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AUTHOR
Azadan, Rod J.; Agha, Nadia H.; Kunz, Hawley E.; et al.

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The effects of normoxic endurance exercise on erythropoietin (EPO) production and the impact of selective β_1 and non-selective $\beta_1 + \beta_2$ adrenergic receptor blockade

Rod J Azadan[1] · Nadia H Agha[2] · Hawley E Kunz[3] · Forrest L Baker[1,4] · Preteesh L Mylabathula[1,4] · Tracy A Ledoux[1] · Daniel O'Connor[1] · Charles R Pedlar[5,6,] · Richard J Simpson[1,4]

[1] Laboratory of Integrated Physiology, Department of Health and Human Performance, University of Houston, 3875 Holman Street, Houston, TX 77204, USA
[2] Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX, USA
[3] Division of Endocrinology, Diabetes, Metabolism, and Nutrition, Mayo Clinic, Rochester, MN, USA
[4] Department of Nutritional Sciences, University of Arizona, Tucson, AZ, USA
Department of Pediatrics, University of Arizona, Tucson, AZ, USA
Department of Immunobiology, University of Arizona, Tucson, AZ, USA
[5] Faculty of Sport, Health and Applied Science, St Mary's University, Twickenham, England, UK
[6] Institute of Sport, Exercise and Health, University College London (UCL) London, England, UK

ORCID

Rod J Azadan 0000-0001-7160-1557
Nadia H Agha 0000-0001-5011-766X
Hawley E Kunz 0000-0002-4291-4455
Forrest L Baker 0000-0001-5338-7905
Preteesh L Mylabathula 0000-0002-8428-0251
Tracy A Ledoux 0000-0003-4935-4822
Daniel O'Connor
Charles R Pedlar 0000-0002-3075-9101
Richard J Simpson 0000-0002-7064-6881 (corresponding author: rjsimpson@arizona.edu)

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Consent to participate: Prior to testing, instructions outlining the experimental procedures were given and written informed consent to participate was obtained from each participant.

Consent for publication: Written informed consent that the results of this study may be published in a professional and/or scientific journal without mention of their name or personal information was obtained from each participant.

Availability of data and material: Supporting data are available from the corresponding author upon request.

Code availability: SPSS (IBM, Armonk, NY, USA) versions 25 and 26 (RRID: SCR_002865) was used for all statistical analyses and Prism (GraphPad, San Diego, CA, USA) version 8 (RRID: SCR_002798) was used to create all figures. No custom code was used in this research.

Abstract

Purpose Habitual endurance exercise results in increased erythropoiesis, which is primarily controlled by erythropoietin (EPO), yet studies demonstrating upregulation of EPO via a single bout of endurance exercise have been equivocal. This study compares the acute EPO response to 30 minutes of high versus 90 minutes of moderate intensity endurance exercise and whether that response can be upregulated via selective adrenergic receptor blockade.

Methods Using a counterbalanced, cross-over design, fifteen individuals (age 28±8) completed two bouts of running (30-minute, high intensity vs 90-minute, moderate intensity) matched for overall training stress. A separate cohort of fourteen individuals (age 31±6) completed three bouts of 30-minute high intensity cycling after ingesting the beta antagonist bisoprolol, nadolol or placebo. Venous blood was collected before, during and after exercise and EPO response assessed.

Results No detectable EPO response was observed during or after high intensity running, however in the moderate intensity trial EPO was significantly elevated at both during-exercise timepoints (+6.8% ± 2.3% at 15 minutes and +8.7% ± 2.2% at 60 minutes). No significant change in EPO was observed post-cycling or between βAR drug trials.

Conclusion Neither training mode (running or cycling), nor beta-blockade significantly influenced the EPO response to 30 minutes of high-intensity exercise, however 90 minutes of moderate-intensity running elevated EPO during exercise, returning to baseline immediately post exercise. This exercise dependent and transient elevation may explain equivocal data regarding the effect of exercise on serum EPO and allow prescription of exercise to maximize EPO response for both performance and clinical applications.

Keywords: erythropoietin – EPO – endurance exercise – running – βAR – beta blockade

Abbreviations

βAR	Beta Adrenergic Receptor	PLT	Platelets
BMI	Body Mass Index	RBC	Red Blood Cells
CI	Confidence Interval	RDW	Red-Cell Distribution Width
EDTA	Ethylenediaminetetraacetic Acid	RPE	Rating of Perceived Exertion
EPO	Erythropoietin	SAO_2	Arterial Hemoglobin Oxygen Saturation
HCT	Hematocrit	SPO_2	Peripheral Capillary Oxygen Saturation
HGB	Hemoglobin	VO_2	Volume of Oxygen
HIF	Hypoxia Inducible Factor	VO_2max	Maximum Rate of Oxygen Consumption
LT	Lactate Threshold	VO_2peak	Peak Rate of Oxygen Consumption
MCH	Mean Corpuscular Hemoglobin	VCO_2	Volume of Carbon Dioxide
MCHC	Mean Corpuscular Hemoglobin Concentration	VT	Ventilatory Threshold
MCV	Mean Corpuscular Volume		
MPV	Mean Platelet Volume		

Introduction

The glycoprotein hormone erythropoietin (EPO) is the primary regulator of erythropoiesis through division and differentiation of CD34+ hematopoietic stem cells (Jelkmann 2016; Kaneko et al. 2017), a process which tightly governs the maintenance of blood hemoglobin levels (Jelkmann 2007; Eggold and Rankin 2019). Accordingly, with the increased oxygen demands of habitual endurance exercise, this homeostatic hemoglobin setpoint can be increased, resulting in a concomitant increase in maximum oxygen utilization capacity (VO_2max) (Heinicke et al. 2001). However, although the training-induced increase in hemoglobin mass among endurance athletes is well known (Ciekot-Soltysiak et al. 2018), as is the essential role of EPO in its regulation (Eggold and Rankin 2019), the mechanism of EPO stimulation via endurance exercise has not yet been well characterized (Gunga et al. 2007; Montero and Lundby 2018).

The primary regulatory mechanism for EPO production in humans is an arterial oxygenation sensing mechanism within the nephrotic tissue involving the stabilization of hypoxia-inducible-factors HIF-1 and HIF-2 (Semenza and Wang 1992; Wang and Semenza 1993; Kapitsinou et al. 2010), as well as increased expression, mRNA stabilization and accumulation of the regulatory subunit HIF-1α (Pialoux et al. 2009b; Pialoux et al. 2009a; Tae Woo Kim 2010). Accumulation of the regulatory subunit HIF-1α is mediated via adrenergic system activation of β_2 agonist receptors (β_2AR), which results in increased EPO mRNA transcription (Cheong et al. 2016). However, even though the regulatory pathway is modestly understood, research into the mechanism of EPO stimulation via endurance exercise is lacking. A recent review by Montero and Lundby highlights that the initial increase in plasma volume following exercise may be the stimulus to upregulate EPO via the homeostatic drive to tightly control circulating red blood cell concentration, explaining early training adaptations as plasma volume is maximized after approximately 4 weeks of endurance training (Montero and Lundby 2018). Further EPO regulation may involve exercise induced hypoxemia (EIH) (Constantini et al. 2017a), mirroring the physiological response to hypoxic environments such as high altitude, which is a reliable EPO stimulus (Wehrlin et al. 2016; Constantini et al. 2017b; Zelenkova et al. 2019), though to date the extent to which EIH is involved in EPO regulation is unclear.

Previous studies have demonstrated acute EPO stimulation via a multitude of conditions that acutely induce hypoxemia. Studies include hemorrhage (Gleiter et al. 1997), high altitude exposure (both with and without exercise) (Schobersberger et al. 2000; Roecker et al. 2006; Pialoux et al. 2009c; Govus et al. 2017; Schmidt 2002), and normoxic exercise (Schwandt et al. 1991; Roberts and Smith 1999; Roecker et al. 2006; Montero et al. 2017), however the results of normoxic exercise have been most equivocal. A rise in serum EPO concentration was observed after 37-42.2km of running (Schwandt et al. 1991; Roecker et al. 2006), as well as 60 minutes of cycling (Montero et al. 2017), but other studies showed no significant rise in serum EPO after ≤60 minutes of running or cycling (Schmidt et al. 1991; Bodary et al. 1999; Klausen et al. 1993). Thus far, a clear understanding of the most effective normoxic training protocol to increase EPO production has been elusive. Nevertheless, based on previous research, it's reasonable to postulate that an extended hypoxemic stress elicited via longer duration (≥60 minute) exercise may be needed to stimulate a response from the nephrotic sensing mechanism to result in increased serum EPO levels and that this EIH would be moderated via β_2AR activation.

Therefore, the aims of this study were two-fold. First, to investigate the effect of intensity adjusted exercise duration on plasma EPO concentrations in healthy human participants. Second, to determine if the EPO response to exercise can be upregulated via selective adrenergic receptor blockade. We hypothesized that longer duration (90 minute), moderate intensity exercise would result in greater serum EPO levels relative to shorter (30 minute), higher intensity exercise, and that selective β_1AR blockade would increase the exercise induced EPO response in a β_2AR dependent manner.

Methods

Effect of exercise duration on serum EPO

Participants

Fifteen (9 men and 6 women) healthy, physically active individuals participated in this study (mean ± SD for age: 28±8 years, height: 173±9 cm, weight: 73±13 kg, BMI Index: 24±3, VO_2peak: 50±8 ml/kg/min). Participants physical activity level was determined by completion of the Jackson PA-R questionnaire and self-reported regular engagement of moderate to vigorous aerobic exercise (Andrew S.J. 1990). Prior to testing, instructions outlining the experimental procedure were given and written informed consent was obtained from each participant. All participants were non-smokers, not taking any prescription medications and familiar with treadmill running. All participants were advised to avoid caffeine and strenuous exercise for the 24 hours prior to each exercise session. Food and drink consumed before the first exercise session were recorded and participants were advised to maintain the same nutritional practices prior to the subsequent exercise trials. Participants provided informed consent and study approval was granted by Committee for the Protection of Human Participants (CPHS) at the University of Houston in accordance with the Declaration of Helsinki.

Baseline Testing

Participants performed one baseline testing session which included anthropomorphic measurements, as well as a ventilatory threshold test using a motorized treadmill (Woodway Desmo, Waukesha, WI, USA) to standardize subsequent exercise testing intensity across all participants. The first ventilatory threshold (VT_1) was used instead of lactate threshold (LT_1) to allow for uninterrupted running, which best emulates a typical run training session. The first ventilatory threshold (VT_1) has also been shown to be significantly correlated with the first lactate threshold (LT_1) (Gaskill et al. 2001). The test procedure used to determine ventilatory threshold was a graded exercise test of increasing intensity using 2-minute stages. The starting speed of the treadmill was 6.4 km/hr at a 1% incline, with increases in speed of 0.8 km/hr for each stage until an R-Value of greater than 1.1 was achieved, after which the treadmill incline was increased by 2% every minute until volitional exhaustion. The first ventilatory threshold was determined using the V-slope method (Beaver et al. 1986). The exercise parameters of heart rate, speed, incline and Rating of Perceived Exertion (RPE) were recorded.

Experimental Overview

Using a randomized, counterbalanced, cross-over design, participants completed two bouts of running (short vs long duration) matched via intensity for overall training stress. The short duration trial included 30 minutes of high-intensity running and the long duration trial included 90 minutes of moderate-intensity running, both using a motorized treadmill (Woodway Desmo, Waukesha, WI, USA) at a 1% incline. The short (high-intensity) trial was set at a treadmill speed of 10% above individual ventilatory threshold (VT_1), and the long (moderate-intensity) trial was set at 10% below VT_1, though adjustments were made when necessary if participants were having trouble completing the trial. Running speed was individualized via VT_1 to ensure that relative exercise intensity was consistent across individuals. And because exercise difficulty increases with duration, trial intensities were chosen to balance overall training stress of the exercise bout (i.e., the short and long trials were equally challenging yet achievable). Participants drank water *ad libitum* and the exercise parameters of heart rate, pace, incline and RPE were recorded during each trial, which were scheduled at least 7 days apart.

Peripheral Blood Sample Collection

Peripheral venous blood samples were collected at 6 time-points for the short duration trial, and 7 time points for the long duration trial: at rest prior to exercise, 15 minutes into (i.e., during) exercise, 60 minutes into (i.e., during) exercise (long duration trial only), immediately post exercise, 1 hour post exercise, 2 hours post exercise, and 3 hours post exercise. Venous blood samples were collected from the participant's arm using a catheter and 6ml Vacutainers containing either EDTA or serum gel (BD, Franklin Lakes, NJ, USA). EDTA vacutainers were used for hematology analysis and the serum gel vacutainers were used for EPO analysis. A catheter was used to facilitate collection of during exercise samples, and during exercise samples, as well as the immediately post-exercise samples were taken while the participant was standing on the treadmill, while all other samples were collected while seated.

Participants

Fourteen healthy, physically active male adults participated in this study (mean ± SD for age: 31±6 years, height: 175±7 cm, weight: 81±11 kg, BMI: 27±4, LT_1: 137±32 w). Participants physical activity level was determined by completion of the Jackson PA-R questionnaire and self-reported regular engagement of moderate to vigorous aerobic exercise (Andrew S.J. 1990). Prior to testing, instructions outlining the experimental procedure were given and written informed consent was obtained from each participant. All participants were non-smokers,not taking any prescription medications and familiar with cycling exercise. All participants were advised to avoid caffeine and strenuous exercise for the 24 hours prior to each exercise session. Food and drink consumed before the first exercise session were recorded and participants were advised to maintain the same nutritional practices prior to the subsequent exercise trials. Participants provided informed consent and study approval was granted by Committee for the Protection of Human Participants (CPHS) at the University of Houston in accordance with the Declaration of Helsinki.

Baseline Testing

Participants performed one baseline testing session to collect anthropomorphic measurements, as well as perform a blood lactate threshold test using either the participant's own bicycle on a cycle ergometer (Racermate, Computrainer® Pro, Seattle, WA, USA) or standalone cycle ergometer (Racermate, Velotron®, Seattle, WA, USA). Individual lactate threshold testing was used to standardize subsequent exercise intensity across all participants. The test procedure used to determine lactate threshold was a discontinuous graded exercise test of increasing intensity using 3-minute stages with increases in power from 15-25 watts for each stage, depending on the participant's level of cycling ability. At the end of each stage, participants stopped cycling for 10-15 seconds while capillary blood samples were collected from the earlobe and immediately analyzed for blood lactate level (Analox Instruments, LM5 Lactate Analyzer, Stourbridge, UK). The starting wattage on the exercise ergometer was also individualized based on cycling ability at 50±25 watts to ensure that blood lactate levels did not rise prematurely, and that enough data-points were captured to achieve an adequate lactate profile. Lactate threshold was defined as the first upward inflection of the blood lactate profile after the rise above baseline; this point in the lactate curve is often referred to as LT_1 (Gordon et al. 2017). Lactate threshold baseline testing was concluded one stage after the participant's blood lactate concentration rose above 4.0 mmol/dl. Resting heart rate and blood pressure, as well as the exercise variables of heart rate, power and RPE were recorded.

Experimental Overview

The experimental structure utilized was a randomized, double-blind, placebo controlled cross-over design where each participant completed three trials (placebo, bisoprolol, nadolol) with a 7-14 day washout period between trials. The three trials included 30 minutes of cycling using a cycle ergometer (Racermate, Computrainer® Pro or Velotron®, Seattle, WA, USA) set to a resistance of 110% of lactate threshold (LT_1) power as determined for each individual during baseline testing. 110% of lactate threshold power was used because it is sufficient to elicit a catecholamine response (Graff et al. 2018), and most recreationally active but non-competitive individuals are able to sustain the intensity for 30-minutes. During each exercise trial, breath-by-breath gas exchange variables such as VO_2 and VCO_2 were recorded via calibrated metabolic cart (Cosmed® Quark CPET, Rome Italy) along with the exercise variables of heart rate, power and RPE. The three experimental conditions used for testing during these exercise sessions included randomized assignment to ingest a beta-adrenergic receptor blocking drug (nadolol or bisoprolol fumarate) or placebo three hours prior to the exercise trial. During the exercise trials, participants were not able to consume water due to the use of the face mask necessary to collect breath-by-breath analysis with the metabolic cart. The exercise variable of intensity (i.e., power) and duration were kept constant across all trials for each participant.

βAR Antagonist Administration

The oral β-receptor antagonist drugs used for this study were 10mg of bisoprolol fumarate (β_1AR selective) or 80mg of nadolol (β_1AR + β_2AR non-selective) for adrenergic receptor blockade. Three hours before each exercise trial the participants were given a capsule containing either bisoprolol, nadolol or placebo in double-blind random order. Both the participant and the investigator supervising the testing were blinded to drug assignment. These drugs were chosen because they are well tolerated by healthy participants and have attained commercial approval by the FDA for clinical use. The capsules were ingested three hours prior to the exercise trial to ensure the drug exerted its full effect based on pharmacokinetic data showing peak plasma levels are reached in 2-4 hours (Le Coz et al. 1991; Kostis et al. 1984). Dosages were standardized across all participants at the highest recommended adult prescription dosage with a minimum required washout period of 7 days between trials.

Peripheral Blood Sample Collection
Peripheral venous blood samples were collected at 4 time-points for each exercise trial: 3 hours prior to exercise, at rest prior to exercise, immediately post exercise, and 1-hour post exercise. Pre-exercise samples were collected after five minutes seated rest and all samples were collected in the seated position. Venous blood samples were collected from the participant's arm using an 18-gauge butterfly needle or catheter and 6ml Vacutainers containing either EDTA or serum gel (BD, Franklin Lakes, NJ, USA). EDTA vacutainers were used for hematology analysis and the serum gel vacutainers were used for EPO analysis.

Sample Analysis
Whole blood was analyzed for complete blood cell counts in duplicate using a hematology analyzer (BC3200, Mindray North America, Mahwah, NJ, USA). Serum was frozen at -80°C until analysis and then analyzed in duplicate using commercially available ELISA kits (Invitrogen™, Human EPO Platinum ELISA (short incubation) #BMS2035, Carlsbad, CA, USA) and a 96-well microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis
SPSS versions 25 and 26 (IBM, Armonk, NY, USA) were used for all statistical analyses. Data are presented as means and one standard deviation (SD) from the mean in tables and graphically as 95% Confidence Intervals (CI) in figures. One-way ANOVA with post hoc pairwise analysis using Bonferroni correction (where there were more than two groups) was used to assess significant differences in exercise measures (Table 1 & Table 3). A maximum likelihood linear mixed effects model was used to compare hematology values at each timepoint and between trial conditions (Table 2 & Table 4). A maximum likelihood linear mixed effects model was also used to compare absolute serum EPO values, as well as change in EPO from pre-exercise values at each timepoint and between trial conditions (Figures 1-4). The exercise induced change from baseline value of peripheral blood EPO concentration was included to investigate the effect of the exercise stimulus while accounting for individual variation in baseline values as well as individualized response and was calculated by comparing baseline (pre-exercise) values with subsequent time points for each individual. The model for the exercise duration trials (Figure 1 and Figure 2) included main effects for time (pre-exercise, 15 minute during-exercise, 60 minute during-exercise (change in EPO only), immediately Post, 1-hour Post, 2-hour Post and 3-hour Post), gender (male vs female) and trial type (short, high intensity vs long, moderate intensity). The model for the βAR trials (Figure 3 and Figure 4) included main effects for time (pre-exercise, immediately Post and 1-hour Post) and drug trial (placebo, bisoprolol or nadolol). Post hoc contrasts with Bonferroni correction were used to compare exercise time points and 95% confidence intervals were used to identify timepoints which were significantly different from the baseline, pre-exercise condition. Statistical significance was set at $p \leq 0.05$.

Results

Physiological responses to the short and long duration running protocols

The exercise measures for the exercise duration trials are listed in Table 1. Average running velocity was significantly higher in the short compared to the long duration trial (12.3 ± 1.9 vs 10.3 ± 1.6 km/h; p = 0.003). Average RPE was significantly higher in the short compared to the long duration trial (16.1 ± 2.5 vs 14.1 ± 1.5; p = 0.016) but was matched by the end of the exercise bouts due to the increasing difficulty of extended exercise (17.8 ± 2.8 vs 16.5 ± 2.1; p = 0.173). Average heart rate (HR) was significantly higher in the short compared to the long duration trial (177.8 ± 7.7 vs 168.0 ± 10.6 bpm; p = 0.007) as well as during the last 5 minutes (182.1 ± 8.1 vs 173.7 ± 10.4 bpm; p = 0.020), showing that the target intensities were achieved and sustained. Accordingly, average percent max HR was significantly higher in the short relative to the long duration trials (92.7 ± 4.7 vs 87.5 ± 4.9%; p = 0.007) as well as during the last 5 minutes (94.9 ± 5.4 vs 90.5 ± 4.9%; p = 0.025), showing the relative difference in exercise intensities was also achieved and maintained.

Table 1. Exercise measures for subjects in the exercise duration trials (n=15). Data are mean ±SD. P-values are for main effect by group.

Measure	Short	Long	p value
Duration Completed (minutes)	29.7±1.3	90±0	<0.001
Treadmill Incline (%)	1	1	1
Starting Velocity (km/h)	12.7±1.8	10.4±1.5	0.001
Average Velocity (km/h)	12.3±1.9	10.3±1.6	0.003
Average Rating of Perceived Exertion (RPE; 6-20 scale)	16.1±2.5	14.1±1.5	0.016
Average RPE, Last 10 min. (6-20 scale)	17.8±2.8	16.5±2.1	0.173
Average Heart Rate (bpm)	177.8±7.7	168.0±10.6	0.007
% Max Heart Rate, Trial Average	92.7±4.7	87.5±4.9	0.007
Average Heart Rate, Last 5 min. (bpm)	182.1±8.1	173.7±10.4	0.020
% Max Heart Rate, Last 10 min.	94.9±5.4	90.5±4.9	0.025

Hematological responses to the short and long duration running protocols

The changes in hematology parameters for the exercise duration trials are listed in Table 2. There was a significant main effect of time on HGB [F (6, 195) = 2.897, p = 0.010], RBC [F (6, 195) = 3.067, p = 0.007], HCT [F (6, 195) = 4.772, p < 0.001], PLT [F (6, 195) = 15.676, p < 0.001] and MPV [F (6, 193) = 2.225, p = 0.042]. Pairwise comparisons reveled a significant difference in HCT at 15-minute during-exercise and post-exercise values relative to 3-hours post exercise in the short duration trial (42.3 ± 4.66; p = 0.035 and 42.3 ± 3.69; p = 0.041 vs 38.0 ± 4.19, respectively) and a significant increase of PLT post-exercise (short trial) and 60-minute during-exercise and post-exercise (long trial) relative to pre-exercise values (263 ± 63.3 vs 194 ± 46.0; p = 0.001 and 266 ± 51.8; p < 0.002 and 280 ± 56.9; p < 0.001 vs 199 ± 36.1). There was a significant main effect of exercise trial for MCHC [F (1, 195) = 6.964, p = 0.009], with a significant difference between short and long duration trials at the 15-minute during-exercise timepoint (35.5 ± 3.91 vs 32.8 ± 5.78; p = 0.002) but no other main effect of exercise trial or interaction effects.

Moderate but not high intensity running elevated serum EPO levels during exercise

The total serum EPO concentration over time for all participants is shown in Figure 1. There was no significant difference in the EPO response between exercise trials or as a function of timepoint within the trials, however there was a trial by time interaction effect [F (5, 21.57) = 3.566, p < 0.017]. Post hoc analysis revealed a significant increase in EPO at the 15' during-exercise timepoint in the Long trial relative to pre-exercise values (p = 0.018). The exercise induced change in serum EPO concentration was investigated to evaluate individual response as well as the 60' during-exercise timepoint (Long Duration trial only) and is shown in Figure 2. Analysis of the change in EPO displayed a significant main effect of time [F (5, 15) = 5.485, p = 0.005], gender [F (1, 15) = 13.367, p = 0.002] and trial by time interaction effect [F (4, 15) = 3.158, p = 0.045], showing an elevated serum EPO at both during-exercise timepoints in the long duration, moderate intensity running trial (+6.8% ± 2.3% at 15 minutes and +8.7% ± 2.2% at 60 minutes), with no EPO response during or after the short duration, high intensity running trial.

Table 2. Hematology values for subjects in the exercise duration trials. Data are mean ±SD. P values are for main effect by group. Statistical differences between pre-exercise indicated by *, between 3h post-exercise by ^{A}, between trials indicated by $^{\#}$, $p<0.05$.

	Pre-Exercise	*15' During-Exercise*	*60' During-Exercise*	*Post-Exercise*	*1h Post-Exercise*	*2h Post-Exercise*	*3h Post-Exercise*	*Time F (p-value)*	*Trial F (p-value)*	*Interaction F (p-value)*
HGB (g/dL)										
Short	13.88 ± 1.48	14.65 ± 1.80	n/a	14.76 ± 1.30	13.38 ± 1.39	13.67 ± 1.10	13.20 ± 1.66	2.897 (0.010)	0.014 (0.907)	0.608 (0.694)
Long	13.81 ± 1.26	14.30 ± 1.40	14.51 ± 1.48	14.22 ± 1.72	13.46 ± 1.45	13.51 ± 1.58	13.60 ± 1.35			
RBC ($1x10^6$)										
Short	4.35 ± 0.45	4.59 ± 0.54	n/a	4.63 ± 0.38	4.18 ± 0.49	4.29 ± 0.34	4.18 ± 0.45	3.067 (0.007)	0.021 (0.885)	0.710 (0.617)
Long	4.38 ± 0.34	4.53 ± 0.40	4.59 ± 0.40	4.39 ± 0.52	4.27 ± 0.44	4.28 ± 0.45	4.32 ± 0.37			
HCT (%)										
Short	39.74 ± 3.78	42.32 ± 4.66 ^{A}	n/a	42.25 ± 3.69^{A}	38.42 ± 3.29	39.11 ± 2.70	38.00 ± 4.19	4.772 (<0.001)	0.449 (0.503)	0.331 (0.894)
Long	40.26 ± 2.85	42.59 ± 5.56	42.02 ± 3.43	41.38 ± 4.42	39.20 ± 3.73	39.16 ± 4.14	39.55 ± 3.25			
MCV (fL)										
Short	91.52 ± 2.18	92.33 ± 2.42	n/a	92.12 ± 2.62	91.40 ± 2.39	91.31 ± 2.29	90.14 ± 3.82	1.294 (0.262)	0.514 (0.474)	0.314 (0.904)
Long	90.06 ± 7.30	92.07 ± 2.20	91.85 ± 2.16	91.70 ± 2.03	91.92 ± 1.81	91.75 ± 1.88	87.99 ± 15.70			
MCH (pg)										
Short	31.91 ± 1.18	31.85 ± 1.22	n/a	31.48 ± 1.97	31.70 ± 1.17	31.81 ± 1.22	31.51 ± 1.71	2.015 (0.065)	0.053 (0.819)	0.006 (1.000)
Long	31.47 ± 1.38	31.56 ± 1.38	41.26 ± 37.47	31.29 ± 1.69	31.47 ± 1.51	30.93 ± 2.40	31.43 ± 1.75			
MCHC (gL)										
Short	34.88 ± 0.93	35.53 ± 3.91$^{\#}$	n/a	34.57 ± 1.18	34.73 ± 1.14	34.85 ± 1.03	34.64 ± 1.83	0.226 (0.968)	6.964 (0.009)	1.195 (0.313)
Long	34.24 ± 1.28	32.80 ± 5.78$^{\#}$	34.48 ± 1.11	34.23 ± 1.54	34.28 ± 1.47	33.68 ± 2.86	34.41 ± 1.56			
RDW (%)										
Short	13.21 ± 0.36	13.32 ± 0.51	n/a	13.27 ± 0.52	13.09 ± 0.44	13.22 ± 0.45	16.97 ± 14.20	0.952 (0.459)	0.943 (0.333)	1.234 (0.295)
Long	13.49 ± 0.63	13.28 ± 0.62	13.34 ± 0.64	13.28 ± 0.64	13.26 ± 0.55	13.18 ± 0.46	13.23 ± 0.58			
PLT ($1x10^3$μL)										
Short	194 ± 46.0	236 ± 57.4	n/a	263 ± 63.3*	187 ± 38.4	194 ± 43.7	181 ± 40.1	15.676 (<0.001)	2.465 (0.118)	0.237 (0.946)
Long	199 ± 36.1	243 ± 44.5	266 ± 51.8*	280 ± 56.9*	199 ± 42.1	195 ± 41.4	203 ± 38.9			
MPV (fL)										
Short	8.99 ± 0.62	9.16 ± 0.66	n/a	9.17 ± 0.63	8.79 ± 0.69	8.74 ± 0.61	8.62 ± 0.65	2.225 (0.042)	0.019 (0.890)	0.214 (0.956)
Long	8.97 ± 0.72	9.05 ± 0.62	9.15 ± 0.64	9.10 ± 0.59	8.92 ± 0.60	8.92 ± 0.57	8.60 ± 0.62			

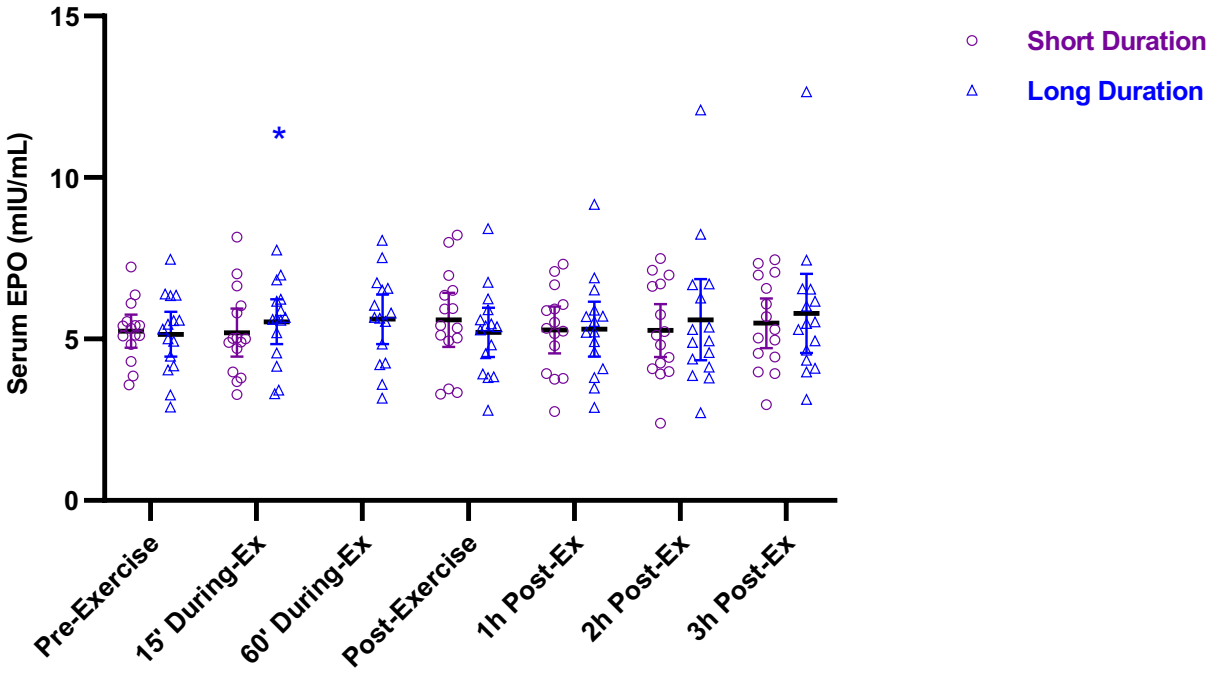

Figure 1 The serum EPO concentration (mlU/ml) at 15' mid-exercise, 60' mid-exercise, immediately post-exercise, 1-hour post exercise, 2-hours post exercise and 3-hours post exercise for the short and long exercise trials. Individual data (n=15) with mean and 95% confidence interval shown for each timepoint. * Indicates significantly different than pre-exercise values ($p<0.05$).

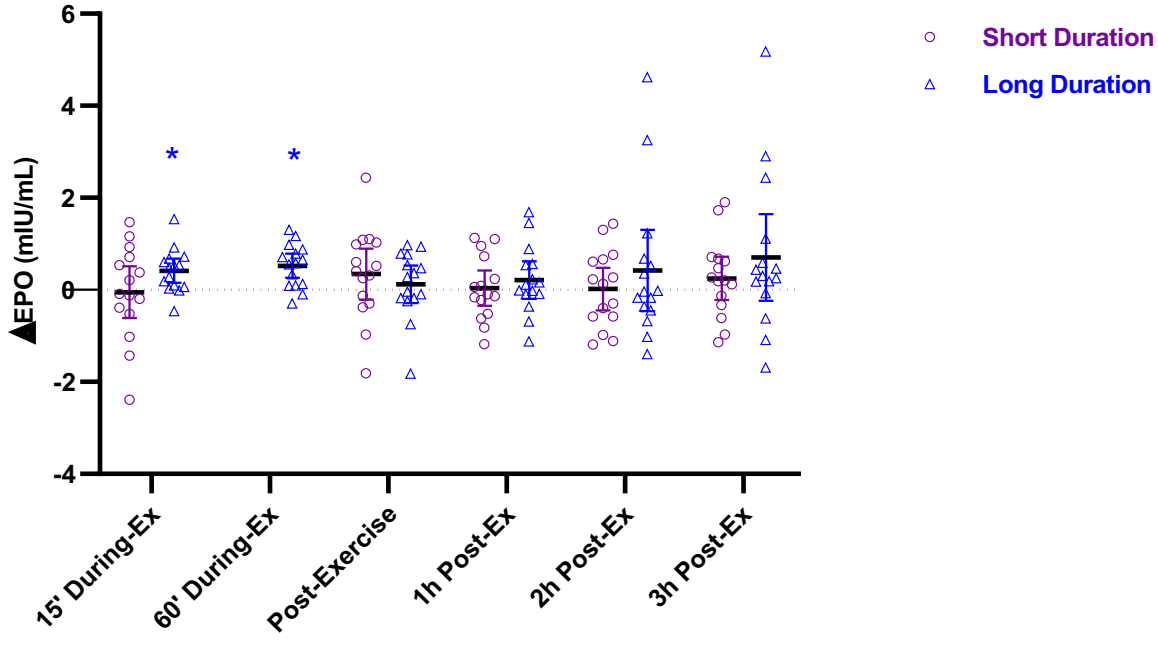

Figure 2 The change in EPO concentration (mlU/ml) from pre-exercise values at 15' mid-exercise, 60' mid-exercise, immediately post-exercise, 1-hour post exercise, 2-hours post exercise, and 3- hours post exercise for the short and long duration exercise trials. Individual data (n=15) with mean and 95% confidence interval shown for each timepoint. * Indicates significantly different than pre-exercise values ($p<0.05$).

βAR Blockade Does not Influence the EPO response to short duration, high intensity cycling exercise

The exercise measures for the βAR trials are listed in Table 3. Time, average cycling power and RPE were the same across all groups. Average HR was significantly lower during bisoprolol and nadolol trials relative to placebo, but not between drug trials (156.7 ± 12.4 vs 121.3 ± 9.2 vs 117.7 ± 10.1 bpm; $p < 0.001$), Placebo vs Bisoprolol vs Nadolol respectively. Average HR during the last 5 minutes was also significantly lower during bisoprolol and nadolol trials relative to placebo (155.6 ± 12.9 vs 121.1 ± 9.0 vs 121.3 ±9.0; $p < 0.001$), showing both a sustained effort and sustained drug effect. Accordingly, average (age predicted) percent maximum HR was significantly lower during bisoprolol and nadolol trials compared to placebo (82.8 ± 6.7 vs 64.2 ± 5.7 vs 62.3 ± 6.2%; $p < 0.001$) as well as during the last 5 minutes (80.9 ± 8.9 vs 64.0 ± 6.2 vs 64.4 ± 7.0%; $p < 0.001$), showing the relative difference was sustained. There was no statistical difference in average relative VO_2, respiratory exchange ratio (RER), or end of exercise blood lactate. The changes in hematology parameters for the βAR trials are listed in Table 4. There was a significant main effect of time on HGB [$F(2, 125) = 14.882$, $p < 0.001$], RBC [$F(2, 125) = 21.067$, $p < 0.001$], HCT [$F(2, 125) = 29.881$, $p < 0.001$], and PLT [$F(2, 125) = 14.812$, $p < 0.001$]. Pairwise comparisons reveled a significant increase in HGB in the Placebo trial post-exercise relative to 1-hour post exercise (15.84 ± 0.99 vs 14.85 ± 0.83; $p = 0.042$), in the Bisoprolol trial post-exercise relative to both pre-exercise and 1- hour post exercise (15.95 ± 1.23 vs 14.85 ± 0.99; $p = 0.017$ and 14.76 ± 1.07; $p = 0.008$, respectively) and in the Nadolol trial post-exercise relative to pre-exercise (16.11 ± 1.23 vs 14.81 ± 1.09; $p = 0.003$). There was also an increase in RBC post-exercise relative to both pre-exercise and 1-hour post exercise in all three trials (Placebo: 5.09 ± 0.23 vs 4.82 ± 0.27; $p = 0.044$ and 4.73 ± 0.25; $p = 0.040$, Bisoprolol: 5.10 ± 0.33 vs 4.75 ± 0.32; $p = 0.004$ and 4.74 ± 0.34; $p = 0.003$, Nadolol: 5.17 ± 0.26 vs 4.73 ± 0.31; $p < 0.001$ and 4.86 ± 0.31; $p = 0.014$), an increase in HCT post-exercise relative to pre-exercise and 1-hour post exercise in all three trials (Placebo: 45.1 ± 1.87 vs 42.5 ± 2.10; $p = 0.009$ and 42.1 ± 1.80; $p = 0.002$, Bisoprolol: 45.4 ± 2.70 vs 42.2 ± 2.19; $p = 0.001$ and 41.8 ± 2.56; $p < 0.001$, Nadolol: 45.9 ± 2.42 vs 41.8 ± 2.63; $p < 0.001$ and 42.9 ± 2.27; $p = 0.001$), a significant increase in PLT post-exercise in the Placebo trial post-exercise relative to both pre-exercise and 1-hour post exercise (257 ± 40.0 vs 208 ± 38.1; $p = 0.040$ and 205 ± 31.1; $p = 0.031$, respectively), in the Bisoprolol trial post-exercise relative to pre-exercise (282 ± 61.4 vs 215 ± 48.5; $p = 0.003$) and in the Nadolol trial post-exercise relative to pre-exercise (277± 53.5 vs 219 ± 43.1; $p = 0.012$), but no main effect of drug trial or interaction effects. Total serum EPO concentration over time for all participants is shown in Figure 3. There was no significant difference in the EPO response between drug trials or as a function of timepoint within the trials. The exercise induced change in serum EPO concentration is shown in Figure 4. There was no significant difference in the EPO response between drug trials or as a function of timepoint within the trials.

Table 3. Exercise measures for subjects in the βAR trials (n=14). Data are mean ±SD. P values are for main effect by group. * Indicates significantly different than placebo.

Measure	Placebo	Bisoprolol	Nadolol	p value
Duration Completed (minutes)	30	30	30	1
Cycling Power (watts)	151.8±34.8	151.8±34.8	151.8±34.8	1
RPE, Exercise End (6-20 scale)	14.4±1.7	15.5±2.4	15.5±1.9	0.268
Blood Lactate, Exercise End (mM)	2.8±1.0	3.6±1.2	2.8±0.8	0.054
Average Heart Rate (bpm)	156.7±12.4	121.3±9.2*	117.7±10.1*	<0.001
% Max Heart Rate, Trial Average (bpm)	82.8±6.7	64.2±5.7*	62.3±6.2*	<0.001
Average Heart Rate, Last 5 min (bpm)	155.6±12.9	121.1±9.0*	121.3±9.0*	<0.001
% Max Heart Rate, Last 5 min (bpm)	80.9±8.9	64.0±6.2*	64.4±7.0*	<0.001
Average Relative VO_2 (ml/min/kg)	31.9±7.3	31.8±7.6	30.9±6.1	0.910
Relative VO_2, Last 5 min (ml/min/kg)	32.5±7.1	31.5±7.5	31.2±5.9	0.863
Average Respiratory Exchange Ratio (RER)	0.91±0.03	0.92±0.03	0.94±0.04	0.146
Average RER, Last 5 min	0.90±0.03	0.91±0.03	0.93±0.04	0.056

Table 4. Hematology values for subjects in the βAR study. Data are mean ±SD. P values are for main effect by group. Statistical differences between pre-exercise indicated by *, between 1h post-exercise indicated by #, p<0.05.

	Pre-Exercise	*Post-Exercise*	*1h Post-Exercise*	*Time F (p-value)*	*Trial F (p-value)*	*Interaction F (p-value)*
HGB (g/dL)						
Placebo	14.95 ± 1.04	15.84 ± 0.99#	14.85 ± 0.83	14.882 (<0.001)	0.424 (0.655)	0.327 (0.859)
Bisoprolol	14.85 ± 0.99	15.95 ± 1.23*#	14.76 ± 1.07			
Nadolol	14.81 ± 1.09	16.11 ± 1.23*	15.21 ± 1.11			
RBC ($1x10^6$)						
Placebo	4.82 ± 0.27	5.09 ± 0.23*#	4.73 ± 0.25	21.067 (<0.001)	0.411 (0.664)	0.712 (0.585)
Bisoprolol	4.75 ± 0.32	5.10 ± 0.33*#	4.74 ± 0.34			
Nadolol	4.73 ± 0.31	5.17 ± 0.26*#	4.86 ± 0.31			
HCT (%)						
Placebo	42.5 ± 2.10	45.1 ± 1.87*#	42.1 ± 1.80	29.811 (<0.001)	0.120 (0.887)	0.550 (0.769)
Bisoprolol	42.2 ± 2.19	45.4 ± 2.70*#	41.8 ± 2.56			
Nadolol	41.8 ± 2.63	45.9 ± 2.42*#	42.9 ± 2.27			
MCV (fL)						
Placebo	88.4 ± 4.53	88.8 ± 4.44	89.1 ± 3.51	0.057 (0.944)	0.009 (0.991)	0.066 (0.992)
Bisoprolol	88.9 ± 4.28	89.1 ± 4.51	88.4 ± 4.43			
Nadolol	88.6 ± 4.58	88.9 ± 4.58	88.6 ± 4.56			
MCH (pg)						
Placebo	31.0 ± 2.12	31.1 ± 2.06	31.3 ± 1.49	0.016 (0.984)	0.030 (0.970)	0.081 (0.988)
Bisoprolol	31.2 ± 2.11	31.3 ± 2.08	31.1 ± 2.06			
Nadolol	31.3 ± 2.21	31.4 ± 2.13	31.1 ± 2.10			
MCHC (gL)						
Placebo	35.1 ± 1.12	35.1 ± 1.04	35.2 ± 0.83	0.014 (0.986)	0.264 (0.769)	0.183 (0.947)
Bisoprolol	35.1 ± 0.98	35.1 ± 0.91	35.2 ± 0.85			
Nadolol	35.4 ± 1.07	35.3 ± 0.89	35.1 ± 0.87			
RDW (%)						
Placebo	13.0 ± 0.76	13.1 ± 0.60	12.9 ± 0.45	0.243 (0.785)	0.164 (0.849)	0.086 (0.987)
Bisoprolol	13.0 ± 0.71	13.1 ± 0.60	13.1 ± 0.68			
Nadolol	13.0 ± 0.60	13.1 ± 0.57	13.0 ± 0.67			
PLT ($1x10^3$µL)						
Placebo	208 ± 38.1	257 ± 40.0*#	205 ± 31.1	14.812 (<0.001)	2.050 (0.133)	0.198 (0.939)
Bisoprolol	215 ± 48.5	282 ± 61.4*	236 ± 93.4			
Nadolol	219 ± 43.1	277 ± 53.5*	232 ± 50.2			
MPV (fL)						
Placebo	8.6 ± 0.81	8.7 ± 0.71	8.40 ± 0.78	1.316 (0.272)	0.478 (0.621)	0.039 (0.997)
Bisoprolol	8.7 ± 0.79	8.8 ± 0.80	8.60 ± 0.75			
Nadolol	8.7 ± 0.74	8.9 ± 0.68	8.60 ± 0.68			

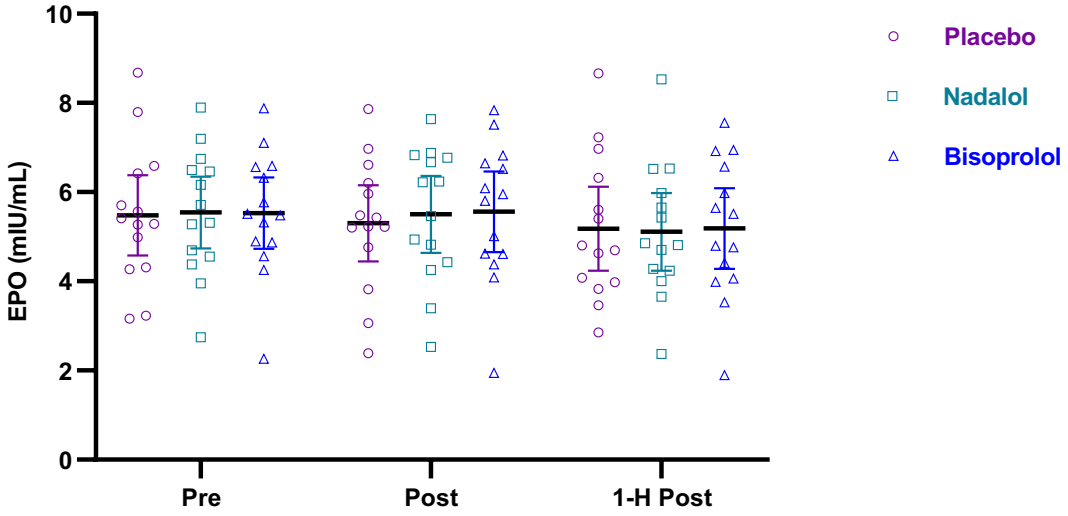

Figure 3 Total EPO concentration (mlU/ml) immediately pre exercise, immediately post exercise and 1-hour post exercise, following ingestion of bisoprolol, nadolol or placebo. Individual data (n=14) with mean and 95% confidence interval shown for each timepoint. There was no significant time or drug effect ($p<0.05$).

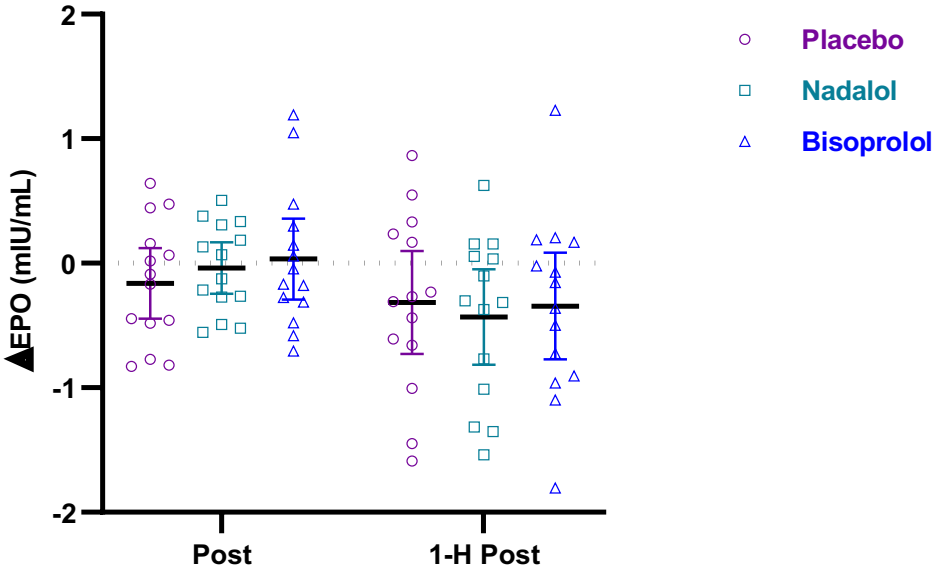

Figure 4 The change in EPO concentration (mlU/ml) immediately post exercise and 1-hour post exercise, following ingestion of bisoprolol, nadolol or placebo. Individual data (n=14) with mean and 95% confidence interval shown for each timepoint. There was no significant time or drug effect ($p<0.05$).

Discussion

Habitually endurance-trained individuals exhibit greater total hemoglobin mass and associated oxygen carrying capacity (Schmidt and Prommer 2008; Otto et al. 2013), yet efforts to demonstrate upregulation of endogenous EPO via various forms of acute, normoxic exercise have thus far been mixed (Montero and Lundby 2018). The aim of this study was to investigate the effect of exercise type, intensity adjusted duration, and β-adrenergic receptor blockade on plasma EPO concentrations in healthy human participants. The primary finding was that the initiation of 90 minutes

of moderate intensity running resulted in a significant during-exercise elevation of serum EPO levels, whereas 30 minutes of high intensity running showed no alteration in serum EPO throughout the time-period sampled (up to 3 hours post-workout). Our secondary finding was that there was no effect of 30 minutes of high intensity cycling on serum EPO with or without either β1 specific or β1 & β2 (non-specific) adrenergic receptor blockade. These results demonstrate that acute exercise in normoxic conditions evokes a modest change in serum EPO levels with long (moderate intensity), but not short (high intensity) running or cycling.

Though mixed results have been reported, our primary finding that long duration, moderate intensity running can be an effective stimulus to increase serum EPO aligns with previous research (Roecker et al., 2006; Schwandt et al., 1991), however due to the early (during exercise) EPO response observed, this may be due to the inhearant (moderate) intensity and/or mindset necessary to complete longer duration exercise and not just the exercise duration. Schwandt et al. reported an increase in serum EPO 3 hours after 37-38km (~2.5 hour) runs (Schwandt et al. 1991), Roecker et al. reported increased serum EPO in runners 3 days after a 42km marathon (Roecker et al., 2006) and Montero et al. observed a rise in serum EPO after 60 minutes of moderate intensity cycling (Montero et al. 2017). In contrast, studies using shorter (<60 minute exercise), higher intensity exercise failed to elicit an exercise induced rise in serum EPO (Bodary, Pate, Wu, & McMillan, 1999; Klausen, Breum, Fogh-Andersen, Bennett, & Hippe, 1993; Schmidt, Eckardt, Hilgendorf, Strauch, & Bauer, 1991). Two studies did observe an increase in EPO 24 hours after 3-minutes of high intensity cycling, however they were performed at simulated altitudes of 1000m and 2100m (Roberts et al. 2000; Roberts and Smith 1999). Interestingly, the studies by Roberts & Smith found no EPO response to resting hypoxic exposure via simulated altitude at similar levels of the internal hypoxemia (≤91% peripheral capillary oxygen saturation (SpO_2)) experienced during the exercise trials (Roberts et al. 2000), indicating that exercise may provide a separate EPO stimulus beyond hypoxemia alone. A more recent study by Baker & Parise using a murine model detected EPO release via exercised skeletal muscle post exercise (Baker and Parise 2016), which could explain the differential effect of altitude induced hypoxemia versus exercise induced hypoxemia (EIH).

The β-adrenergic system also plays a pivotal role in EPO regulation. Though pharmacological stimulation of β_2-adrenergic receptors (β_2AR) alone does not affect serum EPO concentration (Berglund et al. 2002), in conjunction with hypoxemia β_2AR signaling has been shown to moderate the EPO response (Gleiter et al. 1997; Fink et al. 1975). More recently it was shown that the mechanism behind this moderation involves stabilization and accumulation of the HIF-1α regulatory subunit (Cheong et al. 2016). Unfortunately, in our study using 30 minutes of high intensity cycling as the EIH stimulus, we were unable to detect any change in serum EPO with or without the use of β-antagonists. We hypothesized that selective β_1AR blockade would increase the exercise induced EPO response by diverting the exercise stimulated catecholamines from β_1AR to β_2AR, heightening β_2AR activation, but no such response was observed. However, because no EPO response was observed with the 30 minutes of high intensity running or cycling alone, we may not have achieved the exercise conditions necessary to show an effect of β_2 adrenergic moderation. In this respect, it would be valuable for future studies to evaluate the effect of beta blockade with 90 minutes of moderate intensity running since this was shown to be an effective EPO stimulus.

The value of naturally increasing EPO and associated hemoglobin mass in endurance athletes is clear, but EPO has also shown clinical promise as an adjuvant treatment for a wide range of diseases (Sanchis-Gomar et al. 2014; Sanchis-Gomar et al. 2013). In this study, we observed a novel increase in serum EPO during moderate, but not high intensity exercise. And although this data supports our hypothesis that longer duration, moderate intensity exercise bout would result in greater serum EPO levels relative to shorter, higher intensity exercise, the timing of the EPO elevation (i.e., during exercise) suggests that the intensity and not the duration may be the critical aspect in this case. And because long duration exercise is inherently moderate in intensity (or easier), as high intensity endurance exercise cannot be sustained for extended periods, the efficacy of shorter bouts of moderate intensity exercise should be explored. Furthermore, the increase in EPO observed in this study was modest in relation to altitude research, but was in line with Schwandt et al., who observed a +9.9% average increase in serum EPO 3 hours post-run, relative to the +8.7% average increase at 60 minutes into exercise observed here, however the physiological significance of these values is unknown.

We acknowledge several limitations of our study. Firstly, our results showed increased serum EPO levels response to moderate intensity running, but we did not examine this in relation to moderate intensity cycling. There is evidence that running creates greater exercise induced hypoxemia (EIH) than cycling due to a greater central physiological load (Rice et al. 2000), so it is plausible that it would prove a better EPO stimulus, but beyond the work by Montero et al., there are limited data on longer duration, moderate intensity normoxic cycling on serum EPO. Secondly, our study

design involved long and short duration running performed at different intensities. This was done to match the exercise bouts for overall training stress, with the assumption that higher overall training stress would produce a higher EPO response and that the higher the exercise intensity, the shorter the sustainability. Furthermore, because this study was part of larger parent study (Agha et al. 2018), our sampling protocol concluded at 1 hour post cycling and 3 hours post running, yet an elevation of EPO has been detected as long as 3 days post stimulus (Roecker et al., 2006), so it is conceivable that there may have been a rise in EPO beyond the sampling timeframe. In addition, although it is still unknown if there is a threshold blood oxygen desaturation necessary to stimulate an EPO response, Roberts and Smith have suggested a requisite threshold of <91% SPO_2 (Roberts and Smith 1999) and more recent studies using simulated altitude have demonstrated a direct dose-response relationship between blood oxygen desaturation and EPO response (Torpel et al. 2019). Therefore, another limitation of our study is that we did not collect SPO_2 data and could not evaluate the exercise dose via exercise-induced desaturation level. Along these lines, recent data by Roberts and Smith also shows high individual variation in desaturation during normoxic exercise (Gaston et al. 2016), so no assessment could be made in terms of EIH "responders" vs "non-responders" to better evaluate any EIH dose-response relationship, which may prove valuable in future studies.

In conclusion, the main finding in this study was that 90 minutes of moderate intensity running elevated serum EPO levels during prolonged exercise, but no change in EPO was observed in response to 30 minutes of high intensity running or 30 minutes of high intensity cycling with or without beta blockade. Moreover, because the beta blockade trials concluded at 30 minutes and EPO upregulation was only seen in the long duration (90 minute) trial, longer duration βAR trials are needed to further evaluate the β-adrenergic mediation of EPO upregulation via exercise. Notwithstanding, the exercise dependent and transient EPO elevation observed in this study may explain historically inconsistent findings regarding the effect of exercise on serum EPO, as well as open new avenues of study towards prescription of exercise to maximize EPO response for both performance and clinical applications.

Compliance with ethical standards and acknowledgements
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