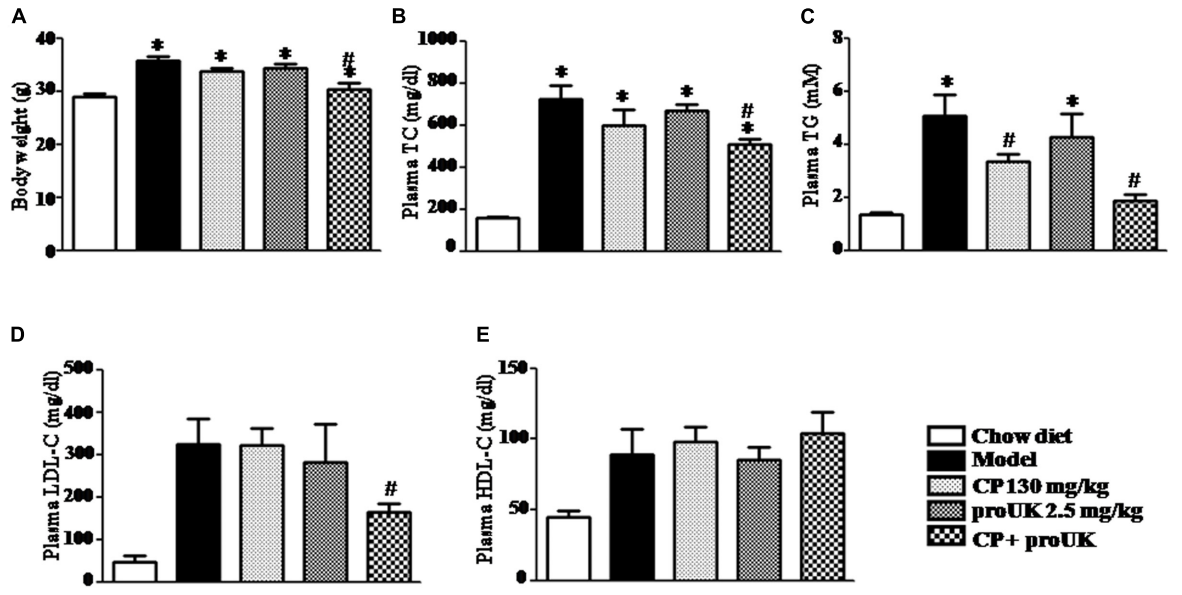

FIGURE 3 | CP plus proUK attenuates the increase in body weight and plasma TG, TC and LDL-C in the high fat diet-induced mice. **(A)** The body weight at the end of experiment. **(B)** Plasma TC level in different groups mice. **(C)** Plasma TG level in different groups mice. **(D)** Plasma LDL-C level in different groups mice. **(E)** Plasma HDL-C level in different groups mice. Total cholesterol, triglycerides, LDL-c, and HDL-c were quantified by enzymatic assays. The values are presented as the means SEM. $n = 10$. $^{*}P < 0.05$ vs. Chow diet mice, $^{\#}P < 0.05$ vs. Model mice.

CP Plus proUK Reduces the Body Weight and Plasma Lipids in High Fat Diet-Fed LDLR$^{-/-}$ Mice

High fat diet resulted in a significantly increase in body weight (**Figure 3A**), as well as in the plasma level of TC, TG, LDL-C, HDL-C in in LDLR$^{-/-}$ mice. Of note, compared with model mice, treatment with CP plus proUK significantly reduced the body weight and the plasma level of TC, TG and LDL-C, respectively, by 29.9, 63.4, and 49.0% (**Figures 3B–D**). CP alone reduced plasma TG levels, but had no influence on the body weight, nor on the plasma TC and LDL-C levels in high fat diet-fed mice. On the other hand, no effect was detected for proUK alone on all the endpoints tested in high fat diet-fed mice. In addition, there was no significant difference in the plasma HDL-C levels among the groups (**Figure 3E**).

CP Plus proUK Decreases Lipids Levels in the Liver of High Fat Dieted LDLR$^{-/-}$ Mice

The biochemical analysis revealed that high fat diet increased levels of hepatic TC and TG in LDLR$^{-/-}$ mice as well, which were attenuated by CP plus proUK, but not by either of the two alone (**Figures 4A,B**). Consistently, the results of transmission electron microscopy showed less lipid droplets and normal ultrastructure in the livers of CP plus proUK treated mice as compared with model (**Figure 4C**). In addition, ELISA revealed no significant differences in plasma ALT and AST levels among all groups (**Figures 4D,E**). Taken together, the results suggested that CP plus proUK decreased hepatic lipids while had no impact on the structure and function of mice liver.

CP Plus proUK Modulates the Lipid Metabolism-Associated Protein Levels in the Liver of High Fat Diet Fed LDLR$^{-/-}$ Mice

In order to explore the underlying mechanism by which CP plus proUK exerted protective effect against atherosclerosis, we further detected the proteins levels that are associated with cholesterol metabolism in the liver. Western blot (**Figure 5A**) revealed that CP alone or CP plus proUK treatment upregulated the protein levels of ABCG5 (**Figure 5G**) and ABCG8 (**Figure 5H**), which participate in hepatic cholesterol efflux to bile. However, no effect was detected for other proteins tested, such as CD36 (**Figure 5B**), SR-A (**Figure 5C**), SR-BI (**Figure 5D**), ABCA1 (**Figure 5I**), ABCG1 (**Figure 5J**), CYP7A1, a rate-limiting enzyme for bile acid synthesis (**Figure 5F**) and HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis) (**Figure 5E**). The levels of proteins (**Figure 6A**) associated with

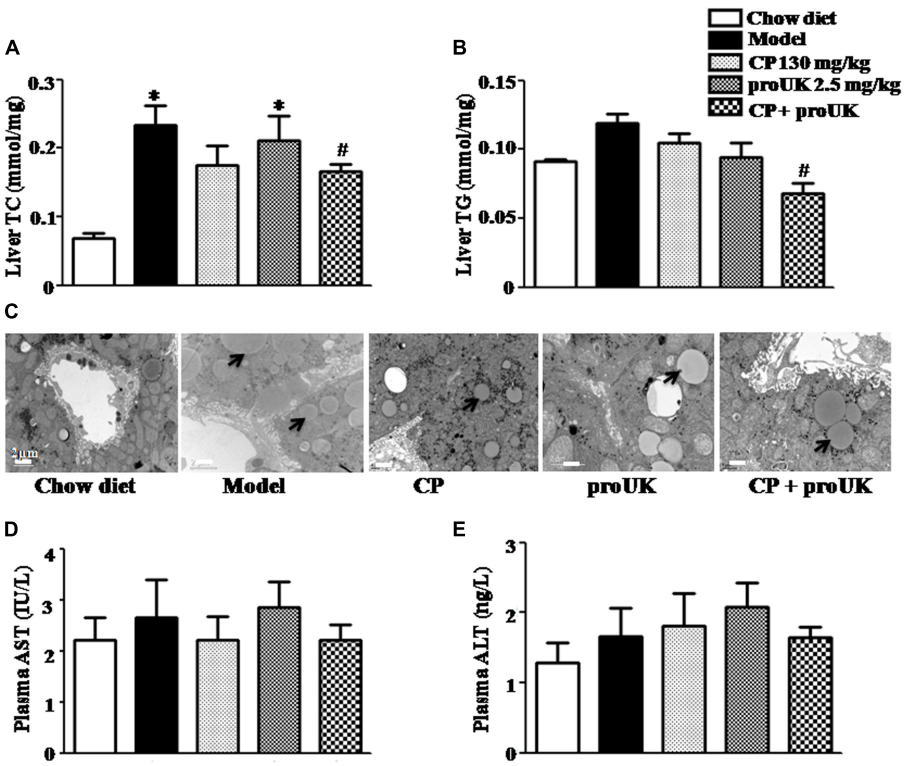

FIGURE 4 | CP plus proUK attenuates hepatic lipid deposition and increase in TC and TG while does not affect liver function in high fat diet-fed LDLR$^{-/-}$ mice. **(A)** Hepatic TG levels in different groups. **(B)** Hepatic TC levels in different groups. **(C)** Transmission electron microscopic images of the sinusoids in different groups. Bar = 2 μm. **(D)** AST activity in plasma from different groups. **(E)** ALT activity in plasma from different group. Data are mean ± SEM (n = 8). The arrow shows the lipid droplet. *P < 0.05 vs. Chow diet mice, $^{\#}P$ < 0.05 vs. Model mice.

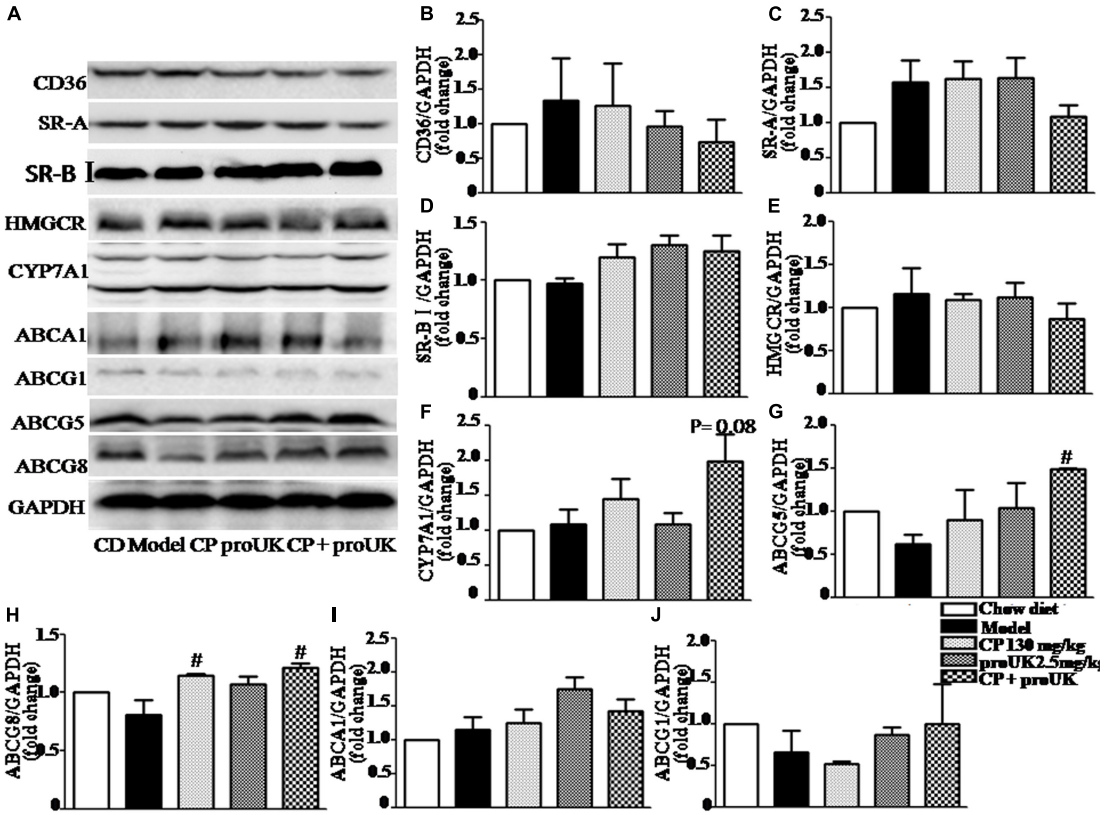

FIGURE 5 | Effect of CP plus proUK on the proteins associated with lipid metabolism in the livers of LDLR^{-}/$^{-}$ mice. **(A)** Western blotting of CD36, SR-A, SR-BI, ABCA1, ABCG1, ABCG5, ABCG8, HMGCR, and CYP7A1 in the livers of LDLR^{-}/$^{-}$ mice from different groups. **(B–D)** The quantitative analysis of the expression of scavenger receptor CD36, SR-A, SR-BI in the liver from different groups. **(E)** The quantitative analysis of the expression of cholesterol synthesis rate-limiting enzyme HMGCR in the liver from different groups. **(F)** The quantitative analysis of CYP7A1 expression in the liver from different groups. **(G)** The quantitation of the ratio betweenABCG5 protein/GAPDH protein in the liver from different groups. **(H)** The quantitation of the ratio betweenABCG8 protein/GAPDH protein in the liver from different groups. **(I)** The quantitative analysis of ABCA1 expression in the liver from different groups. **(J)** The quantitative analysis of ABCG1 expression in the liver from different groups. $n = 5$. $^{\#}P < 0.05$ vs. Model mice.

triglyceride metabolism were evaluated in different groups as well, revealing a significant increase of ATGL (**Figure 6B**), a protein that participates in triglyceride lipolysis, in the livers of the CP plus proUK group compared with model, whereas other proteins related to triglyceride metabolism, such as HSL (**Figure 6C**) and PPARα (**Figure 6D**), did not significantly change among groups.

These results indicated that CP plus proUK increased hepatic cholesterol efflux to bile and triglyceride lipolysis, resulting in decreased TC and TG in the liver and plasma.

CP Plus proUK Reduces Lipid Accumulation by Modulating the Expression of the Proteins Involved in Lipid Metabolism in Aorta of LDLR^{-}/$^{-}$ Mice

In order to observe changes in lipid accumulation caused by CP or/and proUK treatment, we cultured RAW264.7 macrophages in RPIM 1640 medium containing 10% FBS where in 50 μg/ml ox-LDL or 50 μg/ml Dil ox-LDL was added (**Supplementary Method**). As shown in **Figure 7A**, treatment with CP plus proUK significantly reduced lipid droplets in Dil ox-LDL incubated-macrophages. Similarly, CP or/with proUK treatment also attenuated TC and TG accumulation in macrophages (**Figures 7B,C**). Also, we tested the cholesterol efflux by using NBD-cholesterol (a fluorescent analog of cholesterol), the result showed that cholesterol efflux was similar in each group (**Supplementary Figure S4**). Also, ROS levels in RAW 264.7 had no difference in each group (**Supplementary Figure S3**).

The lipid homeostasis in macrophages depends on the balance of influx and efflux of lipid. We thus assessed first the expression of CD36 and SR-A, the two proteins that mediate the phagocytosis in macrophage, in different groups. The result showed a significantly increased expression of the two proteins in high fat diet fed LDLR^{-}/$^{-}$ mice compared with chow diet control, which, however, was noticeably attenuated by treatment with CP plus proUK, but not by either of the two alone (**Figures 7D,F,G**). In addition, ATGL, which mediates neutral cholesterol ester hydrolysis, was upregulated in CP plus proUK group compared with model group (**Figures 7D,H**). Because the free cholesterol hydrolyzed from cholesterol ester transported by ATP-binding cassette

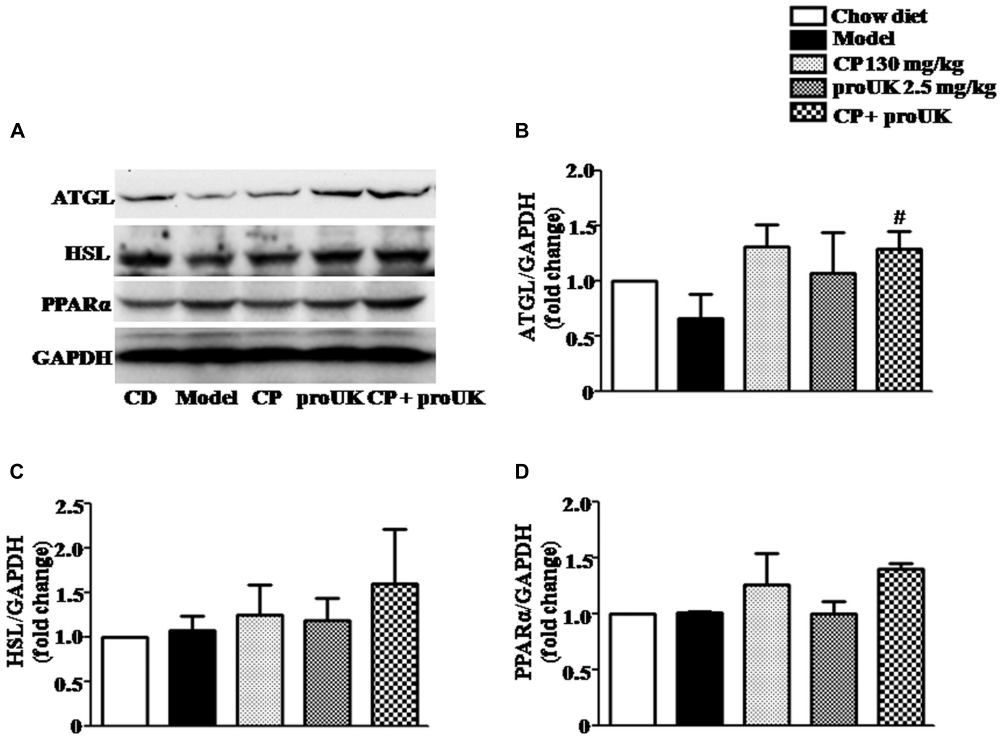

FIGURE 6 | Effect of treatment with CP plus proUK on the expression of hepatic triglyceride metabolism-related proteins in the liver. **(A)** Western blot of the liver protein levels of ATGL, HSL and PPARα. **(B)** The quantitative analysis of ATGL expression in the liver from different groups. **(C)** The quantitative analysis of HSL expression in the liver from different groups. **(D)** The quantitative analysis of PPARα expression in the liver from different groups. Data are presented as the mean ± SEM. $n = 5$. $^{\#}P < 0.05$ vs. Model mice.

A1 (ABCA1), we thus detected the expression of ABCA1but revealing no difference between model group and CP plus proUK group (**Figure 7E**).

CP Plus proUK Inhibits Systemic Inflammation in LDLR$^{-/-}$ Mice

We also explored the effect of CP plus proUK on chronic systemic inflammation in LDLR$^{-/-}$ mice. Flow cytometry and ELISA analysis showed that the concentration of ICAM-1 (**Figure 8A**) and MIP-1α (**Figure 8B**) was significantly lower in the CP plus proUK group compared with the model group, whereas the levels of the other cytokines (IL-1α, IL-1β, IL-6, IL-10, TNF-α, and MCP-1) and MDA detected did not vary significantly among groups (**Figures 8C–H** and **Supplementary Figure S2**). Furthermore, we collected the aorta from LDLR$^{-/-}$ mice and assessed the aortic ICAM-1 levels by ELISA revealing that CP plus proUK significantly decreased aortic ICAM-1 levels (**Figure 8I**), whereas CP or proUK alone did not affect the aortic ICAM-1 levels. Also, CP plus proUK reduced aortic CD68 levels (**Supplementary Figure S1**).

DISCUSSION

The present study provides evidence that CP plus proUK protects against the high fat diet-induced atherosclerosis development in LDLR$^{-/-}$ mice, manifested reduced the areas of atherosclerotic lesions by 34.8%, along with decreased plasma TC, TG, and LDL-C, as well as a reduction in body weight. The beneficial role of CP plus proUK observed is likely mediated by several pathways, as shown by the modulation of the expression of ABCG5, ABCG8, and ATGL in liver, decreased aortic SR-A and CD36 protein levels, and reduced plasma ICAM-1 and MIP-1α after CP plus proUK treatment. Atherosclerosis is a complex pathological process which has not been fully elucidated until now. Previous intervention studies have focused on reducing lipid accumulation and inflammation. Current treatment of atherosclerosis includes statins and antiplatelet drugs, but the clinical feasibility is not completely satisfactory due to the side effects of these medicines. Abnormal lipid metabolism plays a critical role in the occurrence and development of atherosclerosis. An excessive plasma concentration of LDL-C is widely recognized as a causal factor of endothelial dysfunction and atherosclerotic vascular disease (Hartley et al., 2018). CP has been widely applied for prevention and treatment of angina. On the other hand, increasing evidence suggests that CP may interfere in lipid metabolism and pathogenesis of atherosclerosis. To this end, *S. miltiorrhiza*, a main component of CP, was reported to have lipid-lowering activity (Lim et al., 2017), but the underlying mechanism has not been explored yet. It has been reported that CP significantly inhibited the formation of thrombosis formation and platelet function in

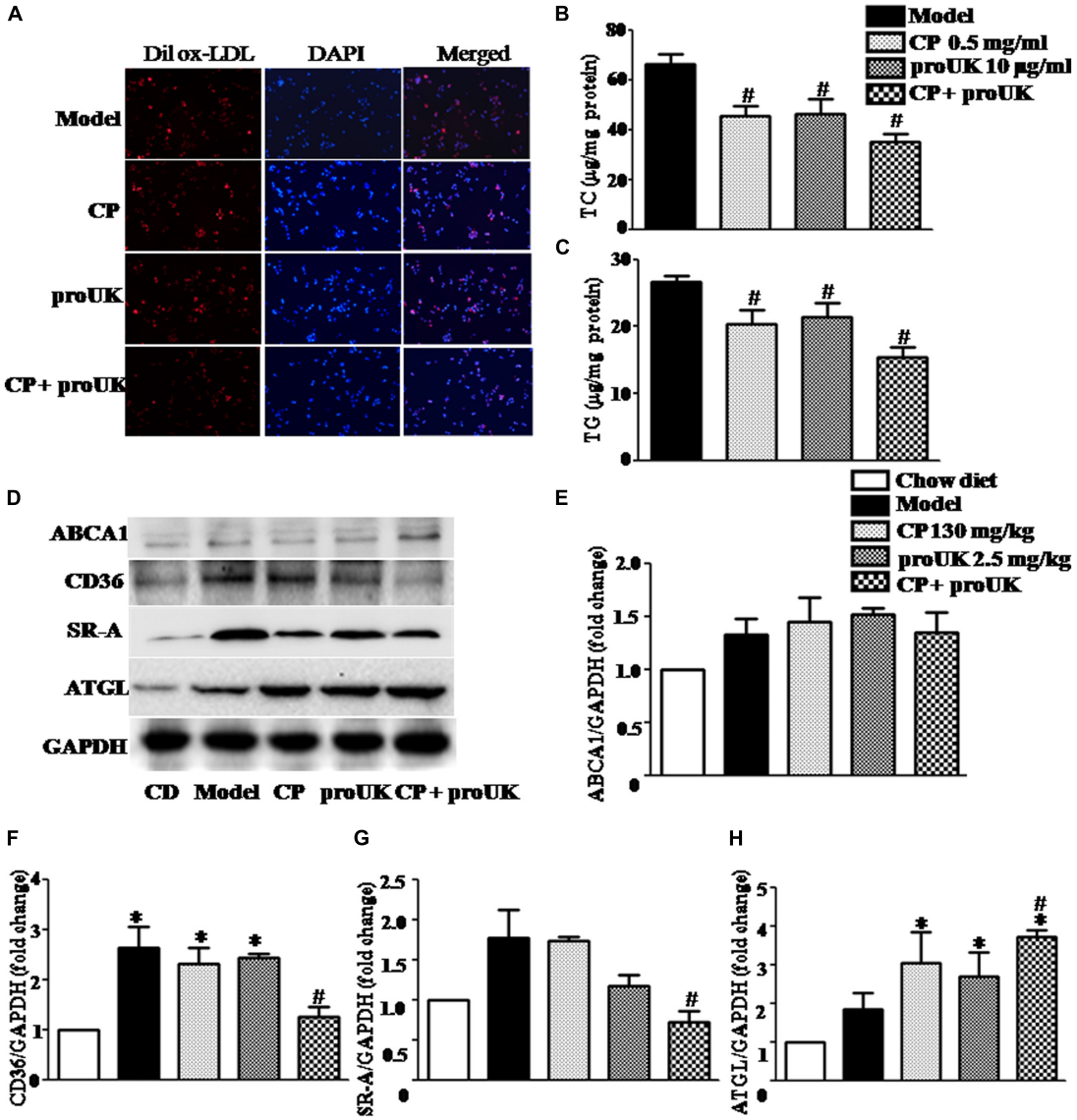

FIGURE 7 | CP plus proUK decreases the accumulation of lipids in RAW264.7 macrophages and the levels of CD36 and SRA in the aorta of LDLR$^{-/-}$ mice. **(A)** The accumulation of lipid droplets in RAW264.7 macrophages. **(B)** The effect of CP or/with proUK on the level of TC in RAW264.7 macrophages. **(C)** The effect of CP or/with proUK on the level of TG in RAW264.7 macrophages. **(D)** Western blot of ABCA1, CD36, SRA1 and ATGL from the aorta in LDLR$^{-/-}$ mice from different groups. **(E)** The quantitative analysis of ABCA1 expression in aorta from different groups. **(F)** The quantitative analysis of CD36 expression in aorta from different groups. **(G)** The quantitative analysis of SR-A expression in aorta from different groups. **(H)** The quantitative analysis of ATGL expression in aorta from different groups. Data are presented as the mean ± SEM. $n = 4$. $^{*}P < 0.05$ vs. Chow diet mice, $^{\#}P < 0.05$ vs. Model mice.

high fat diet-fed dogs (Zhang et al., 2006; Wang et al., 2014). Niu et al. (2000) showed that Tanshinone II-A, an ingredient of *S. miltiorrhiza*, inhibits low density lipoprotein oxidation *in vitro*. In a high cholesterol feeding rabbit model, Chen et al. (2006) reported that *S. miltiorrhiza* significantly inhibits intimal hyperlipidemia and improved Ass. In the present study, treatment with CP alone for 4 weeks decreased plasma TG level while increased ABCG8 expression in high fat diet fed LDLR$^{-/-}$ mice, consistent with the reports above. However, CP alone did not show any significant effect on the progression of atherosclerosis, nor on other parameters evaluated. These results reflect the complexity of atherosclerosis pathogenesis, to cope with which more strategy should be included in addition to lowering plasma lipids. proUK preferentially activates plasminogen on the fibrin surface, induces fibrin-selective clot lysis and thus is used as a thrombolytic agent. On the other hand, increasing evidence suggests the potential of proUK to interfere in the progression of atherosclerosis. Fibrin is known as a consistent component of human atherosclerotic plaque and it may contribute to plaque growth by stimulation of cell proliferation and by the binding and accumulation of low density lipoprotein (Thompson and Smith, 1989). It is thus

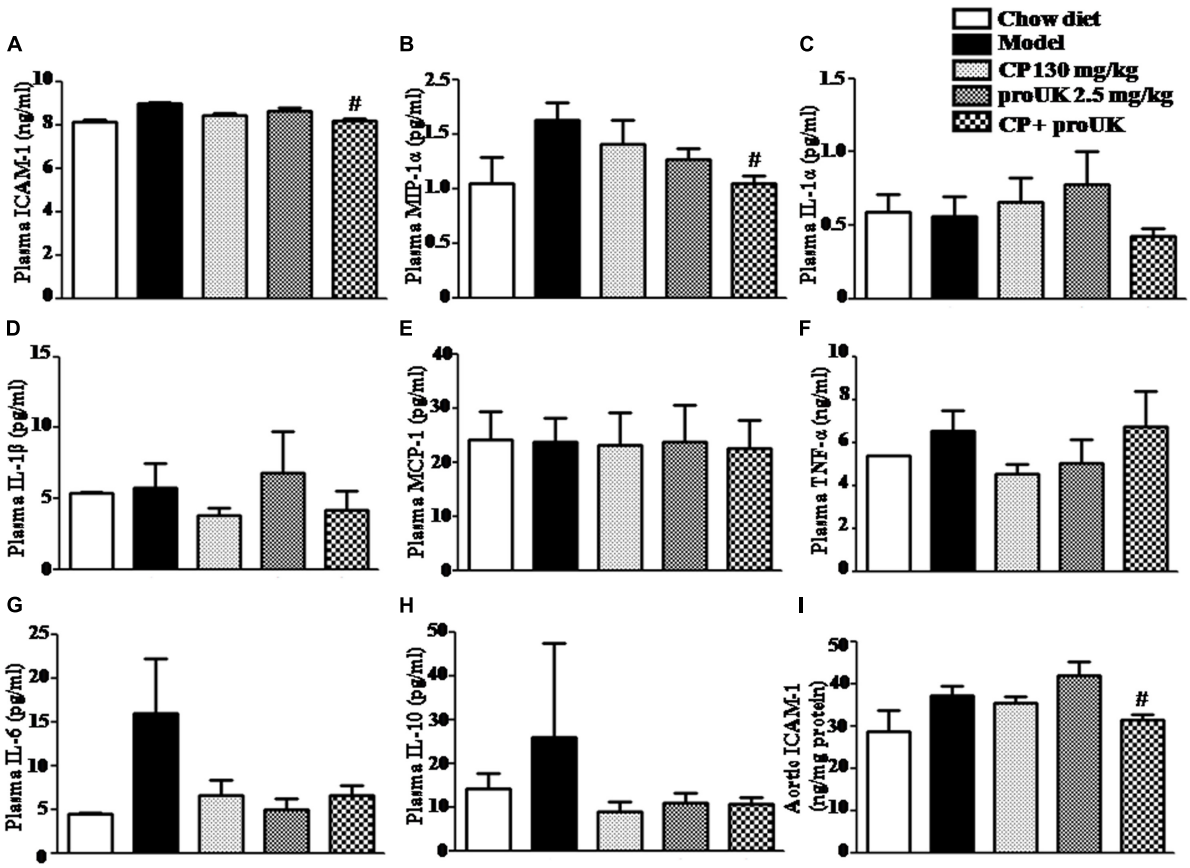

FIGURE 8 | Effect of CP plus proUK on plasma inflammatory cytokine levels in LDLR$^{-/-}$ mice. **(A)** Plasma ICAM-1 detected by ELISA in LDLR$^{-/-}$ mice from different groups. **(B)** Plasma MIP-1α in LDLR$^{-/-}$ mice from various groups. **(C)** Plasma IL-α in LDLR$^{-/-}$ mice from various groups. **(D)** Plasma IL-1β in LDLR$^{-/-}$ mice from various groups. **(E)** Plasma MCP-1 in LDLR$^{-/-}$ mice from various groups. **(F)** Plasma TNF-α in LDLR$^{-/-}$ mice from various groups. **(G)** Plasma IL-6 in LDLR$^{-/-}$ mice from various groups. **(H)** Plasma IL-10 in LDLR$^{-/-}$ mice from various groups. **(B–H)** All the data were acquired by Flow cytometer. **(I)** The concentrations of aortic ICAM-1 protein measured by ELISA in LDLR$^{-/-}$ mice from various groups. The values are presented as the means ± SEM, n = 8–12. $^{\#}P < 0.05$ vs. Model mice.

anticipated that fibrin hydrolysis by proUK may help impede the progression of atherosclerosis. In addition, study showed that uPA plays a pivotal role in the regulation of cell adhesion, migration, and proliferation during tissue remodeling including plaque formation (Tkachuk et al., 2009). Despite these, to our knowledge, no study is reported as to the role of proUK in protection of atherosclerosis progression. In the present study, we found no effect of proUK alone on the atherosclerosis plaque area in high fat diet fed LDLR$^{-/-}$ mice, nor on other endpoints tested. However, treatment with proUK in combination of CP surprisingly revealed a significantly protective effect on plaque formation and plasma lipid. Of notice, for most of the variables tested, CP plus proUK is more effective than either one of the two alone. On the other hand, for some variables such as liver TC and liver TG, ABSG5, ATGL, SR-A, Plasma MIP-1α, and aortic ICAM-1, only CP plus proUK exhibited effect while either of the two alone showed no effect, suggesting they may have a synergistic effect. This result highlights CP plus proUK as a potential combination in protection of atherosclerosis progression.

Studies were further conducted to gain insight into the possible pathways that mediate the role of CP plus proUK. The results suggested that at least three pathways are implicated in the effect of CP plus proUK. (1) Lipid metabolism, which was evidenced by the finding that ABCG5 and ABCG8, the proteins that promote hepatic cholesterol efflux to bile (Graf et al., 2002; Repa et al., 2002; Yu et al., 2002, 2005), increased after CP plus proUK treatment, and that the expression of aortic ATGL was elevated by CP plus proUK, which participate in triglyceride lipolysis. It is likely that it is the ABCG5, ABCG8, and ATGL work coordinately that lower the plasma lipid lever. (2) Development of foam cells – We found an reduced lipid droplets in RAW264.7 macrophages, and a decreased expression of CD36and SR-A after CP plus proUK treatment, which belong to the scavenger receptor family and mediate the uptake of modified LDLs by macrophages thus take part in the development of foam cells (de Winther et al., 1999; Herijgers et al., 2000). (3) Inflammation – We observed a reduced expression of pro inflammatory cytokines ICAM-1 and MIP-1αby CP plus proUK compared with high fat diet fed LDLR$^{-/-}$ mice, implying involvement of anti-inflammation in

the effect of CP plus proUK treatment. Whether or not other pathway (s) are implicated in the effect of CP plus proUK requires further study. Nevertheless, plasma lipid, foam cells, and inflammation are recognized as the major contributors to the formation of atherosclerosis plaque. CP plus proUK exerts effect through the three pathways suggesting this combination to be a multi targeting strategy. However, more study is required to elucidate how these two medicines orchestrate to activate or strength these pathways and what is the contribution of each of the ingredient contained in CP to the lipid lowering effect.

CONCLUSION

The present study showed that CP plus proUK significantly ameliorated the development of atherosclerosis, this effect was associated with modulation of plasma lipids, prevention of the development of foam cells and protection of inflammation in high fat diet-fed mice. The results of the present study suggest CP plus proUK as a novel strategy for preventing AS progression. Nevertheless, more study is needed for clinic translation of the present study results, especially the study on whether or not they have adverse side effect.

ETHICS STATEMENT

All surgical procedures performed on animals were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (LA2010001). Procedures involving animals and their care were conducted in conformity with international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept).

AUTHOR CONTRIBUTIONS

J-ND and J-YH designed the experiments. J-ND, KS, C-SP, B-HH, XC, HL, and GL carried out the experiments. J-ND and QL analyzed the experimental results and data. J-ND, QL, J-YF, and J-YH wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01128/full#supplementary-material

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frontiers in Physiology

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Assessment of Exercise Stroke Volume and Its Prediction From Oxygen Pulse in Paralympic Athletes With Locomotor Impairments: Cardiac Long-Term Adaptations Are Possible

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The determinants of cardiac output (CO) during exercise, i.e., stroke volume (SV) and heart rate (HR), could differ in Paralympic athletes (PAthl) with spinal cord injury (SCI) with respect to PAthl with locomotor impairments caused by different health conditions (HCs). The purposes of the present study were the comparisons of two groups of PAthl, one with SCI and the other with either amputation (AMP) or post poliomyelitis syndrome (PM), assessing the (1) peak cardiorespiratory responses and determinants (SV and HR) of CO during maximal and submaximal arm cranking exercise (ACE), respectively; (2) correlations between peak oxygen uptake (VO_{2peak}) and the highest SV obtained during submaximal exercise; and (3) correlations between oxygen pulse (O_2 pulse, ratio between VO_2 and HR) and both SV and O_2 arterio-venous difference [(a-v)O_2diff]. Each athlete (19 PAthl with SCI, 9 with AMP, and 5 with PM) completed a continuous incremental cardiopulmonary ACE test to volitional fatigue to assess peak responses. In a different session, CO was indirectly measured through carbon dioxide (CO_2) rebreathing method at sub-maximal exercise intensities approximating 30, 50, and 70% of the VO_{2peak}. There were no significant differences between the PAthl groups in age, anthropometry, and VO_{2peak}. However, peak HR was significantly lower, and peak O_2 pulse was significantly higher in PAthl with AMP/PM compared to those with SCI. During sub-maximal exercise, PAthl with AMP/PM displayed significantly higher SV values (154.8 ± 17.60 ml) than PAthl with SCI (117.1 ± 24.66 ml). SV correlated significantly with VO_{2peak} in both PAthl with SCI (R^2 = 0.796) and AMP/PM (R^2 = 0.824). O_2 pulse correlated significantly with SV in both PAthl with SCI (R^2 = 0.888) and AMP/PM (R^2 = 0.932) and in the overall sample (R^2 = 0.896). No significant correlations were observed between O_2 pulse and (a-v)O_2diff. It was concluded that in PAthl with different

HCs: (1) significant differences, as a consequence of the different HC, exist in the determinants of CO at maximal and submaximal ACE; (2) SV is a significant determinant of VO_{2peak}, suggesting cardiac adaptations possible also in PAthl with SCI; and (3) SV can be predicted from O_2 pulse measurements during submaximal exercise in both groups of PAthl.

Keywords: spinal cord injury, lower limb amputation, cardiac output, Paralympic sport, lower limb poliomyelitis

INTRODUCTION

Aerobic fitness levels have been well documented in recreational (Abel et al., 2008) and Paralympic Athletes (PAthl) with locomotor impairments (Baumgart et al., 2018) competing in summer (Bernardi et al., 2010) and winter sports (Bernardi et al., 2012), particularly those with spinal cord injury (SCI; Bernard et al., 2000; Bhambhani, 2002; van der Woude et al., 2002). Cross-sectional evidence indicates that trained individuals with SCI (tetraplegia and paraplegia) have significantly higher peak oxygen uptake (VO_{2peak}) values compared to their sedentary counterparts (Flandrois et al., 1986; Hopman et al., 1996; Huonker et al., 1998; Schumacher et al., 2009) and that the changes in aerobic fitness in PAthl with locomotor impairments are determined by the practiced sport (Bhambhani, 2002; Bernardi et al., 2010, 2012; Baumgart et al., 2018). Possible factors that are associated with the higher VO_{2peak}, i.e., the enhanced ability to transport, deliver, and utilize oxygen, are both central and peripheral in nature. Central factors, which enhance systemic oxygen availability, include significant increases in blood volume and hemoglobin concentration in endurance trained athletes with paraplegia (Schumacher et al., 2009). A primary circulatory factor that elevates their cardiac output (CO) is a significant increase in stroke volume (SV) attained in the trained state. Peripheral factors that contribute to a greater increase in maximal O_2 extraction [i.e., $(a\text{-}v)O_{2diff}$] include greater capillary and mitochondrial density, as well as enhanced activity of enzymes in the aerobic metabolic pathways (Bhambhani, 2002).

Previous studies have documented that SV during arm cranking exercise is significantly lower in individuals with motor complete tetraplegia (C5–C7 lesions) and complete paraplegia (T4–T11 lesions) compared to able-bodied individuals (Jacobs et al., 2002; Hostettler et al., 2012). Reduced SV was also evident in trained individuals with complete SCI between T6 and T12 compared to those who were wheelchair dependent due to permanent hip or knee injuries (Hopman et al., 1993). The primary reason for the reduced SV in individuals with SCI, particularly those with cervical and high thoracic lesions, is: (1) reduction in cardiac preload resulting from "venous blood pooling" in the abdomen and/or lower extremities, due to the absence of the skeletal muscle pump and vasoconstriction below the level of lesion (Hopman, 1994; Hopman et al., 1998); (2) reliance on relatively small upper body muscles during exercise (Theisen, 2012); and (3) reduced blood volume (Houtman et al., 2000). However, it should be noted that in individuals with a SCI above T4, blood volume significantly increases with lower limb functional electrical stimulation cycling training (Houtman et al., 2000). Comparisons among individuals with high and low paraplegia indicate that venous return from the splanchnic vasculature depends on the lesion level (Thijssen et al., 2009). Evidence also indicates that in 10 sedentary individuals with a SCI from T1 to T12, reduced venous return was the most possible cause of blunted metaboreflex-induced blood pressure and SV post exercise responses when compared to able-bodied controls (Crisafulli et al., 2009). In addition, the reduced cardiac preload, coupled with the autonomic dysfunction and sedentary lifestyle of individuals with SCI over several years, could result in chronic changes in the myocardium, thereby reducing its mass and contractility (Washburn et al., 1986; Jacobs and Nash, 2004; de Groot et al., 2006; Palmieri et al., 2010; Williams et al., 2019).

Although there are numerous studies pertaining to the metabolic and cardiorespiratory responses to exercise in athletes with SCI (Price, 2010; Theisen, 2012), research that has documented exercise SV responses in PAthl with locomotor impairments derived from lower limb amputation (AMP) and post poliomyelitis syndrome (PM) is lacking. Generally, the acute cardiorespiratory responses in the individuals with these two impairments are not physiologically altered during exercise, as the central (brainstem) control of the sympathetic innervation to their myocardium is unaffected (Birk and Pitetti, 2009). Even though the reduced muscle mass in AMP and PM could result in some decrease in venous return and therefore cardiac preload during exercise, it is unlikely that this reduction will be as high as that seen in PAthl with SCI. The greater degree of muscle atrophy resulting from chronic SCI could reduce venous return (cardiac preload) by a larger extent than that observed in PAthl with AMP and PM because of the attenuated muscle pump action during exercise.

In able-bodied individuals, the oxygen pulse (O_2 pulse; calculated as the ratio between absolute VO_2 and heart rate – HR) has been shown to be a valid predictor of SV during submaximal exercise (Whipp et al., 1996). Mathematically, O_2 pulse is the product of the Fick equation variables, namely, SV and the mixed arterio-venous oxygen difference $\{(a\text{-}v)O_{2diff}\}$. Whipp et al. (1996) used indwelling catheters to directly measure the $(a\text{-}v)O_{2diff}$ during incremental cycle exercise in healthy subjects and reported a Pearson correlation of 0.80 ($p < 0.01$) between the SV calculated from the Fick equation and that predicted from O_2 pulse. Regression equations have been developed for predicting SV during cycling exercise in untrained and trained men (Bhambhani et al., 1994; Crisafulli et al., 2007) and during upper and lower body exercise in untrained men and women (Bevegard et al., 1966; Stenberg et al., 1967; Bhambhani, 1995). While O_2 pulse is a valid predictor of SV during exercise in healthy individuals, research that has examined the relationship between these two variables in individuals with locomotor impairments is lacking.

From an athletic performance standpoint, high levels of aerobic fitness (i.e., high VO_{2peak}) were correlated with faster

simulated wheelchair distance racing performance in athletes with tetraplegia (Bhambhani et al., 1995). As well, wheelchair racing performance was significantly correlated with both SV and CO in these athletes. Since SV is one of the two determinants of CO, it is important that some simple techniques for evaluating these variables during exercise be available to comprehensively monitor sport performance in athletes. Although HR can be easily recorded during exercise using wireless instruments, the measurement of SV is more challenging. Non-invasive techniques such as electrical impedance, carbon dioxide rebreathing, and acetylene rebreathing, currently used for the measurement of CO and SV, require specialized instrumentation, which may not be available in the laboratory. Furthermore, these techniques require participants to perform specific maneuvers, which may be difficult during exercise.

In light of the physiological and pathological differences between individuals with SCI and AMP/PM, comparing the maximal and submaximal cardiorespiratory responses to exercise between these two groups of PAthl is of interest to sport scientists, coaches, and athletes because they compete against each other in several classes of Paralympic sports. As well, exercise physiologists, physiatrists, cardiologists, and sports medicine physicians would find this information useful from a clinical standpoint in prescribing effective exercise programs. Therefore, the objectives of this study were to compare the: (1) peak cardiorespiratory responses and determinants (SV and HR) of CO during maximal and submaximal arm cranking exercise, respectively, in PAthl with SCI and AMP/PM; (2) magnitude of the correlation between VO_{2peak} and highest sub-maximal SV in both groups of athletes; and (3) magnitude of the correlation between the O_2 pulse and both SV and (a-v)O_{2diff} during submaximal arm cranking exercise in these two groups of athletes. It was hypothesized that: (1) the peak cardiorespiratory responses would be significantly higher in the AMP/PM athletes compared to those with SCI; (2) the highest SV measured at sub-maximal exercise intensities would be significantly greater in athletes with AMP/PM compared to those with SCI; (3) SV would be significantly correlated with VO_{2peak} in both groups of athletes; and (4) O_2 pulse would be significantly correlated with SV in both groups of athletes.

METHODS

Design and Setting

All athletes completed two exercise tests on different days: an incremental graded exercise test to voluntary fatigue to determine their VO_{2peak}, and three submaximal exercise tests at different intensities relative to their VO_{2peak} to assess their highest SV. Maximal data were collected in the Institute of Sport Medicine and Science of the Italian National Olympic Committee (CONI), Rome, Italy. Sub-maximal data were collected at the exercise physiology laboratory in the School of Specialty of Sports Medicine of the "Sapienza," University of Rome (Italy). Both test sessions were carried out in accordance with the guidelines of the CONI and Italian Paralympic Committee (CIP).

Participants

Written informed consent was obtained from two groups of elite athletes who represented Italy in the Paralympic Games over several years: 20 PAthl with SCI and 16 PAthl with lower limb impairments derived from health conditions other than SCI: AMP (N = 10) and PM (N = 6). All the testing procedures were approved by CONI and CIP for athlete testing and by the "Santa Lucia Foundation" Ethical Committee. The PAthl with SCI had complete lesions ranging from thoracic (T)4 to lumbar (L)1. Eighteen of them had a complete lesion according to the American Spinal Injury Association (ASIA) Impairment Scale (AIS) (AIS A), while two of them had an AIS C (Kirshblum et al., 2011). In all of them, the motor and the sensory levels corresponded. PAthl with SCI competed in the following Paralympic sports: three in shooting (neurological level of injury – NLI – equal to T7, T8, and T10), five in athletics (three in field events –throwing sports – one with NLI at T4 and the other two at T9 and two in track events with NLI both at T12), two in table tennis (NLI at T4 and L1, respectively), three in wheelchair fencing [two with NLI at T6 and T7, respectively, and the third one with NLI at L2 with AIS C who was excluded from the study (see the last part of the methods)], three in archery (one with NLI at T7 and the other 2 at T12), one in wheelchair tennis (NLI at T11), one in hand bike (NLI at T9), one in Alpine skiing, and one in Nordic skiing (both PAthl competing as sitting skiers, the former with NLI at T11, and the latter at L1 with AIS C). Among the PAthl with AMP (all of them with lower limb AMP), one PAthl had double thigh AMP (who was excluded from the study), three PAthl had single trans-femoral AMP, two PAthl had trans-pelvic AMP, and four PAthl had single trans-tibial AMP. The sport participation of the PAthl with AMP was as follows: one in track events (the one excluded from the study), two in field events (one in high and one in long jump), one in both wheelchair fencing and wheelchair basketball, one in cycling, one in Nordic skiing (as sitting skier), and four in Alpine skiing (all of them as standing skiers). The six athletes with PM acquired the disorder within 6 years after birth (1.92 ± 2.06, range 0.5–6 years). Three PAthl with PM had impairments in both lower limbs, while the other three had monolateral lower limb impairment. All of them were able to walk without assistive devices. They participated in the following sports: one each in archery, table tennis, shooting, Nordic skiing (as sitting skier), fencing (the athlete was excluded from the study), and field events. In summary, the subdivision of the PAthl within the sport types (Bernardi et al., 2010; Pelliccia et al., 2016) was as follows: 8 PAthl with SCI and 3 PAthl with PM competing in skill sports, 4 PAthl with SCI, 1 with PM and 6 AMP competing in power sports, 4 PAthl with SCI, 1 AMP and 1 with PM competing in intermittent sports, and 4 PAthl with SCI and 4 PAthl with AMP/PM competing in endurance sports. The pertinent age and physical characteristics of all the PAthl are summarized in **Table 1** [because three PAthl were excluded from the study (see section "Statistical Analysis" at the end of the methods), they are not included in the table].

TABLE 1 | Pertinent physical characteristics of Paralympic athletes (PAthl) with locomotor impairments (Mean ± SD).

Variable	SCI (*N* = 19)	AMP (*N* = 9) PM (*N* = 5) AMP/PM (*N* = 14)	Probability (P) level
Age (years)	36.8 ± 4.50	AMP 31.8 ± 7.26	SCI vs. AMP ($p = 0.03$)
		PM 45.0 ± 4.24	SCI vs. PM ($p = 0.001$)
		AMP/PM 36.5 ± 9.01	SCI vs. AMP/PM ($p = 0.904$)
Height (cm)	178.9 ± 8.59	AMP 178.5 ± 7.52	SCI vs. AMP ($p = 0.906$)
		PM 168.0 ± 12.47	SCI vs. PM ($p = 0.032$)
		AMP/PM 174.1 ± 10.47	SCI vs. AMP/PM ($p = 0.223$)
Body mass (kg)	70.7 ± 10.1	AMP 68.2 ± 9.9	SCI vs. AMP ($p = 0.213$)
		PM 71.0 ± 11.31	SCI vs. PM ($p = 0.720$)
		AMP/PM 69.2 ± 10.09	SCI vs. AMP/PM ($p = 0.794$)
Injury time period (years)	17.4 ± 7.5	AMP 16.4 ± 7.30	SCI vs. AMP ($p = 0.127$)
		PM 43.2 ± 3.70	SCI vs. PM ($p < 0.001$)
		AMP/PM 25.8 ± 14.89	SCI vs. AMP/PM ($p = 0.04$)

SCI, PAthl with spinal cord injury; AMP/PM, PA with amputation/poliomyelitis; PM, poliomyelitis.

Cardiorespiratory and Metabolic Measurements

The cardiorespiratory measurements during all the exercise tests were obtained using a stationary metabolimeter (Quark b^2, COSMED, Italy), which was interfaced with an electrocardiograph (Delta 640, Cardioline, Italy) for the incremental exercise test. The metabolimeter was calibrated according to the manufacturer's specifications. The oxygen and carbon dioxide analyzers were calibrated using commercially available precision gases: 15% oxygen and 5% carbon dioxide, balance nitrogen. The volume turbine was calibrated using a 3 L syringe. The calibrations were verified following each test to ensure the accuracy of the data.

Session 1: Incremental Arm Cranking Ergometer Maximal Exercise Test

The purpose of this test was to determine the VO_{2peak} of each athlete during an incremental arm cranking ergometer (ACE) maximal exercise test on an iso-power ergometer (Ergometrics 800, Ergoline GmbH, Bitz, Germany) under standardized laboratory conditions (Bernardi et al., 2010, 2012). All athletes were tested while sitting. The athletes were allowed to choose their own wheelchair or they could use the chair with a high back rest and comfortable seat provided by Ergometrics 800. Strappings were allowed, so as to replicate the specific sport conditions. The test consisted of a 3-min warm-up phase at a constant power ranging from 30 to 50 W at a cadence of 50–70 rpm, followed by increments of either 10 or 15 W every minute until voluntary fatigue; i.e., until the athletes were unable to maintain the desired cadence despite constant encouragement. In both phases, the selected power depended upon the estimated aerobic fitness and the functional classification of the athlete (Bernardi et al., 2010). These protocols were designed to complete the test in about 10 min (van der Woude et al., 2002; Bernardi et al., 2012). However, if a leveling off or decline in the VO_2 with increasing power output was not evident at the point of fatigue, the following two criteria for VO_{2peak} (Bernardi et al., 2007) had to be attained during the exercise phase of the test: (1) HR equivalent to at least 95% (Sawka, 1986) of their age predicted maximum (220 – age, years), and (2) respiratory exchange ratio (RER) $\geq$ 1.10 (Bernardi et al., 2010). On line, the breath by breath data file (CPET Software Suite, Version 10.0, Cosmed, Italy) was examined to eliminate artifacts using typical cutoff for respiratory frequency, tidal volume, and oxygen and carbon dioxide expiratory fractions. Upon completion of each test, after having eliminated possible further evident artifacts, the data were averaged every seven breaths with a passing filter to smooth the curve and then around the highest identified VO_2 values an average over 10 s was carried out to assess and quantify the VO_{2peak}. The VO_2 at each exercise stage was plotted against the power output for each athlete. From these data, ventilatory threshold (VT) and respiratory compensation point (RCP) were identified using the following Wasserman's gas exchange criteria (Wasserman et al., 1999): lack of linearity in the relationship between carbon dioxide production (VCO_2) and VO_2 (i.e., non-linear increase in the RER), due to a greater increase of VCO_2 with respect to VO_2, and systematic decrease in end tidal CO_2 ($PetCO_2$) with concomitant increases in the ventilatory equivalent of the CO_2 {pulmonary ventilation (VE) divided by VCO_2}, respectively. Details of the methods used for the assessments (including other methods to validate the RCP assessment) were carried out with the previous mentioned customized software and are reported elsewhere (Patacchioli et al., 2015). From these incremental metabolic data, VO_2 values that corresponded to 30, 50, and 70% of the VO_{2peak} were used as reference points for the CO measurements during the subsequent testing session.

Session 2: Measurement of Cardiac Output

This submaximal test was administered on the subsequent day at approximately the same time in order to allow the participant at least 24 h rest between successive testing sessions. The CO of each athlete was determined non-invasively using the CO_2 rebreathing technique (Collier, 1956) during seated steady-state exercise using the same ACE. The power outputs corresponding to 30, 50, and 70% of the VO_{2peak} were calculated using a formula previously assessed with a Fleish ACE and later confirmed with the same ACE used in the present study (Adami et al., 2015). The CO was measured at intensities lower than 70% of the VO_{2peak} if the preset VO_2 value exceeded that of the RCP. This was because a physiological steady state in the

$PetCO_2$ would not be possible as this is an essential criterion for a valid measurement of CO using this non-invasive technique (Jones, 1988). During each stage of the test, the breath-by-breath gas exchange responses were visualized in real time, and if the data indicated that the athlete had exceeded the RCP on the basis of the criteria identified above, then the exercise intensity was lowered slightly so as to ensure that it was below the RCP. None of the athletes exceeded the RCP during the 30 and 50% exercise stages. However, at 70% VO_{2peak}, four athletes exceeded the RCP, and in these cases, the work rate was reduced by approximately 5–10 W to ensure that the intensity was below the RCP.

The CO_2 rebreathing method to assess CO is based on the Fick equation applied to CO_2:

$$VCO_2 = CO \times C_{(v-a)}CO_2, \text{or } CO = VCO_2 / C_{(v-a)}CO_2$$

The athlete exercised for 8–9 min at each of the prescribed power outputs on the ACE before the CO assessment. The rebreathing maneuver was performed during the last 30 s of the exercise stage, while the subject was in a physiological steady state condition (i.e., no significant change in pulmonary ventilation, VO_2, VCO_2, and HR during the previous 2 min of exercise). In accordance with the Collier equilibrium method (Collier, 1956), the subject hyperventilated from a 3, 5, or 7 L anesthesia bag containing a mixture with CO_2 of 9, 11, or 13% (balance oxygen), until an equilibrium was attained between the gas in the bag and the lungs. The gas concentration selected was based on the VO_2 and $PetCO_2$ criteria proposed by Jones (1988). The volume of the mixture was equal to approximately 1.5 times the tidal volume of the participant in the minute before the measurement. The $PetCO_2$ tension was considered to be representative of arterial pCO_2, while the bag CO_2 was assumed to be indicative of mixed venous pCO_2. The downstream correction factor proposed by Jones (1988) was utilized to correct the cardiac output values. The criterion used by the computer program for identifying pCO_2 equilibrium was a change of less than 1 mm Hg pressure over a 5 s interval. In cases where the software was unable to detect this equilibrium point, the value was extrapolated from the line joining the points for expired $PetCO_2$ at 6 to 10 s of rebreathing to that at 20 s (the software was customized to assess this point). This value has been reported to be within ±2 mm Hg of the equilibrium value. From these measurements, the SV was calculated as the ratio between CO and HR at each of the three submaximal exercise intensities. The intensity at which the highest SV was attained for each participant was then used to calculate the following variables: (1) O_2 pulse, ml•beat^{-1} = VO_2/HR and (2) $(a\text{-}v)O_{2diff}$, ml/100 ml = VO_2/CO%. The validity of this technique in able-bodied individuals during submaximal ACE exercise has been previously established (Hopman et al., 1994). In accordance with these authors, when the steady state condition used for the CO measurement corresponded to an intensity in which RER was higher than 1, most likely between VT and RCP, a bicarbonate correction factor was adopted. An intra-class correlation of 0.85 has been reported (Myers et al., 2007) for the test-retest reliability of these measurements during submaximal ACE exercise in individuals with SCI.

Statistical Analysis

The mean values of the pertinent physical characteristic variables in the two groups of PAthl were compared using independent "*t*" tests. The Bonferroni correction factor was used to reduce the degree of Type 1 error. One-way ANOVA was used to compare the highest SV found in the three groups of PAthl (with SCI, AMP, and PM). Two-way repeated measures ANOVA (one factor repetition) followed by all pairwise multiple comparison procedures (Student-Newman-Keuls method) was used to compare the SV values at 30, 50, and 70% of VO_{2peak} in the SCI and AMP/PM athletes. Pearson correlations were used to examine the relationships between the highest SV attained during the submaximal exercises and: (1) VO_{2peak} obtained in the ACE incremental maximal exercise test for each group of PA and (2) the corresponding values of O_2 pulse and the latter with $(a\text{-}v)O_{2diff}$. Linear regression analyses were used to develop regression equations for assessing if SV was a significant determinant of VO_{2peak} and for predicting SV and $(a\text{-}v)O_{2diff}$ from O_2 pulse in both groups of PAthl. Slopes and intercepts of the linear regression curves for SV vs. VO_{2peak} and SV vs. O_2 pulse of the two groups of PAthl were compared using analysis of covariance (ANCOVA) to test for possible differences between PA with SCI and PA with AMP/PM. Bland-Altman analysis (Bland and Altman, 1986) was used to examine the validity of predicting SV from the calculated O_2 pulse in both groups of PAthl. Briefly, the difference between the measured and predicted values of SV (*y* axis) was plotted against the average of these two values (*x* axis) for each individual athlete. Data points that were above or below the 95% confidence intervals (i.e., ± 2 standard deviations) were considered to be outliers. Validity between the measured SV and predicted SV was further verified using the two-way mixed effect model of the intra-class correlation coefficient (ICC). The results were considered to be significant at α value lower than 0.05. Data analyses were completed using SPSS computer program (Version 17.0). The results of three athletes were excluded from the statistical analysis and therefore from the study for the following reasons: (1) two fencers (one with incomplete SCI at L2 and the other with single lower limb PM) because they did not attain at least two of the three criteria previously described for a maximal cardiopulmonary exercise test and (2) one track athlete with double thigh AMP because he was declared ineligible for participation in the Paralympic Games due to cardiovascular disorders (Pelliccia et al., 2016).

RESULTS

Peak Cardiorespiratory Responses

The peak cardiorespiratory responses of the two groups of athletes (PAthl with SCI vs. PAthl with AMP/PM) during the incremental ACE maximal exercise test are summarized in **Table 2**. In spite of a similar VO_{2peak}, PAthl with SCI displayed peak HR values higher (+6.03%) than PAthl with AMP/PM (p = 0.015). Peak O_2 pulse was higher in PAthl with AMP/PM than those with SCI by 17.54%. Comparisons between PAthl with AMP and those with PM (i.e., comparison within

TABLE 2 | Peak exercise responses of Paralympic athletes with locomotor impairments (Mean ± SD).

Variable	SCI (*N* = 19)	AMP/PM (*N* = 9/5)	% Difference
Power output (W)	126.3 ± 41.0	145.2 ± 30.54	13.05 ($p = 0.155$)
Oxygen uptake (L/min)	2.24 ± 0.581	2.56 ± 0.593	12.59 ($p = 0.128$)
Oxygen uptake (ml/kg/min)	32.7 ± 10.90	37.2 ± 7.23	11.94 ($p = 0.196$)
Heart rate (beats/min)	186 ± 11.0	175 ± 12.6	6.03 ($p = 0.015$)
Oxygen pulse (ml/beat)	12.0 ± 3.08	14.6 ± 3.41	17.54 ($p = 0.031$)
Pulmonary ventilation (L/min)	97.1 ± 28.88	111.7 ± 25.82	13.10 ($p = 0.143$)
Respiratory exchange ratio	1.23 ± 0.079	1.22 ± 0.103	−0.80 ($p = 0.760$)

SCI, PAthl with spinal cord injury; AMP/PM, PAthl with amputation/poliomyelitis. The % difference was calculated as follows: {(AMP/PM – SCI)/AMP/PM} × 100.

the group of PAthl without SCI) revealed no significant differences in the following peak values: power output = 151.4 ± 29.6 vs. 134.0 ± 32.1 W; VO_2 = 2.62 ± 0.62 vs. 2.46 ± 0.59 l•min^{-1}; 38.1 ± 4.6 vs. 35.5 ± 11.0 ml•kg^{-1}•min^{-1}; HR = 179 ± 11.4 vs. 169 ± 13.4 beats•min^{-1}; O_2 pulse = 14.8 ± 3.9 vs. 14.4 ± 2.7 ml•beat^{-1}; VE = 118.0 ± 25.6 vs. 100.4 ± 24.6 l•min^{-1}; and RER = 1.22 ± 0.10 vs. 1.19 ± 0.11.

Submaximal Cardiorespiratory Responses

The submaximal steady state intensities (%VO_{2peak}) at which the CO measurements were undertaken corresponded to 36.3 ± 4.3%, 54.1 ± 6.1%, and 69.8 ± 5.8% in the PAthl with SCI and 34.9 ± 8.1%, 52.9 ± 10.1%, and 69.5 ± 13.8% in the PAthl with AMP/PM. The results of the two-way ANOVA for the SV indicated that there was significant interaction between the two factors implying that the overall trend (highest values at 70% of VO_{2peak}) was similar in the SCI and AMP/PM athletes, with PAthl with SCI with lower values at each intensity. The comparison of the values at the three intensities indicated that the SV was significantly higher at 70% than 50% of VO_{2peak} and 50% higher than 30% in both groups of athletes ($p < 0.001$). The mean values of the submaximal measures for both groups are shown in **Table 3**. There was no significant difference between the two groups in the intensity levels at which the highest SV was reached: 68.3 ± 9.7 and 68.2 ± 9.8% VO_{2peak} in PAthl with SCI and PAthl with AMP/PM, respectively. Although CO at these intensities was not significantly different between the two groups of athletes, significant differences were observed between the groups of PAthl for SV, HR, and O_2 pulse. SV was significantly lower in the PAthl with SCI than the PAthl with AMP/PM, but there was no difference between the PAthl with AMP (SV: 160 ± 18.2 ml) and those with PM (SV: 145.4 ± 12.78 ml). The corresponding HR was significantly higher in PAthl with SCI than those with AMP/PM. The corresponding O_2 pulse was significantly higher in the PAthl with AMP/PM than the PAthl with SCI athletes. The CO/VO_2 ratio and the (a-v)O_{2diff} were not significantly different between the two groups of PAthl at this submaximal exercise intensity.

Relationship Between Peak Aerobic Power and Stroke Volume

Significant correlations were observed between the VO_{2peak} assessed in the continuous maximal exercise test and the highest SV found at the steady state sub-maximal intensities in both groups of athletes. **Figure 1** illustrates the scatterplots and regression curves with the respective confidence intervals (95%) for predicting VO_{2peak} from the highest SV measured at the sub-maximal intensities in each group of PA. The relative equations (Eq.) are described below:

TABLE 3 | Cardiorespiratory and metabolic responses during submaximal exercise in Paralympic athletes with a locomotor impairment (Mean ± SD).

Variable	SCI (*N* = 19)	AMP/PM (*N* = 14)	% Difference
Power output (W)	75.6 ± 33.12	82.4 ± 21.17	8.17 ($p = 0.512$)
Oxygen uptake (L/min)	1.53 ± 0.481	1.73 ± 0.350	11.29 ($p = 0.209$)
Oxygen uptake (ml/kg/min)	22.3 ± 8.00	25.2 ± 5.20	11.61 ($p = 0.242$)
Oxygen uptake (% Peak)	68.3 ± 9.68	68.2 ± 9.98	−0.12 ($p = 0.982$)
Cardiac output (L/min)	16.7 ± 4.4	19.5 ± 3.1	14.29 ($p = 0.051$)
Cardiac output/oxygen uptake	11.1 ± 1.1	11.4 ± 0.8	2.81 ($p = 0.361$)
Heart rate (beats/min)	142 ± 15.8	126 ± 13.7	−13.19 ($p = 0.004$)
Stroke volume (ml/beat)	117.1 ± 24.7	154.8 ± 17.6	24.31 ($p < 0.001$)
Oxygen pulse (ml/beat)	10.8 ± 2.8	13.7 ± 2.3	21.63 ($p = 0.003$)
Mixed (a-v)O_{2diff} (ml/100 ml)	9.12 ± 0.89	8.82 ± 0.59	−3.40 ($p = 0.282$)

Cardiac output was determined by carbon dioxide (CO_2) rebreathing method at approximately 30, 50, and 70% of the VO_{2peak}. The intensity at which the highest stroke volume was attained in each athlete was selected to calculate the mean value for the two athlete groups.
SCI, PAthl with spinal cord injury; AMP/PM, PAthl with amputation/poliomyelitis. The % difference was calculated as follows: {(AMP/PM – SCI)/AMP/PM} × 100.

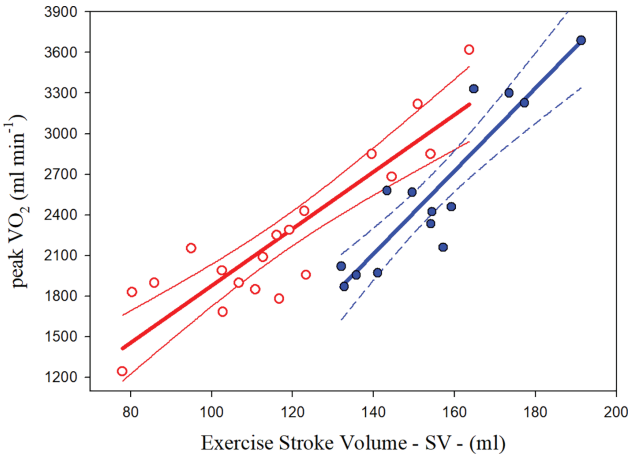

FIGURE 1 | Relationships between oxygen uptake peak (VO_{2peak}) and highest sub-maximal exercise stroke volume in Paralympic athletes with spinal cord injury (red scatterplot and curves) and Paralympic athletes with amputation and poliomyelitis (blue scatterplot and curves). The relative equations [Eqs. (1) and (2)] for each curve are reported in the text.

$$\text{PAthl with SCI}: VO_{2peak} = 21.03\,SV - 222.5;\quad SEE = 269.80; R^2 = 0.796 \tag{1}$$

$$\text{PAthl with AMP / PM}: VO_{2peak} = 30.62\,SV - 2176.2;\quad SEE = 258.96; R^2 = 0.824 \tag{2}$$

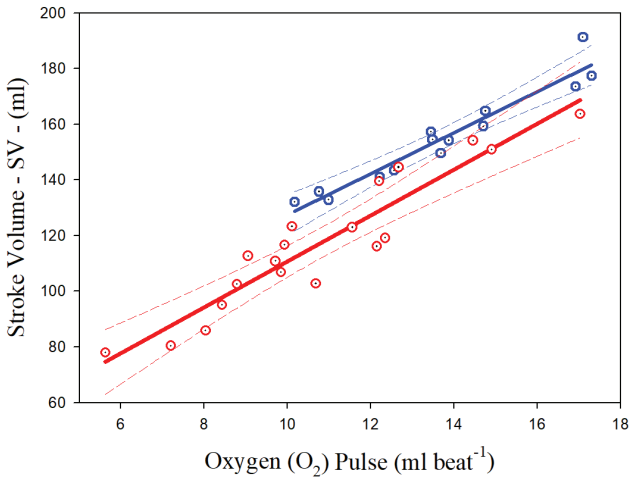

FIGURE 2 | Relationships between highest sub-maximal exercise stroke volume and oxygen pulse in Paralympic athletes with spinal cord injury (red scatterplot and curves) and Paralympic athletes with amputation and poliomyelitis (blue scatterplot and curves). The relative equations [Eqs. (3) and (4)] for each curve are reported in the text.

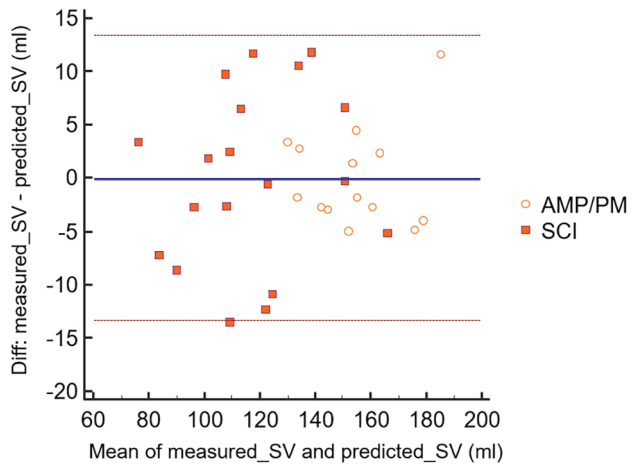

FIGURE 3 | Bland-Altman analysis of measured highest sub-maximal exercise stroke volume and predicted highest sub-maximal exercise stroke volume (SV) from oxygen pulse. The scatter points are related to both Paralympic athletes with spinal cord injury (SCI) [computed with Eq. (3)] and Paralympic athletes with amputation and poliomyelitis (AMP/PM) [computed with Eq. (4)]. Mmeans, mean values of measured highest sub-maximal exercise stroke volume and predicted highest sub-maximal exercise stroke volume; Diff, difference between measured highest sub-maximal exercise stroke volume and predicted highest sub-maximal exercise stroke volume. The dashed lines indicate the 95% confidence intervals for these data points.

The statistical analysis showed that the two curves differed significantly for the slopes and intercepts.

Prediction of Stroke Volume From Oxygen Pulse

Significant relationships were observed between the O_2 pulse and highest SV measured at the sub-maximal intensities in both groups of athletes. The common variance of the overall data (both groups together) was 89.30%. The scatterplots and regression curves with the respective confidence intervals (95%) for predicting the SV from the O_2 pulse values are illustrated in **Figure 2** for both the PAthl with SCI (in red) and PAthl with AMP/PM (in blue). The specific equations (Eq.) are described below:

$$\text{PAthl with SCI}: SV = 8.26\,O_2\text{ pulse} + 28.14;\quad SEE = 8.45; R^2 = 0.888 \tag{3}$$

$$\text{PAthl with AMP/PM}: SV = 7.37\,O_2\text{ pulse} + 53.73;\quad SEE = 4.78; R^2 = 0.932 \tag{4}$$

The statistical analysis showed that the two curves differed for the slopes and intercepts. Considering the whole group of PAthl, the O_2 pulse was significantly correlated with the SV (R^2 = 0.896) but not with the (a-v)O_{2diff}, where the overall common variance was only 24.9%.

The Bland-Altman analysis for the PAthl with SCI and PAthl with AMP/PM is illustrated in **Figure 3**. It is evident that all the predicted values of SV from the O_2 pulse measurements were within the 95% confidence intervals. Therefore, the null hypothesis that there was no proportional bias between the two measurements was accepted. These findings, in conjunction with the excellent ICC of 0.984, which had lower and upper bound confidence intervals of 0.967 and 0.992, respectively, further attested the validity of this prediction.

DISCUSSION

This study compared the peak cardiorespiratory responses and the CO determinants (HR and SV) during submaximal arm cranking exercise in PAthl with a thoracic or lumbar level spinal cord injury (PAthl with SCI) and PAthl with a single/double lower limb amputation or poliomyelitis (PAthl with AMP/PM). A further objective was to examine whether the SV could be predicted from the O_2 pulse measurements in these groups of athletes. The main findings were: (1) PAthl with SCI and AMP/PM had similar values for peak aerobic fitness and CO, but the former group had significantly higher peak HR and significantly lower peak O_2 pulse values than the latter group; (2) the highest SV measured during submaximal exercise intensities (at around 70%VO_{2peak}) was significantly lower in PAthl with SCI than those with AMP/PM; (3) in both groups of PAthl, SV was a significant determinant of the VO_{2peak}; and (4) SV values were highly correlated with the O_2 pulse assessed at the same exercise intensity, implying that the latter variable could be used to predict in both groups of PAthl. The physiological basis and practical implications of these findings are discussed below.

Peak Cardiorespiratory Responses

The VO_{2peak} of the PAthl with SCI (32.7 ml•kg^{-1}•min^{-1}) is comparable to the values reported by Hopman et al. (1993) for competitive athletes with paraplegia (30 ml•kg^{-1}•min^{-1})

during arm cranking, as well as the overall mean (31.5 ml•kg^{-1}•min^{-1}) of athletes with high and low lesion paraplegia evaluated by Bernard et al. (2000) during wheelchair ergometry. The high standard deviation (± 10.9 ml•kg^{-1}•min^{-1}) of the VO_{2peak}, and therefore the wide range of VO_{2peak} values, is consistent with the fact that these PAthl competed in sports with a wide range of energy expenditure (Bernardi et al., 2010; Price, 2010) and therefore their aerobic fitness varied widely (Bernardi et al., 2012; Baumgart et al., 2018; Kouwijzer et al., 2018). Some of the lower VO_{2peak} values in the PAthl with SCI (see **Figure 1**) can also be explained by their lesion level. It has been reported indeed that the VO_{2peak} is inversely related to the lesion level in individuals with paraplegia (Bernard et al., 2000; Janssen et al., 2002; Nightingale et al., 2019), i.e., the higher the lesion level the lower the VO_{2peak}. In the current study, the lesion level of the 19 PAthl with SCI ranged from T4 to L1. Three of these PAthl had high level paraplegia (from T4 to T6), 11 had mid-level paraplegia (from T7 to T12), and five had a low-level paraplegia (L1) with one athlete in this group having an incomplete lesion. The PAthl with high- and mid-level paraplegia had limited ability to control the trunk due to their SCI. Since trunk stabilization is important during upper body exercise (Sawka, 1986), it is likely that the performance of some of these athletes, despite using "strapping," was compromised during the ACE, thereby resulting in a reduced CO and therefore a lower VO_{2peak}. By contrast, the AMP/PM athletes were able to fully utilize their trunk musculature during ACE which enabled them to attain a higher power output and VO_{2peak}.

The VO_{2peak} of athletes with AMP and PM has not been well documented. In many studies, their results have been combined with data of PAthl with other health conditions, mostly SCI, and impairments (Bernardi et al., 2010, 2012). van der Woude et al. (2002) reported a value of 35.6 ml•kg^{-1}•min^{-1} in six elite athletes with AMP during wheelchair ergometry, but details of the lesion (unilateral vs. bilateral AMP; trans-femoral vs. trans-tibial AMP) were not provided. These values were slightly lower than those found in the PAthl with AMP in the present study (see section "Peak Cardiorespiratory Responses"). Kriz et al. (1992) reported pre-training VO_{2peak} values of 17.7 ml•kg^{-1}•min^{-1} during arm cranking exercise in sedentary individuals with PM. These values were also considerably lower than the subgroup with PM tested in the present study. The current data on PAthl with AMP and PM fill the void that currently exists in the literature and could be useful to sport scientists, trainers, and athletes involved in Paralympic sports.

The current findings indicated that the VO_{2peak} was similar in the PAthl with AMP/PM compared to the PAthl with SCI. However, in the PAthl with SCI, these VO_{2peak} values were attained with a significantly higher HR and lower O_2 pulse (suggesting a lower maximal SV, see discussion below) during peak exercise. This was despite the fact that in both groups of athletes, the peak HR was within their age predicted maximal HR (220 – age, ± 8 beats min^{-1}), suggesting that autonomic control of the myocardium was not altered in both groups (Hopman, 1994; Bhambhani, 2002; Birk and Pitetti, 2009). The attainment of the age predicted maximal HR was also evident in the three athletes with SCI at levels between T4 and T6. This observation needs to be interpreted with caution as no autonomic assessments were done on any of the athletes who participated in this study. It is important to stress that while PAthl with AMP/PM reached the 95.7 ± 5.09% of their age predicted maximal HR, PAthl with SCI reached HR values equal to 101.7 ± 6.13%. Five PAthl with SCI (mean age of 34.2 years) showed extremely high peak HR values (range between 193 and 206 beats•min^{-1}) at the end of the maximal ACE tests (i.e., at the VO_{2peak} values). If we had not observed these extremely high HR values in these five PAthl with SCI, it is likely that there would have been no significant difference between the PAthl with SCI and with AMP/PM (**Table 2**). These findings suggest that this high chronotropic reserve (Palmieri et al., 2010), which allows athletes to sustain high performance with high HR and high VO_2 values (Bernardi et al., 2010), may be a long-term central adaptation of athletes with SCI who train and compete at high intensities (Bernardi et al., 2013) over several years (see the following discussion on isokinetic circulation).

Submaximal Cardiorespiratory Responses

Currently there is limited research that has examined the CO and SV responses during ACE exercise in PAthl. Hopman et al. (1993) measured CO by CO_2 rebreathing (similar to this study) at 50, 70, and 80% of the peak power output during arm cranking in well-trained individuals with paraplegia. They reported no significant increase in SV between 50 and 80% of the peak power output (i.e., a plateau at approximately 50% of peak power output), a trend which was similar to that observed in untrained able-bodied individuals. In the current study, CO was evaluated at intensities between 30 and 70% of VO_{2peak}, and the highest sub-maximal exercise SV was attained at 68.3 ± 9.7% in the PAthl with SCI and 68.2 ± 10.0% in the PAthl with AMP/PM (**Table 3**). The highest SV among the three intensities was selected even if the difference between the measurements was minimal. The higher SV at 70% of VO_{2peak} than that observed at 50% could be due to the fact that the present study included elite athletes who did considerable amount of endurance training to participate in Paralympic sport competitions. However, this cannot be confirmed on the basis of the current cross-sectional evidence from this study. Hopman et al. (1993) reported that although CO was not significantly different between well-trained individuals with paraplegia and able-bodied controls at the different exercise intensities (i.e., isokinetic circulation), the manner in which it was attained was quite different. In the able-bodied individuals, CO was attained by a lower HR and higher SV, whereas in the athletes with paraplegia, the reverse was true. This disparity between the two groups was attributed to the "venous blood pooling" in the abdomen and in the lower extremities of the individuals with higher levels of SCI, which would reduce their cardiac preload, thereby decreasing the SV. As a result, the HR had to demonstrate a compensatory increase to maintain the CO. Although Jacobs et al. (2002) confirmed that SV during submaximal arm cranking exercise at the same absolute work rate was significantly lower in untrained individuals with

paraplegia and HR was significantly higher than able-bodied individuals, they reported that CO measured by bioelectrical impedance was significantly lower in the individuals with paraplegia. They claimed that the HR was unable to fully compensate for the reduced SV in individuals with paraplegia during exercise, thereby resulting in a hypokinetic circulation. These apparently contrasting results suggest that only trained individuals with SCI display the isokinetic circulation.

In the present study, able-bodied control individuals were not evaluated for comparative purposes, but the PAthl with AMP/PM could be considered a surrogate control group as their autonomic nervous system and its central (brainstem) control were intact. From another point of view, the present control group is ideal because these PAthl compete in many sports against each other. When these three variables (i.e., CO, HR, and SV) were compared between the two groups of PAthl (**Table 3**), there were no significant differences in CO and the CO/VO_2 ratio between the two groups. However, the SV was significantly lower, and the HR was significantly higher in the PAthl with SCI. These physiological differences between the two groups of athletes were most likely due to the reduced muscle pump action and lack of vasomotor control below the lesion level in the PAthl with SCI. These findings support the observations of Hopman et al. (1993) discussed above and collectively suggest that notwithstanding the differences in the technique of measuring CO, well-trained individuals with paraplegia demonstrate an isokinetic circulatory pattern during submaximal and maximal arm cranking exercise. To further investigate this issue, however, the two groups should be matched for age and aerobic fitness, measuring the two variables at different exercise intensities. Additionally, measurements of cardiac dimensions need to be conducted to establish whether differences in long-term myocardial adaptations between these groups of athletes could confirm these observations.

Relationship Between Peak Aerobic Power and Stroke Volume

This study showed that SV, measured during submaximal testing, is a significant determinant of VO_{2peak} in PAthl with SCI and AMP/PM. The common variance between these two variables was 79.6 and 82.4% in the PAthl with SCI and PAthl with AMP/PM, respectively. Previous studies (Huonker et al., 1998; Jacobs and Nash, 2004; Palmieri et al., 2010; Ordonez et al., 2013; Rosety-Rodriguez et al., 2014; Nightingale et al., 2017) have consistently demonstrated the cardiorespiratory benefits of regular upper limb aerobic exercise that accrue in untrained individuals with SCI. The present findings demonstrate that long-term central cardiac adaptation occurs in both PAthl with AMP/PM and with SCI, but to a different extent. As illustrated in **Figure 1**, PAthl with AMP/PM tend to have SV values higher than those with SCI of about 30 ml at low levels of VO_{2peak} (less fit athletes) and about 15 ml at high VO_{2peak} levels (most trained athletes). This greater long-term adaptation in PAthl with AMP/PM is probably due to their ability to walk and, in some cases, participation in a standing sport. The reduced SV observed in the PAthl with SCI compared to those with AMP/PM during exercise was most likely due to a reduction in their cardiac preload as a result of chronic venous blood pooling in the lower extremities (Hopman, 1994; Hopman et al., 1998; Jacobs et al., 2002; Theisen, 2012) and reduction in blood volume (Kinzer and Convertino, 1989; Houtman et al., 2000). These data and the related equations show that PAthl with SCI rely on peripheral adaptations to exercise to increase their VO_{2peak} to a greater extent than PAthl without SCI. Reduced cardiac preload as well as several years of reduced physical activity could induce chronic changes in the myocardium resulting in reduced cardiac wall thickness and/or heart cavity dimensions, thereby influencing the myocardial contractility and SV of these athletes (Washburn et al., 1986; Palmieri et al., 2010; Williams et al., 2019). Animal research has indicated that the cardiac atrophy is dependent on the severity of the SCI (Squair et al., 2018). In this study, no echocardiographic measurements were undertaken to study heart dimensions, and therefore, it is difficult to identify whether these factors could account for the differences in SV between the two groups of PAthl. Further research is needed to elucidate the myocardial changes that occur from long-term aerobic detraining and training and their effect on cardiovascular dynamics and aerobic fitness in PAthl. However, the current physiological assessment has demonstrated an important clinical use of the relationship between highest SV at submaximal exercise intensities and VO_{2peak}. One athlete with double thigh AMP was excluded from the study because, few years following this assessment, he was declared ineligible for Paralympic sport due to dilated cardiomyopathy (Pelliccia et al., 2016). This athlete was definitely an outlier in this relationship, because his SV was more than two standard deviations lower than that predicted for his aerobic fitness. His relatively high VO_{2peak} was mainly due to peripheral adaptations to exercise. However, his predicted SV from the O_2 pulse measurement (data not shown in the present paper) was fairly accurate (see next section of the paper).

Prediction of Stroke Volume From Oxygen Pulse

The current findings supported our hypothesis that the O_2 pulse would be a valid predictor of SV during arm cranking exercise in the PAthl with SCI and AMP/PM [Eqs. (3) and (4) and **Figure 2**]. These findings corroborate previous reports on healthy, able-bodied individuals, which have shown that O_2 pulse is a valid predictor of SV during arm cranking (Bevegard et al., 1966; Stenberg et al., 1967; Bhambhani, 1995) and leg cycling (Bhambhani et al., 1994; Whipp et al., 1996). The common variance (R^2) between these two variables (Stenberg et al., 1967; Bhambhani, 1995) using the CO_2 rebreathing technique (73%) was lower than those found in the present study for PAthl with SCI and AMP/PM. The current findings demonstrate that the common variance between the O_2 pulse and SV was 88.8 and 93.2% in the SCI and AMP/PM athlete groups, respectively. This was despite the differences in: (1) the determinants of their CO during submaximal exercise; (2) the reduced muscle mass available for recruitment in the athletes with AMP/PM; and (3) impaired ability to recruit musculature below the lesion level in the PAthl with SCI. The latter two points would increase the degree of venous blood pooling, which would normally occur during seated upper body exercise (Sawka, 1986), thereby affecting differently the cardiac

preload and the SV. The Bland-Altman analysis in **Figure 3** indicated that all data points were within the 95% limits of agreement in both the athlete groups, supporting the validity of these predictive equations. Two additional points should be noted: (1) because SV remains fairly constant between 50 and 80% of peak power output during arm cranking exercise (Hopman et al., 1993), these regression equations enable sport scientists to predict the SV within this exercise range without the need for additional tests, and (2) since O_2 pulse was not significantly correlated with the (a-v)O_{2diff} during submaximal arm exercise (the Bland-Altman analysis failed), it is likely that the changes in O_2 pulse, which occur with training primarily, reflect modifications in the SV (Bhambhani, 1995). However, this hypothesis needs to be further investigated with appropriately designed research studies.

LIMITATIONS OF THE STUDY

The following limitations should be considered when interpreting the results of this study. First, all the athletes were tested during arm cranking and not the exercise mode that was specific to their competitive sport. It is possible that this could have influenced the cardiorespiratory responses to some extent. However, if we had chosen to test the athletes during wheelchair ergometry, an exercise mode that is routinely used to test athletes with locomotor impairments, this could have biased the results of the athletes who used wheelchairs for training and competition; e.g., track and tennis athletes in the SCI and the AMP/PM groups (Abel et al., 2008). Second, the PAthl with AMP/PM group consisted of a small number of athletes with each of these disorders and therefore different impairments. However, we felt justified in combining these two impairments because neither of them alters autonomic function, which was very important to control for this type of exercise study. Third, there was some heterogeneity in the PAthl with AMP who participated in this study. Five of them had single trans-femoral amputation and four had single trans-tibial amputation. Nevertheless, it is unlikely that differences in muscle mass of the lower extremities would have influenced their performance during arm cranking exercise, because all these athletes had complete control of their trunk and upper body musculature. Finally, there were significant differences in the age of the athletes with SCI compared to the two groups of athletes with AMP and PM, which could influence the cardiovascular responses during exercise. The SCI Athletes were significantly older than the athletes with AMP by 4.3 years and significantly younger than the athletes with PM by 8.2 years. However, when the ages of the athletes with AMP and PM were pooled, there was no significant difference between the athletes with SCI and AMP/PM. It should be noted that both groups of athletes attained their age predicted maximum heart rate (220 – age), as indicated in **Table 2**, and the mean HR value was significantly different between the two groups of athletes. Furthermore, the peak cardiorespiratory responses of the athletes with AMP and PM were not significantly different (see section "Results") despite a significant difference in their age, suggesting that the differences between the athletes with SCI and AMP/PM were most likely due to their health conditions. Despite these limitations, the ability to predict SV from the O_2 pulse was robust in both the groups of athletes.

CONCLUSIONS

In two groups of elite PAthl with different locomotor impairments, but with similar aerobic fitness (VO_{2peak}), the cardiac output determinants were significantly different. PAthl with SCI displayed significantly higher HR and lower O_2 pulse values during peak arm cranking exercise than PAthl with AMP/PM. The ability to reach high HR values in PAthl with SCI who had no neural disruption to the myocardium was most likely due to their chronic aerobic training. The highest SV found at submaximal intensities (at around 70% VO_{2peak}) in PAthl with SCI was significantly lower than that measured in PAthl with AMP/PM. The corresponding HR values were significantly lower in PAthl with AMP/PM than those with SCI. When assessing training status of PAthl, HR values should be carefully considered in conjunction with their specific impairment. In both groups of athletes, SV was significantly correlated with VO_{2peak}. The O_2 pulse was a valid predictor of SV in both the athlete groups and was not significantly correlated with the mixed (a-v)O_{2diff}. O_2 pulse is a useful physiological variable to evaluate changes in SV in PAthl when direct measurement is not feasible. Further research is needed to examine whether the changes in SV resulting from long-term aerobic training can be tracked by evaluating the changes in O_2 pulse in these athletes.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available because they are related to a private database. Requests to access the datasets should be directed to marco.bernardi@uniroma1.it.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Italian National Health service. All the testing procedures were approved by the Italian National Olympic Committee and Italian Paralympic Committee for athlete testing and by the "Santa Lucia Foundation" Ethical Committee. Written informed consent was obtained from all subjects. All participants gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MB, VC, FS, and YB contributed to conception and design of the work. MB, EG, and AR participated in data acquisition. MB, EG, AR, DD, IP, FS, and YB analyzed and interpreted the data. MB, EG, and VC contributed to recruitment of athletes. MB and VC contributed to clinical and functional classification (scales) of the subjects. MB, EG, DD, FS, and YB drafted the manuscript. MB, AR, IP, and YB revised the manuscript. MB

and EG contributed to clinical evaluation of subjects. DD and IP performed the statistics of the work. MB, EG, AR, DD, VC, IP, FS, and YB approved the final version of the manuscript.

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