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Blood flow restricted resistance exercise and reductions in oxygen tension attenuate mitochondrial H_2O_2 emission rates in human skeletal muscle

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Key Points

- Blood flow restricted resistance exercise (BFR-RE) is capable of inducing comparable adaptations to traditional resistance exercise (RE), despite a lower total exercise volume.
- It has been suggested that an increase in reactive oxygen species (ROS) production may be involved in this response, however oxygen partial pressure (pO_2) is reduced during BFR-RE, and the influence of pO_2 on mitochondrial redox balance remains poorly understood.
- In human skeletal muscle tissue, we demonstrate that both maximal and submaximal mitochondrial ROS emission rates are acutely decreased 2 hours following BFR-RE, but not RE, occurring along with a reduction in tissue oxygenation during BFR-RE.
- We further suggest that pO_2 is involved in this response, as *in vitro* analysis revealed that reducing pO_2 dramatically decreased mitochondrial ROS emissions and electron leak to ROS.
- Altogether, these data indicate that mitochondrial ROS emission rates are attenuated following BFR-RE, a response which is likely influenced by reductions in pO_2.

Author Profile

Heather Petrick completed her BSc in Biomedical Science at the University of Guelph in 2017, and is currently an MSc student in the University of Guelph Human Health and Nutritional Science Department. She is pursuing a PhD in the same laboratory, where her research will focus on understanding the regulation of mitochondrial bioenergetics in response to various dietary and exercise interventions.

Abstract

Low-load blood flow restricted resistance exercise (BFR-RE) training has been proposed to induce comparable adaptations to traditional resistance exercise (RE) training, however the acute signaling events remain unknown. While a suggested mechanism of BFR-RE is an increase in reactive oxygen species (ROS) production, oxygen partial pressure (pO_2) is reduced during BFR-RE, and the influence of O_2 tension on mitochondrial redox balance remains ambiguous. We therefore aimed to determine if skeletal muscle mitochondrial bioenergetics were altered following an acute bout of BFR-RE or RE, and to further examine the role of pO_2 in this response. To study this, muscle biopsies were obtained from 10 males at rest and 2 hours after performing 3 sets of single-leg squats (RE or BFR-RE) to failure at 30% 1-RM. We determined that mitochondrial respiratory capacity and ADP sensitivity were not altered in response to RE or BFR-RE. While maximal (succinate) and submaximal (non-saturating ADP) mitochondrial ROS emission rates were unchanged following RE, BFR-RE attenuated these responses ~30% compared to pre-exercise, occurring along with a reduction in skeletal muscle tissue oxygenation during BFR-RE ($p<0.01$ vs. RE). In a separate cohort of participants, evaluation of mitochondrial bioenergetics *in vitro* revealed that mild O_2 restriction (50μM) dramatically attenuated maximal (~4-fold) and submaximal (~50-fold) mitochondrial ROS emission rates and the fraction of electron leak to ROS compared to room air (200μM). Combined, these data demonstrate that mitochondrial ROS emissions are attenuated following BFR-RE, a response which may be mediated by a reduction in skeletal muscle pO_2.

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Abbreviations: 1-RM, 1-repetition maximum; ADP, adenosine diphosphate; AMPK, adenosine monophosphate-activated protein kinase; BFR-RE, blood flow restricted resistance exercise; ETC, electron transport chain; Hb, hemoglobin; H_2O_2, hydrogen peroxide; JO_2, mitochondrial O_2 flux; K_m, Michaelis-Menten constant; LOP, lowest effective occlusive pressure; NIRS, near-infrared spectroscopy; Mb, myoglobin; O_2, oxygen; pO_2, oxygen partial pressure; RCR, respiratory control ratio; ROS, reactive oxygen species; RE, resistance exercise; TSI, tissue saturation index.

Introduction

While high-load resistance exercise (RE) training is known to promote skeletal muscle hypertrophy (Tesch, 1988) and mitochondrial adaptations (Pesta *et al.*, 2011; Porter *et al.*, 2015; Holloway *et al.*, 2018*a*), evidence now suggests that RE in the presence of blood flow restriction (BFR-RE) is capable of inducing comparable metabolic outcomes, despite a lower volume of work (Farup *et al.*, 2015; Groennebaek *et al.*, 2018). While this well-found notion suggests that ischemia induced by BFR-RE is sufficient to augment the metabolic perturbations of exercise, the mechanisms underlying cellular adaptations to RE and BFR-RE remain poorly understood, particularly when both are performed to repetition failure.

As a traditional metabolic event associated with aerobic exercise, activation of AMPK in response to reductions in energy status is known to regulate mitochondrial ATP production, substrate breakdown, and the exercise-induced increase in mitochondrial content (Hawley, 2009). However, since AMPK is capable of inhibiting anabolic pathways (Hawley, 2009), acute RE is suggested to be a less potent activator of AMPK compared to aerobic exercise (Atherton *et al.*, 2005; Lundberg *et al.*, 2014), and recent evidence suggests that a greater increase in AMPK phosphorylation is not present following BFR-RE performed to repetition failure (Groennebaek *et al.,* 2018). As a result, alternative cellular mechanisms are likely involved in mitochondrial adaptations to RE and BFR-RE. One such event which may be implicated in this response is the acute exercise-mediated regulation of skeletal muscle mitochondrial bioenergetics. Specifically, it is well established that mitochondrial sensitivity to ADP is acutely impaired in response to aerobic exercise (Perry *et al.*, 2012; Dohlmann *et al.*, 2018; Barbeau *et al.*, 2018; Petrick & Holloway, 2019), an effect which appears required for cellular adaptations to occur (Miotto & Holloway, 2018). As a closely linked control point (Holloway, 2017), evidence also suggests that an increase in mitochondrial reactive oxygen species (ROS) emission rates in the presence of physiological ADP concentrations occurs acutely in response to aerobic exercise (Barbeau *et al.*, 2018; Miotto & Holloway, 2018). While it has

been speculated that ROS production is an important mechanism involved in the acute responses to BFR-RE, direct evidence in skeletal muscle is lacking, as previous studies have mainly examined plasma markers of redox balance (Takarada *et al.*, 2000; Goldfarb *et al.*, 2008; Neto *et al.*, 2017). However, in support of a potential role of mitochondrial-specific ROS emissions in mediating the training responses to BFR-RE, temporary occlusion induced by BFR is known to decrease tissue oxygenation (Ganesan *et al.*, 2015), which could influence mitochondrial bioenergetics, especially since decreasing O_2 availability renders the ETC overly reduced and optimal for superoxide production (Chance & Williams, 1955; Clanton, 2007). Altogether, it appears plausible that alterations in mitochondrial ROS production may be implicated in the acute responses to BFR-RE and RE, however, to date, this remains to be directly determined.

Therefore, in the present study, we aimed to examine if mitochondrial respiration and ROS emission rates were altered in permeabilized muscle fibers following an acute bout of BFR-RE or RE performed to failure. To further examine the influence of pO_2 on mitochondrial bioenergetics *in vitro*, we simultaneously measured mitochondrial respiration and ROS emission rates in the presence of room air (~200μM O_2) and mild O_2 restriction (~50μM O_2). We provide evidence that both an acute bout of BFR-RE and *in vitro* O_2 restriction attenuated skeletal muscle mitochondrial ROS emission rates, suggesting that a reduction in skeletal muscle pO_2 may be an important mechanism influencing ROS emissions, and subsequent metabolic adaptations, in response to BFR-RE.

Methods

Participants

Ten healthy males (24±1 y, 1.77±0.01 m, 78±6 kg) were recruited to examine the acute responses to BFR-RE and RE, while a second subset of 6 healthy males (25±2 y, 1.86±0.04 m, 76±4 kg) were

recruited to determine the *in vitro* effect of O_2 tension on mitochondrial bioenergetics. Prior to enrollment in the study, participants completed health questionnaires to determine eligibility. The experimental procedures and risks were thoroughly explained, and written informed consent was given by each participant as approved by the Ethics Committee at the University of Guelph (REB#17-12-005) or the Medical Ethical Committee of Maastricht University^{+} (METC#153010) in accordance with the Declaration of Helsinki.

Mitochondrial bioenergetic responses to resistance exercise

Study design

To control for potential between-subject confounding factors, a within-person, unilateral model was used to investigate the acute responses of non-occluded RE and occluded BFR-RE single-leg squats. Participants arrived to the laboratory for all visits having refrained from lower body exercise for 72 hours prior, alcohol consumption for 24 hours prior, and food and caffeine for 12 hours prior to all measurements. At least 3 days before the experimental day, participants' 1-repetition maximum (1-RM) was determined on each leg using a Smith Machine. The leg not performing exercise was supported by a band and the participants were instructed to refrain from contacting the ground or applying force into the band during each repetition. The load was progressively added based on approximately 20% of participant's body mass and each attempt was separated by 3-5 minutes. Once a failed attempt occurred, the load was decreased by 10% body mass. If this was/was not made, 2 kg was added/decreased each repetition until participants successfully reached their 1-RM.

On the acute exercise day, a single resting skeletal muscle biopsy (*m. vastus lateralis)* was performed between 7:00-8:00 hours using the Bergström technique, in a randomized manner (RE or BFR-RE leg) for each participant. The sample was immediately placed in ice-cold BIOPS (50mM MES, 7.23mM K_2EGTA, 2.77mM CaK_2EGTA, 20mM imidazole, 0.5mM dithiothreitol, 20mM taurine,

5.77mM ATP, 15mM PCr, 6.56mM $MgCl_2 \cdot H_2O$; pH 7.1) preservation buffer for preparation of permeabilized muscle fibers for mitochondrial bioenergetic experiments. After the resting muscle biopsy was obtained, participants conducted a warm-up on a treadmill for 5-10 minutes at a self-directed pace. Legs were randomized to RE or BFR-RE, and 3 sets of single-leg squats to repetition failure were performed at 30% 1-RM, with 100 seconds recovery between sets, and a 5-10 minute rest before performing repetitions on the second leg. The lowest effective occlusive pressure (LOP), defined as the lowest pressure required to occlude arterial blood flow (Masri *et al.,* 2016), was determined for the occluded leg using the personalized tourniquet system containing LOP calculation software (11cm cuff, Delfi Inc., Vancouver, Canada) placed on the upper thigh while the participants remained seated. Pressure was set to 60-70% LOP (151±6mmHg). Following completion of both legs, participants consumed a small granola bar (100 calories; 23% energy as fat, 72% energy as carbohydrate, 5% energy as protein), and remained sedentary for 2 hours before a muscle biopsy was taken on each leg and immediately placed in ice-cold BIOPS preservation buffer for mitochondrial bioenergetic experiments.

Preparation of permeabilized muscle fibers and assessment of mitochondrial bioenergetics

Vastus lateralis muscle samples in ice-cold BIOPS were trimmed of non-muscle tissue, separated with fine-tipped forceps under a microscope, and incubated in saponin prior to performing mitochondrial respiration experiments using high-resolution respirometry (Oroboros Oxygraph-2K: Oroboros Instruments, Innsbruck, Austria), as previously described (Barbeau *et al.*, 2018; Miotto & Holloway, 2018). Briefly, ADP was titrated (concentrations of 25, 100, 175, 250, 500, 1000, 2000, 4000, 6000, 8000, 10000, 12000 µM ADP) in the presence of 5mM pyruvate and 1mM malate, and 10mM glutamate and 10mM succinate were sequentially added following ADP titrations to determine maximal complex I and complex I/II-linked respiration. 10µM cytochrome c was added at the end of all experiments, and an increase in respiration less than 10% was used to confirm the

integrity of the mitochondrial membrane. Respiratory control ratios (RCR) were calculated as the ratio of pyruvate-malate supported respiration in the presence and absence of ADP to demonstrate the mitochondrial coupling. Mitochondrial ROS emission rates were determined in permeabilized muscle fibers by measuring the rate of H_2O_2 release using Amplex Red fluorescence quantification (Invitrogen, Carlsbad, CA, USA) at 37ºC, as previously described (Barbeau *et al.*, 2018). 5µM blebbistatin was added to all experiments to inhibit myosin ATPase, which has previously been shown to be more indicative of the *in vivo* environment when modelling ADP kinetics (Perry *et al.,* 2011). After completion of experiments, fiber bundles were recovered and freeze-dried to normalize all data to fiber weight.

Western blotting

Freeze-dried muscle fibers were digested in a lysis buffer containing 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS in 62.5mM Tris-HCl, and 0.01% bromophenol blue, for 1hr at 65ºC with gentle shaking (Barbeau *et al.*, 2018). Samples were vortexed briefly every 15 minutes to improve digestion, and 5µL of digested lysate was then loaded onto SDS-polyacrylamide gels for Western blotting protein quantification. Proteins were separated by electrophoresis at 150V for 1 hour, and transferred at 100V for 1 hour to polyvinylidene difluoride membranes. Commercially available antibodies were used to detect COXIV (Invitrogen-A21347), OXPHOS (Mitosciences-ab110413), VDAC (Abcam-ab14734), Mi-CK (Abcam-ab131188), SOD2 (Abcam-ab13533) and α-tubulin (Abcam-ab7291). All samples for each protein were transferred on the same membrane to limit variability, and quantified via chemiluminescence using a FluorChem HD imaging system (Alpha Innotech, Santa Clara, US).

Near-infrared spectroscopy (NIRS)

A NIRS device (PortaMon, Artinis, The Netherlands) was used to estimate muscle oxygenation by measuring the concentration of oxygenated and deoxygenated hemoglobin (Hb) and myoglobin (Mb). The device was placed on the vastus lateralis at approximately 50% femur length from the lateral epicondyle and greater trochanter, and measured at a penetration depth of ~2.5 cm (McManus *et al.,* 2018). The NIRS was secured and protected from outside light, and the location was marked pre-exercise to ensure minimal movement of the device during exercise. Participants remained upright with minimal movement during baseline and rest periods, and exercise was performed as explained above. Tissue saturation index (TSI) was calculated by the equation:

$$\text{Tissue Saturation Index (TSI)} = \frac{\text{Oxy(Hb + Mb)}}{\text{Oxy(Hb + Mb) + Deoxy(Hb + Mb)}} \times 100\ \%$$

In vitro assessment of mitochondrial bioenergetics in response to O_2 restriction

Study design

In a second subset of experiments, we examined the impact of pO_2 on mitochondrial bioenergetics using skeletal muscle biopsies obtained from 6 individuals who participated in a larger study (Nederlands Trial Register #NTR5111) examining the impact of protein ingestion on responses to exercise. However, importantly, all analysis in the current study was performed prior to participants receiving their nutritional interventions. On the evening before the experimental trial, participants consumed a pre-packaged standardized meal containing 55% energy as carbohydrate, 30% as fat, and 15% as protein before 20:00 hours, after which they remained fasted. The following morning a single muscle biopsy sample was collected from the *m. vastus lateralis* using the Bergström technique following 90 minutes of exercise (60% W_{max}) on a cycle ergometer. The collected muscle tissue was freed from any visible blood and non-muscle tissue, and rapidly placed in BIOPS preservation buffer for analysis of mitochondrial bioenergetics.

Mitochondrial bioenergetic analysis

O_2 consumption and H_2O_2 emission were simultaneously determined in Buffer Z using an Oxygraph-2K with a fluorometry modular attachment (Oroboros Instruments) as previously reported (Holloway *et al.*, 2018*a*). However, in the present study pO_2 was fluctuated between room air (~200μM O_2, ~150mmHg) and O_2 restriction (~50μM O_2, ~40mmHg) by lifting the stopper and rapidly injecting 100% nitrogen (to decrease O_2 concentration) or 100% O_2 gas (to increase O_2 concentration back to room air conditions). The rate of H_2O_2 emission was calculated from a standard curve of known concentrations of H_2O_2 established with the same reaction conditions in room air saturation using DatLab software (Oroboros Instruments, Innsbruck, Austria) after subtracting fiber background. As standard curves performed in room air and O_2 restriction were highly correlated (R^2=0.9997), all data was analyzed using a standard curve generated at room air saturation. Oxygen consumption (JO_2) was calibrated to a standard curve performed at a range of O_2 concentrations (0μM – 200μM O_2). All fibers were weighed in Buffer Z before the experiments to normalize data to fiber wet weight.

Statistics

All data are presented as means±SEM. Repeated-measures ANOVA was used for exercise volume and NIRS data comparing RE and BFR-RE (group) at each set/timepoint (time) with Bonferroni multiple comparison post-hoc analysis. Paired-samples Student's t-tests were carried out at similar timepoints between RE and BFR-RE. Repeated-measures ANOVA was used to compare mitochondrial bioenergetics between rest, RE, and BFR-RE legs with Fisher's least significant difference post-hoc analysis. ADP titrations were analyzed using Michaelis-Menten kinetics with a constraint of 100 in Prism 8 (GraphPad Inc., La Jolla, CA, USA). The influence of pO_2 was analyzed using a two-tailed paired-samples Student's t-test in Prism 8. Statistical significance for all data was set at p<0.05.

Results

Acute resistance exercise

Participant's single-leg 1-RM was similar between legs randomly assigned to RE (76.6±5.5 kg) and BFR-RE (75.2±5.2 kg). Therefore, during the acute exercise bout, the load corresponding to 30% 1-RM was comparable between legs. However, BFR caused a reduction in the number of repetitions performed, and therefore the total exercise volume (load × repetitions) was ~50% lower while performing BFR-RE (Figure 1). This study design was specifically chosen as exercise to failure appears instrumental in the metabolic adaptations to RE (Burd *et al.*, 2012*b*; Farup *et al.*, 2015; Holloway *et al.*, 2018*b*). As fatigue occurs earlier when conducting BFR-RE, a work-matched study design may therefore compromise the metabolic stimulus of traditional RE and prevent a direct comparison between associated acute signaling events.

Mitochondrial protein content and function in response to RE and BFR-RE

On a cellular level, we aimed to determine if RE and BFR-RE acutely influenced mitochondrial bioenergetics. We first characterized markers of mitochondrial content and function, and established there were no differences in the content of mitochondrial ETC proteins (CI, CII, CIII, CV; COXIV), proteins involved in ADP transport (VDAC, MiCK), or antioxidant proteins (SOD2) (Figure 2A,B) before or 2 hours after acute RE or BFR-RE. Functionally, RE and BFR-RE did not alter mitochondrial respiratory capacity in the presence or absence of maximal ADP (10mM ADP), or various substrates (glutamate, succinate), providing further evidence mitochondrial respiratory capacity was not different between legs, or following resistance exercise (Figure 2C).

Mitochondrial ADP sensitivity in response to RE and BFR-RE

As moderate (Perry *et al.*, 2012) and high (Dohlmann *et al.*, 2018) intensity aerobic exercise are known to acutely impair mitochondrial ADP sensitivity in human permeabilized muscle fibers, we examined if resistance exercise, with or without occlusion, influenced this response. However, ADP sensitivity was not altered by RE or BFR-RE (Apparent ADP K_m = 1138µM and 1134µM ADP, respectively), compared to pre-exercise (Apparent ADP K_m = 1038µM ADP) (Figure 2D,E).

Mitochondrial ROS emissions in response to RE and BFR-RE

Given the role of ROS in acute exercise-mediated signaling events, we examined mitochondrial H_2O_2 emission rates following RE and BFR-RE. While maximal succinate-supported H_2O_2 emission rates were not altered in response to RE (79.6 pmol min^{-1} mg dry wt^{-1}), mitochondrial H_2O_2 emission was attenuated ~25% from pre-exercise levels following BFR-RE (65.5 pmol min^{-1} mg dry wt^{-1}, p=0.01) (Figure 3A). A similar response was evident with respect to submaximal H_2O_2 emission in the presence of 100µM ADP, as rates were decreased ~35% following BFR-RE compared to pre-exercise (p=0.03). In contrast to the changes in absolute rates of mitochondrial ROS emission, the capacity of 100µM ADP to suppress maximal H_2O_2 emission rates was not influenced by RE or BFR-RE compared to pre-exercise (Figure 3C).

Skeletal muscle O_2 saturation during RE and BFR-RE

Since O_2 is required for the production of mitochondrial ROS, we next aimed to determine the influence of BFR-RE on skeletal muscle O_2 saturation as a potential factor mediating the observed reduction in H_2O_2 emission rates. To achieve this, participants returned to the laboratory 7-14 days after the initial acute experimental day to estimate muscle oxygenation during identical squats to

repetition failure. This approach revealed that tissue saturation index (TSI) was decreased during all RE and BFR-RE sets compared to pre-exercise; however, the reduction in TSI was more pronounced during BFR-RE compared to RE sets (Figures 4A,B). Moreover, during the recovery periods between sets, TSI returned to baseline levels in the RE leg (Figure 4A,F), while it remained decreased throughout the rest period in the BFR-RE leg (BFR-RE: 46.1±2.0 vs. RE: 60.6±1.1%, $p<0.01$) until cuff release following the third set (Figure 4B,F). This reduction in TSI was driven by ~25% decrease in oxygenated Hb and ~35% increase in deoxygenated Hb, in the absence of changes in total Hb content (Figures 4C-E). Combined, these data suggest that BFR-RE decreased skeletal muscle O_2 saturation throughout the duration of the exercise protocol.

Influence of pO_2 on mitochondrial respiration and H_2O_2 emissions

As tissue oxygenation was consistently lower during BFR-RE, and H_2O_2 emission rates were reduced following BFR-RE, we next aimed to specifically examine the role of pO_2 on mitochondrial bioenergetics *in vitro,* while removing any other confounding factors present during exercise (e.g. hypercapnia). In muscle biopsy samples obtained from a second subset of participants (n=6), we first determined the concentration of O_2 that limited respiration in human permeabilized muscle fibers. To achieve this, maximal respiration (10mM ADP) was initiated at room air saturation (~200μM O_2, 21% O_2) in the presence of pyruvate and malate and respiration was monitored until anoxia (Figure 5A), which leads to a progressive decline in mitochondrial respiration rate. Maximal ADP-stimulated respiration was stable (>95% of maximal respiration) above ~125μM O_2, while a mild impairment in respiration (~30% inhibition) was observed at ~50μM O_2 (Figure 5B). Therefore, to determine the influence of pO_2 on mitochondrial bioenergetics, we simultaneously analysed maximal H_2O_2 emission rates (pyruvate+malate+succinate; absence of ADP), submaximal respiration/H_2O_2 emission (+100μM ADP) and maximal respiration (+10mM ADP) in the presence of room air (~200μM O_2) and mild O_2 restriction (~50μM O_2) (Figure 5C).

With respect to mitochondrial respiratory function, mild O_2 restriction attenuated leak respiration in the absence of ADP (P+M+S) and maximal ADP-stimulated respiration (10mM ADP) by ~50% (Figure 6A). As a result of the similar reductions in each respiratory state, the RCR was not altered by mild O_2 restriction (Figure 6A). In addition, there was a trend ($p=0.06$) for O_2 restriction to reduce submaximal respiration in the presence of 100μM ADP (~25%), a concentration that models the skeletal muscle microenvironment during exercise conditions (Phillips *et al.*, 1996). To ensure repeatability of oxygen consumption (JO_2) measurements with rapid fluctuations in pO_2, submaximal respiration was monitored in separate experiments with repeated cycles of mild O_2 restriction and room air saturation, and the coefficient of variation for both pO_2 values was determined to be <10% (room air: 8±1%, mild O_2 restriction: 7±2%). This suggests that the absence of changes in submaximal ADP-supported respiration with decreased pO_2 was not an artifact of initially stimulating respiration in the presence of ADP while exposed to O_2 restricted conditions.

We next examined the influence of reducing O_2 availability on rates of mitochondrial H_2O_2 emission. Mildly decreasing pO_2 markedly attenuated both maximal succinate-supported mitochondrial H_2O_2 emission rates and submaximal H_2O_2 emission (100μM ADP) (Figure 6B). However, this reduction was more pronounced in the presence of submaximal ADP concentrations, and therefore the ability of 100μM ADP to supress mitochondrial H_2O_2 emission was ~4-fold greater when O_2 was restricted compared to room air (Figure 6C). While reducing pO_2 decreased both respiration and ROS emission rates, this effect was far greater with respect to ROS, and as a result, the fraction of electron leak to ROS was decreased by O_2 restriction (Figure 6D). This was most pronounced when exercising free ADP concentrations were present (100μM ADP), as the fraction of electron leak to ROS during O_2 restriction was almost non-detectable in this situation (<0.02%).

Discussion

In the present study, we established that acute RE and BFR-RE did not alter maximal respiratory capacity or mitochondrial sensitivity to ADP in human permeabilized muscle fibers. In contrast, maximal and submaximal mitochondrial H_2O_2 emission rates in the presence of room air conditions were attenuated following BFR-RE, but not RE, suggesting prolonged changes within the ETC following BFR-RE. Since BFR-RE decreased skeletal muscle oxygen saturation, we also examined the effects of mild reductions in pO_2 on mitochondrial bioenergetics *in vitro*, which revealed an attenuation in leak respiration, maximal respiration, and mitochondrial H_2O_2 emission rates in human permeabilized muscle fibers. Importantly, the effect was far more pronounced with respect to mitochondrial H_2O_2 emission, and therefore the fraction of electron leak to ROS was dramatically reduced during mild O_2 restriction. Altogether, these data suggest that the capacity for mitochondrial-derived ROS is acutely decreased in human skeletal muscle following BFR-RE, and the reductions in pO_2 during BFR-RE may contribute to this response *in vivo*.

Mitochondrial bioenergetic responses to RE and BFR-RE

As resistance exercise with or without blood flow restriction does not elicit a degree of AMPK activation classically associated with aerobic exercise (Groennebaek *et al.,* 2018), alternative mechanisms must be involved in the cellular adaptations to resistance exercise. A well characterized AMPK-independent (Miotto *et al.,* 2017) response to both low (Perry *et al.*, 2012; Barbeau *et al.*, 2018; Miotto & Holloway, 2018) and high (Dohlmann *et al.*, 2018; Petrick & Holloway, 2019) intensity aerobic exercise, in both rodents and humans, is an impairment in mitochondrial ADP sensitivity. Recent evidence in mice suggests this response is necessary for the acute exercise-mediated increase in PGC-1α mRNA expression, and as a result, the chronic mitochondrial biogenic effects of aerobic exercise (Miotto & Holloway, 2018). As similar mitochondrial adaptations have recently been observed following RE and BFR-RE training (Pesta *et al.*, 2011; Farup *et al.*, 2015; Groennebaek *et al.*, 2018), and as acute RE increases skeletal muscle PGC-1α mRNA content (Burd *et*

al., 2012*a*; Ruas *et al.*, 2012) and cellular metabolites indicative of metabolic stress (increased lactate, and reductions in ATP, glycogen, and creatine phosphate) (Tesch *et al.*, 1986; Dudley, 1988), we speculated mitochondrial ADP sensitivity would also be impaired in these situations. However, neither RE nor BFR-RE altered mitochondrial sensitivity to ADP when directly compared between both types of exercise performed to failure. This finding may be due to the brief nature of RE (2-3 minutes total exercise work over 3 sets), while previous research utilizing aerobic exercise was significantly more prolonged (30-120 minutes in duration) (Perry *et al.*, 2012; Dohlmann *et al.*, 2018; Barbeau *et al.*, 2018; Miotto & Holloway, 2018). As muscle time under tension appears important in the hypertrophic effects of RE (Burd *et al.*, 2012*a*), it is also likely that this may influence mitochondrial adaptations to similar types of exercise. While biopsy samples in the current study were obtained 2 hours following exercise, this is not expected to alter the exercise-mediated regulation of mitochondrial ADP sensitivity, as it has previously been shown that moderate-intensity cycling impairs mitochondrial ADP sensitivity for at least 3 hours post-exercise (Perry *et al.*, 2012). However, it remains possible that the effects of RE and BFR-RE on mitochondrial ADP sensitivity are more transient in nature, and the acute regulation of ADP sensitivity was not maintained 2 hours post-exercise.

Several lines of evidence also suggest the ability of ADP to supress mitochondrial H_2O_2 emission rates is closely linked with the impairment in ADP sensitivity following exercise (Miotto & Holloway, 2018), particularly as exercise-mediated reductions in ADP sensitivity are absent in mice with attenuated mitochondrial ROS production (Barbeau *et al.*, 2018). Consistent with this closely linked relationship, in the current study, along with the absence of changes in mitochondrial ADP sensitivity, we observed no differences in the ability of ADP to supress mitochondrial H_2O_2 emission rates. Similar to previous findings in response to aerobic exercise (Barbeau *et al.*, 2018; Miotto & Holloway, 2018), we demonstrate that maximal H_2O_2 production following RE was comparable to pre-exercise. Interestingly, however, maximal (succinate-supported) and submaximal (presence of 100μM ADP) H_2O_2 emission rates were lower in response to BFR-RE. As ROS is proposed to be an

important cellular event mediating responses to exercise (Davies *et al.*, 1982), it remains possible that chronic training-induced adaptations are blunted following BFR-RE compared to RE when performed to fatigue, as opposed to matched for low exercise volume. We should acknowledge that participants consumed a granola bar immediately after the cessation of the exercise protocol to better model nutritional approaches utilized outside the laboratory setting. While unknown, given the low caloric intake (100 kcal), and the single leg model employed (i.e. both legs exposed to the same circulation), the consumption of this granola bar is unlikely to have confounded our interpretation that BFR-RE reduces mitochondrial ROS.

As the decline in mitochondrial ROS emission rates persisted at a 2 hour timepoint following an acute bout of BFR-RE, it is possible that prolonged, post-translational modifications or changes within ROS-producing sites of the electron transport chain occurred. Given the reduction in tissue oxygen saturation at all time points during and between sets in the BFR-RE leg compared to RE leg, we speculated the decrease in pO_2 could be an acute event contributing to this response, and as a result, the attenuated mitochondrial ROS emissions with BFR-RE. However, it remains unknown if this is a direct result of a change within the ETC, or occurred secondary to changes in metabolism, ventilation, or blood flow during exercise.

In vitro assessment of mitochondrial bioenergetics in response to O_2 restriction

As tissue oxygen saturation was decreased during BFR-RE, we also aimed to examine the influence of decreased pO_2 on mitochondrial ROS emissions. Specifically, we evaluated mitochondrial bioenergetics *in vitro* in the absence of other confounding factors that may play a role during exercise. This approach revealed that mild O_2 restriction decreased mitochondrial ROS emission rates in human skeletal muscle compared to room air. As the role of pO_2 in mitochondrial redox balance is conflicting, our findings of a decrease in ROS production support previous work in isolated

mitochondria from rat livers (Hoffman *et al.*, 2007), however they are in contrast to other findings in isolated cell lines in which a reduction in O_2 increased H_2O_2 production and induced oxidative stress (Duranteau *et al.*, 1998; Chandel *et al.*, 2000). The ambiguity in the ability of O_2 to influence mitochondrial bioenergetics arises as O_2 exerts an obligatory role in regulating both oxidative phosphorylation and ROS production. Therefore, as electron flux through the ETC, and thus aerobic respiration, is simultaneously reduced in the presence of lower pO_2, any absolute changes in mitochondrial ROS production may not be representative of relative proportions of electron leak towards ROS. This therefore may reflect a different reducing potential and O_2 affinity of proximal sites within the ETC implicated in ROS production, and the terminal site (Complex IV) mediating aerobic respiration.

Our data supports this supposition, as while the effect of mild O_2 restriction on attenuating mitochondrial H_2O_2 emission rates was extremely pronounced, we only observed a moderate decrease in mitochondrial respiration. Interestingly, while maximal respiration was impaired with mild O_2 restriction, submaximal ADP-stimulated respiration (100μM ADP) was minimally decreased, suggesting that a reduction in pO_2 would only compromise aerobic respiration to a minor extent in the presence of ADP concentrations representative of the skeletal muscle microenvironment. As a result of these mild respiratory impairments, we observed a reduction in the fraction of electron leak to ROS in both maximal and submaximal conditions, suggesting Complex IV possesses a greater affinity for O_2 than proximal sites within the ETC implicated in the production of superoxide radicals.

In vivo implications

The present data imply a reduction in pO_2 decreases mitochondrial ROS, a response which appears evident following BFR-RE, and is in stark contrast to our hypothesis. Interestingly, this effect persisted 2 hours following BFR-RE despite normalized pO_2 *in vivo*, and when assessed in the

presence of room air conditions. This suggests that the reduction in pO_2 during BFR-RE exerts direct acute effects on mitochondrial ROS signaling pathways, in addition to indirect and prolonged post-translational modifications capable of influencing mitochondrial redox balance. Despite the reduction in mitochondrial ROS with mild O_2 restriction and BFR-RE, the present data do not necessarily indicate an improvement in redox balance *in vivo*, but rather suggests that BFR-RE and reductions in pO_2 do not cause an increase in mitochondrial-specific ROS emission 2 hours post-exercise. In this respect, it has been suggested that decreasing pO_2 increases the generation of ROS from NADPH oxidase, xanthine oxidase, and various extra-mitochondrial membranes (e.g. sarcoplasmic reticulum, transverse tubules, and sarcolemma) (Waypa & Schumacker, 2005), pathways which also can influence cellular redox balance in response to exercise (Powers *et al.,* 2011). In addition, it remains possible that ROS emission rates were transiently elevated immediately following exercise, a response which decreased as a compensatory mechanism 2 hours following exercise. However, given the importance of delayed post-exercise signaling events in mediating chronic training adaptations (Perry *et al.,* 2010) a reduction in ROS emission rates 2 hours post-exercise would nonetheless be expected to have important implications for regulating subsequent metabolic responses to BFR-RE. While additional work is clearly required to delineate the metabolic consequences of reductions in skeletal muscle pO_2 *in vivo*, particularly immediately following resistance exercise in a time-course manner, the present data strongly suggests that decreasing pO_2 attenuates the absolute and relative production of mitochondrial-derived H_2O_2 in human skeletal muscle, an event which appears to occur following BFR-RE.

Conclusion

The present data provides evidence that submaximal and maximal mitochondrial H_2O_2 emission rates are acutely decreased in response to BFR-RE, but not RE, in the absence of any changes in mitochondrial respiration following either exercise. This response is likely influenced by the

reduction in tissue oxygenation (i.e. lower pO_2) during BFR-RE, as our *in vitro* data further provides evidence that mild O_2 restriction dramatically attenuates mitochondrial H_2O_2 emission rates and electron leak to ROS. While we observed a comparatively modest decrease in maximal respiratory capacity *in vitro*, this data suggests Complex IV mediating mitochondrial oxidative phosphorylation is more sensitive to O_2 than proximal sites implicated in superoxide production. Altogether, these findings have important implications for understanding the regulation of basic mitochondrial redox biology, particularly as a potential mechanism influencing the training adaptations to BFR-RE and RE. Furthermore, the current results exclude the theory that acute elevations in mitochondrial ROS emission rates following BFR-RE contribute to comparable training adaptations between low-volume BFR-RE and traditional RE training, specifically when both types of exercise are performed to repetition failure.

Author Contributions

HLP, CP, LJvL, JFB, GHG, and GPH designed the study. HLP, CP, PAB, TACV, KMJHD, JFB, GHG, and GPH organized and performed the experiments. All authors analyzed and interpreted the data. HLP and GPH drafted and edited the manuscript, and all authors approved the final version. GPH and GHG contributed equally as guarantors of the study.

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Competing Interests

None of the authors disclose any conflicts of interest.

References

Atherton PJ, Babraj JA, Smith K, Singh J, Rennie MJ & Wackerhage H (2005). Selective activation of AMPK-PGC1a or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation. *FASEB J* **23,** 1–23.

Barbeau P, Miotto PM & Holloway GP (2018). Mitochondrial-derived reactive oxygen species influence ADP sensitivity, but not CPT-I substrate sensitivity. *Biochem J* **475,** 2997–3008.

Burd NA, Andrews RJ, West DWD, Little JP, Cochran AJR, Hector AJ, Cashaback JGA, Gibala MJ, Potvin JR, Baker SK & Phillips SM (2012*a*). Muscle time under tension during resistance exercise stimulates differential muscle protein sub-fractional synthetic responses in men. *J Physiol* **2,** 351–362.

Burd NA, Mitchell CJ, Churchward-Venne TA & Phillips SM (2012*b*). Bigger weights may not beget bigger muscles: evidence from acute muscle protein synthetic responses after resistance exercise. *Appl Physiol Nutr Metab* **37,** 551–554.

Chance B & Williams GR (1955). Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. *J Biol Chem* **217,** 383-393.

Chandel NS, Mcclintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM & Schumacker PT (2000). Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1a during hypoxia. *J Biol Chem* **275,** 25130–25138.

Clanton TL (2007). Hypoxia-induced reactive oxygen species formation in skeletal muscle. *J Appl Physiol* **102,** 2379–2388.

Davies KJA, Quintanilha AT, Brooks GA & Packert L (1982). Free radical and tissue damage produced by exercise. *Biochem Biophys Res Com* **107,** 1198–1205.

Debevec T, Pialoux V, Saugy J, Schmitt L, Cejuela R, Mury P, Ehrstrom S, Faiss R & Millet GP (2015). Prooxidant/antioxidant balance in hypoxia: a cross-over study on normobaric vs. hypobaric "live high-train low ." *PLoS One* **10,** 1–14.

Dohlmann TL, Hindso M, Dela F, Helge JW & Larsen S (2018). High-intensity interval training changes mitochondrial respiratory capacity differently in adipose tissue and skeletal muscle. *Physiol Rep* **6,** e13857.

Dudley GA (1988). Metabolic consequences of resistive-type exercise. *Med Sci Sport Exerc* **20,** S158–S161.

Duranteau J, Chandel NS, Kulisz A, Shao Z & Schumacker PT (1998). Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. *J Biol Chem* **273,** 11619–11624.

Essen-Gustavsson B & Tesch PA (1990). Glycogen and triglyceride utilization in relation to muscle metabolic characteristics in men performing heavy-resistance exercise. *Eur J Appl Physiol* **61,** 5–10.

Farup J, de Paoli F, Bjerg K, Riis S, Ringgard S & Vissing K (2015). Blood flow restricted and traditional resistance training performed to fatigue produce equal muscle hypertrophy. *Scand J Med Sci Sport* **25,** 754–763.

Ganesan G, Cotter JA, Reuland W, Cerussi AE, Tromberg BJ & Galassetti P (2015). Effect of blood flow restriction on tissue oxygenation during knee extension. *Med Sci Sport Exerc* **47,** 185–193.

Goldfarb AH, Garten RS, Chee PDM, Cho C, Reeves GV, Hollander DB, Thomas C, Aboudehen KS, Francois M & Kraemer RR (2008). Resistance exercise effects on blood glutathione status and plasma protein carbonyls Influence of partial vascular occlusion. *Eur J Appl Physiol* **104,** 813–819.

Groennebaek T, Jespersen NR, Jakobsgaard JE, Sieljacks P, Wang J, Rindom E, Musci R V, Bøtker HE, Hamilton KL, Miller BF, Paoli FV De & Vissing K (2018). Skeletal muscle mitochondrial protein synthesis and respiration increase with low-load blood flow restricted as well as high-load resistance training. *Front Physiol* **9,** 1–14.

Hawley JA (2009). Molecular responses to strength and endurance training: Are they incompatible? *Appl Physiol Nutr Metab* **34,** 355–361.

Hoffman DL, Salter JD & Brookes PS (2007). Response of mitochondrial reactive oxygen species generation to steady-state oxygen tension: Implications for hypoxic cell signaling. *Am J Physiol Hear Circ Physiol* **292,** H101–H108.

Holloway GP (2017). Nutrition and training influences on the regulation of mitochondrial adenosine diphosphate sensitivity and bioenergetics. *Sport Med* **47,** 13–21.

Holloway GP, Holwerda AM, Miotto PM, Dirks ML, Verdijk LB & van Loon LJ (2018*a*). Age-associated impairments in mitochondrial ADP sensitivity contribute to redox stress in senescent human skeletal muscle. *Cell Rep* **22,** 2837–2848.

Holloway TM, Morton RW, Oikawa SY, Mckellar S, Baker SK & Phillips SM (2018*b*). Microvascular adaptations to resistance training are independent of load in resistance-trained young men. *Am J Physiol Regul Integr Comp Physiol* **315,** R267–R273.

Lundberg TR, Fernandez-Gonzalo R & Tesch PA (2014). Exercise-induced AMPK activation does not interfere with muscle hypertrophy in response to resistance training in men. *J Appl Physiol* **116,** 611–620.

Masri BA, Day B, Younger ASE & Jeyasurya J (2016). Technique for measuring limb occlusion presure that facilitates personalized tourniquet systems: A randomized trial. *J Med Biol Eng* **36**(5), 644-650.

McManus CJ, Collison J & Cooper CE (2018). Performance comparison of the MOXY and PortaMon near-infrared spectroscopy muscle oximeters at rest and during exercise. *J Biomed Optics* **23,** 015007.

Miotto PM, Steinberg GR & Holloway GP (2017). Controlling skeletal muscle CPT-I malonyl-CoA sensitivity: the importance of AMPK-independent regulation of intermediate filaments during exercise. *Biochem J* **474,** 557-569.

Miotto PM & Holloway GP (2018). Exercise-induced reductions in mitochondrial ADP sensitivity contribute to the induction of gene expression and mitochondrial biogenesis through enhanced mitochondrial H2O2 emission. *Mitochondrion*; DOI: 10.1016/j.mito.2018.03.003.

Neto GR, Novaes JS, Salerno VP, Goncalves MM, Batista GR & Cirilo-Sousa MS (2017). Does a resistance exercise session with continuous or intermittent blood flow restriction promote muscle damage and increase oxidative stress? *J Sport Sci* **36,** 104–110.

Perry CGR, Lally J, Holloway GP, Heigenhauser GJF, Bonen A & Spriet LL (2010). Repeated transient mRNA bursts precede inreases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J Physiol* **588.23,** 4795-4810.

Perry CGR, Kane DA, Lin CT, Kozy R, Cathey BL, Lark DS, Kane CL, Brophy PM, Gavin TP, Anderson EJ & Neufer PD (2011). Inhibiting myosin-ATPase reveals a dynamic range of mitochondrial respiratory control in skeletal muscle. *Biochem J* **437,** 215-222.

Perry CGR, Kane DA, Herbst EAF, Mukai K, Lark DS, Wright DC, Heigenhauser GJF, Neufer PD, Spriet LL & Holloway GP (2012). Mitochondrial creatine kinase activity and phosphate shuttling are acutely regulated by exercise in human skeletal muscle. *J Physiol* **590.21,** 5475–5486.

Pesta D, Hoppel F, Macek C, Messner H, Faulhaber M, Kobel C, Parson W, Burtscher M, Schocke M & Gnaiger E (2011). Similar qualitative and quantitative changes of mitochondrial respiration

following strength and endurance training in normoxia and hypoxia in sedentary humans. *Am J Physiol Regul Integr Comp Physiol* **301,** R1078–R1087.

Petrick HL & Holloway GP (2019). High intensity exercise inhibits carnitine palmitoyltransferase-I sensitivity to L-carnitine. *Biochem J;* DOI: 10.1042/BCJ20180849.

Phillips SM, Green H, Tarnopolsky M, Heigenhauser G & Grant S (1996). Progressive effect of endurance training on metabolic adaptations in working skeletal muscle. *Am J Physiol Endocrinol Metab* **270,** E265–E272.

Picard M, Taivassalo T, Ritchie D, Wright KJ, Thomas MM & Hepple RT (2011). Mitochondrial structure and function are disrupted by standard isolation methods. *PLoS One* **6,** 1–12.

Porter C, Reidy PT, Bhattarai N, Sidossis LS & Rasmussen BB (2015). Resistance exercise training alters mitochondrial function in human skeletal muscle. *Med Sci Sport Exerc* **47,** 1922–1931.

Powers SK, Ji LL, Kavazis AN & Jackson MJ (2011). Reactive oxygen species: Impact on skeletal muscle. *Compr Physiol* **1**(2), 941-969.

Ruas JL, White JP, Rao RR, Kleiner S, Brannan KT, Harrison BC, Greene NP, Wu J, Estall JL, Irving BA, Lanza IR, Rasbach KA, Okutsu M, Nair KS, Yan Z, Leinwand LA & Spiegelman BM (2012). A PGC-1a isoform induced by resistance training regulates skeletal muscle hypertrophy. *Cell* **151,** 1319–1331.

Takarada Y, Nakamura Y, Aruga S, Onda T, Miyazaki S & Ishii N (2000). Rapid increase in plasma growth hormone after low-intensity resistance exercise with vascular occlusion. *J Appl Physiol* **88,** 61–65.

Tesch PA (1988). Skeletal muscle adaptations consequent to long-term heavy resistance exercise. *Med Sci Sport Exerc* **20,** S132–S134.

Tesch PA, Colliander EB & Kaiser P (1986). Muscle metabolism during intense, heavy-resistance exercise. *Eur J Appl Physiol* **55,** 362–366.

Waypa GB & Schumacker PT (2005). Hypoxic pulmonary vasoconstriction: redox events in oxygen sensing. *J Appl Physiol* **98,** 404–414.

Figure legends

Figure 1 – Exercise volume during each set to repetition failure. Participants performed 3 sets of single-leg squats to repetition failure at 30% 1-RM with (BFR-RE) and without (RE) occlusion. *n*=10, values are reported as mean ± SEM. * p<0.05 versus RE.

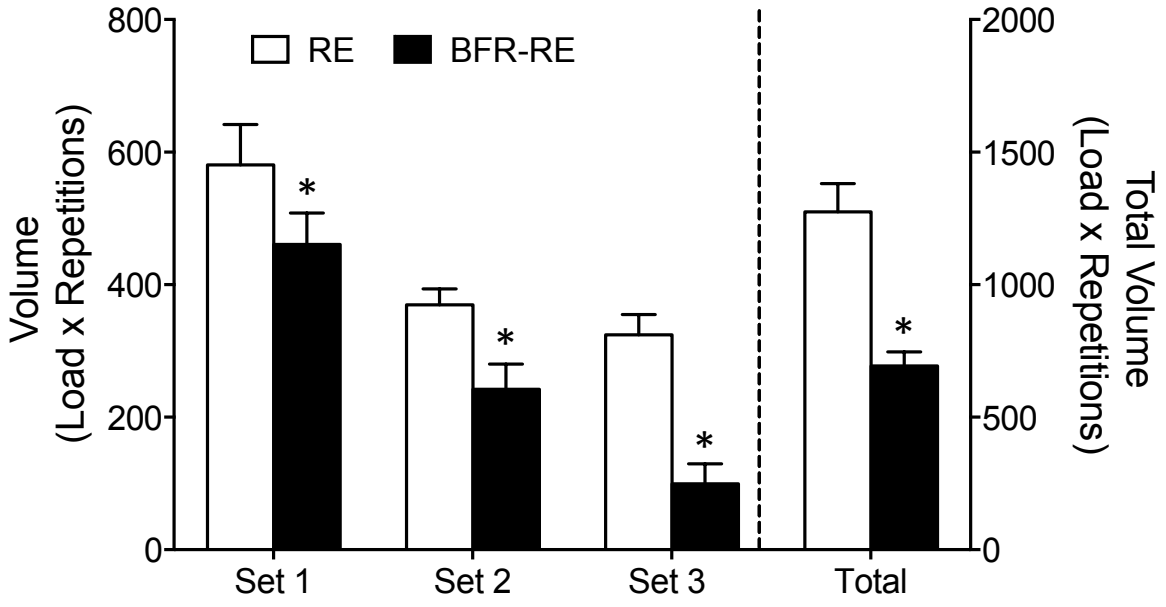

Figure 2 – The influence of acute RE and BFR-RE on mitochondrial protein content and respiratory capacity in permeabilized muscle fibers. Neither RE or BFR-RE altered ETC subunits and mitochondrial protein content in permeabilized muscle fibers (A and B), maximal complex I/II linked respiration (C), or the respiratory sensitivity of mitochondria to ADP titrated in concentrations of 25, 100, 175, 250, 500, 1000, 2000, 4000, 6000, 8000, 10000, and 12000µM ADP (D and E). Michealis-Menten R^2=0.9727 Pre; 0.9745 RE; 0.9704 BFR-RE. PM, pyruvate+malate; +D, PM+ADP; +G, PMD+glutamate; +S, PMDG+succinate; +C, PMDGS+cytochrome C; RCR, respiratory control ratio. *n*=10, values are reported as mean ± SEM.

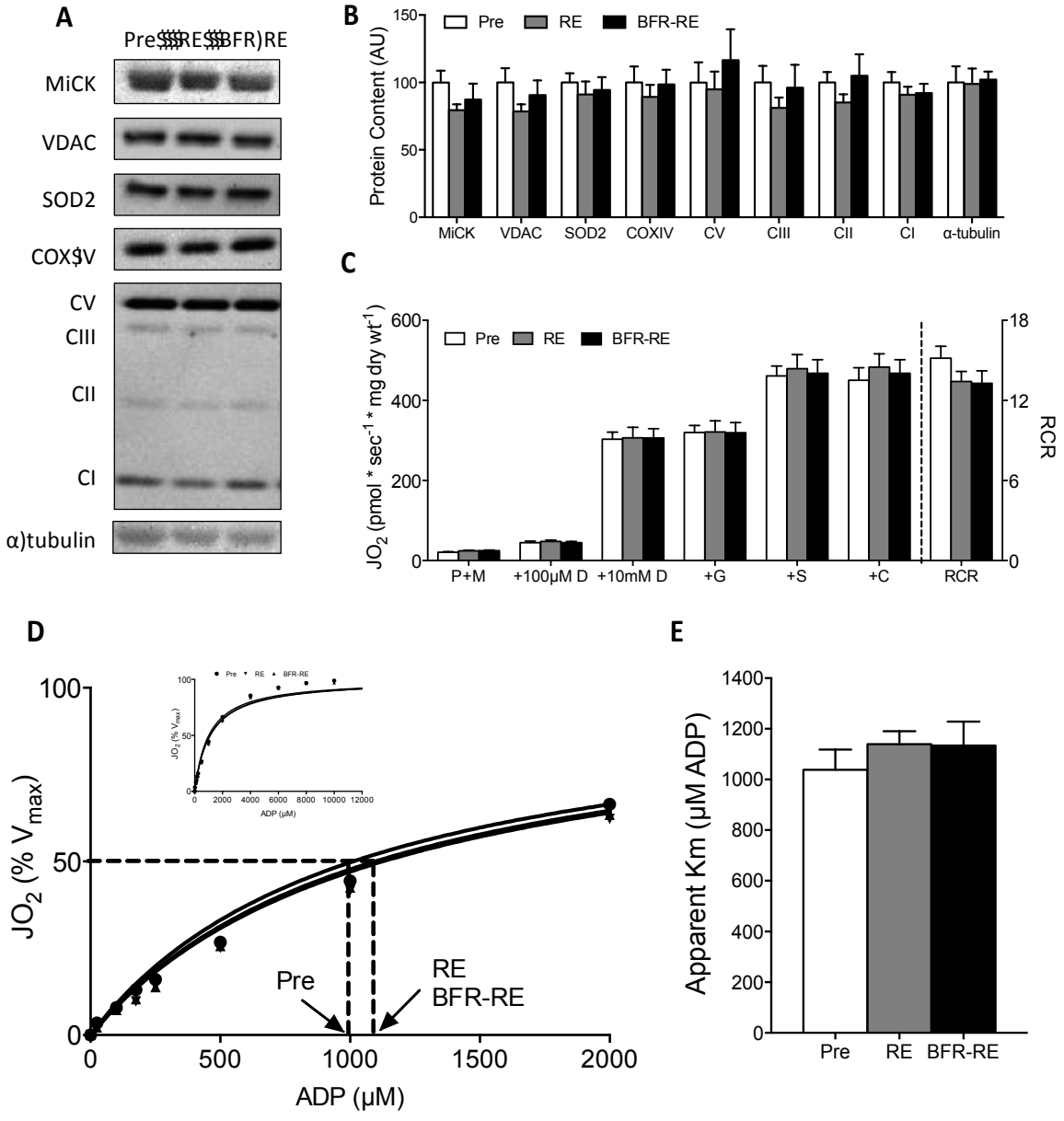

Figure 3 – The effect of RE and BFR-RE on mitochondrial H_2O_2 emission rates in permeabilized muscle fibers. BFR-RE decreased absolute rates of maximal succinate-supported mitochondrial H_2O_2 emission (A) and submaximal (+100μM ADP) mitochondrial H_2O_2 emission rates (B). The ability of 100μM ADP to attenuate maximal H_2O_2 emission rates was not altered by RE or BFR-RE (C). *n*=10, values are reported as mean ± SEM. * p<0.05 versus pre-exercise.

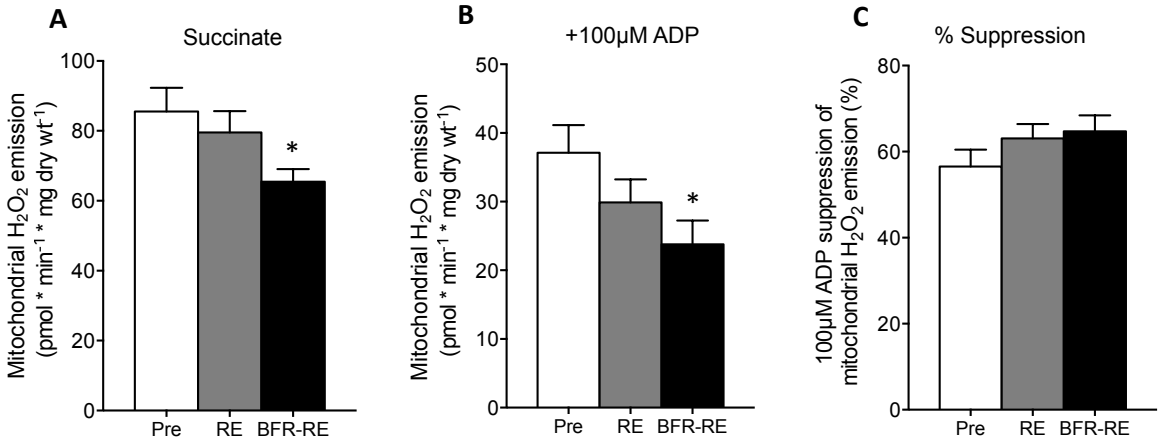

Figure 4 – Estimations of muscle oxygenation during RE and BFR-RE. A NIRS device was placed on the *vastus lateralis* to estimate regional skeletal muscle oxygenation. Representative tracings of tissue saturation index are represented for RE (A) and BFR-RE (B). Averages across all participants are depicted for oxygenated Hb (C), deoxygenated Hb (D), total Hb content (E), and tissue saturation index (F). Hb, hemoglobin. *n*=10, values are reported as mean ± SEM. * p<0.05 versus RE. † p<0.05 versus baseline for respective leg.

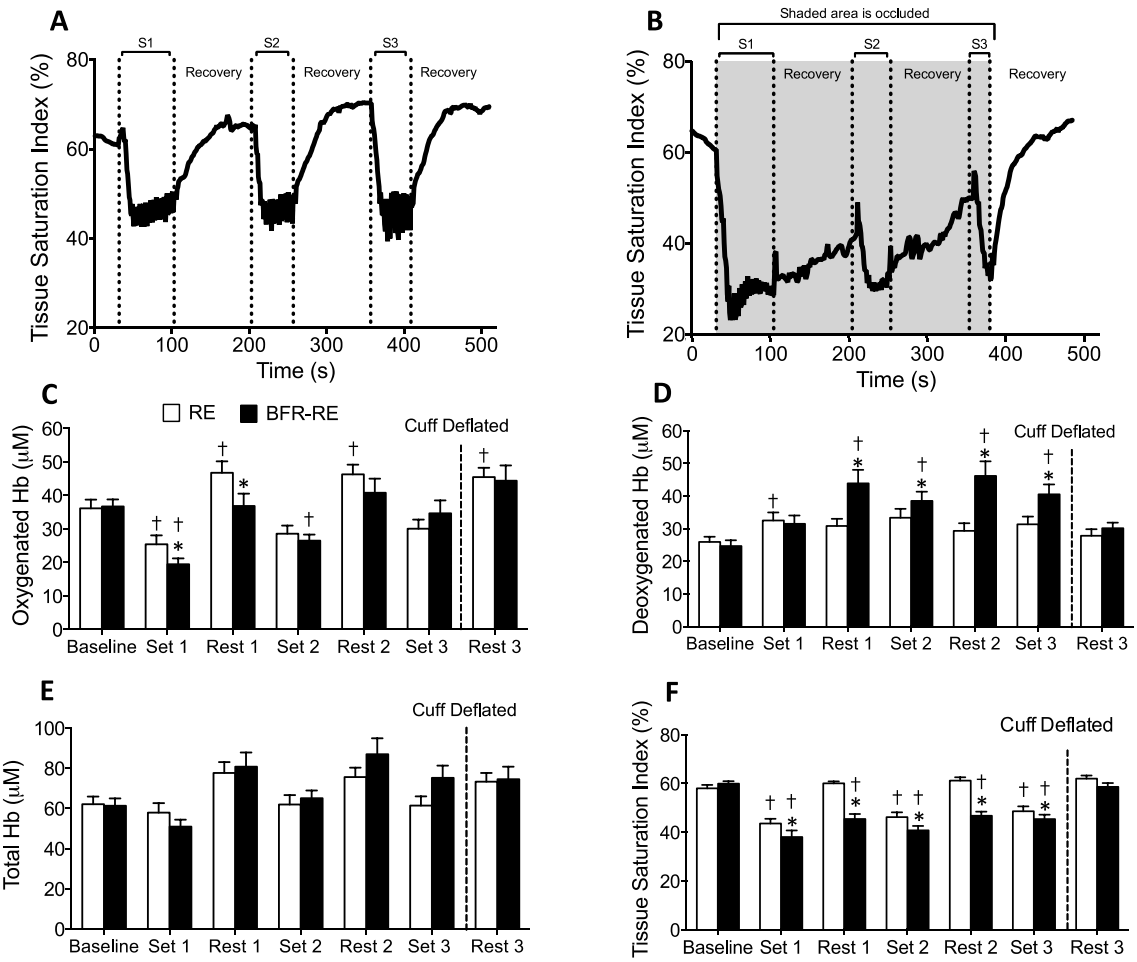

Figure 5 – Establishing a protocol to vary oxygen tension *in vitro* while simultaneously assessing respiration and H_2O_2 emission in permeabilized muscle fibers. Maximal oxidative phosphorylation was first initiated with 10mM ADP and the rate of oxygen consumption (JO_2) monitored until anoxia (A) to directly compare the influence of oxygen concentration on JO_2 (B). In separate experiments, oxygen concentration (C) was decreased while the rate of oxygen consumption (JO_2) and mitochondrial H_2O_2 emission were determined. Leak respiration (P+M+S) and maximal (Max ROS) H_2O_2 were simultaneously measured in the absence of ADP. Submaximal (+100µM D) respiration and H_2O_2 (Submax. ROS) were simultaneously determined in the presence of 100µM ADP, while maximal respiration (+10mM D) was determined in the presence of 10mM ADP. D, adenosine diphosphate (ADP); O_2: oxygen; PM, pyruvate+malate; P+M+S, pyruvate+malate+succinate.

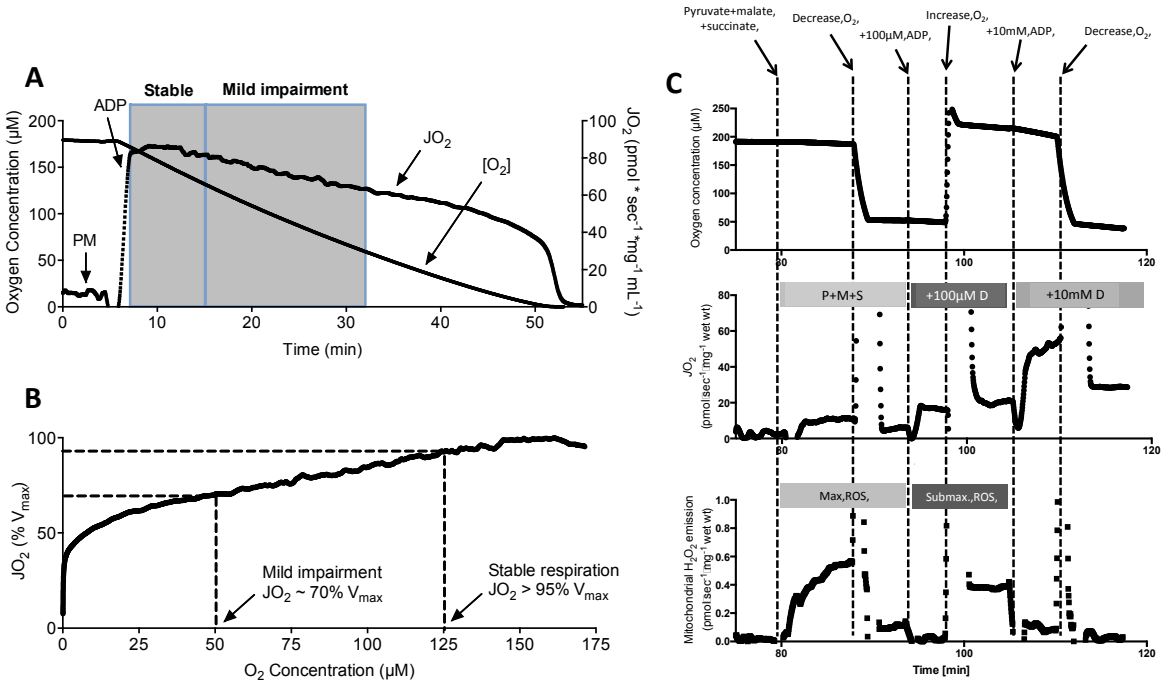

Figure 6 – The effect of decreasing oxygen tension *in vitro* on mitochondrial respiration and H_2O_2 emission rates in permeabilized muscle fibers. Absolute rates of oxygen consumption (A) were determined in room air (~200μM O_2) and oxygen restriction (~50μM O_2) in the absence of ADP (P+M+S) presence of 100μM ADP (submaximal respiration) and 10mM ADP (maximal respiration). Absolute rates of H_2O_2 emission (B) were determined in the absence (Succinate, maximal H_2O_2 emission), and presence of 100μM ADP (Submaximal H_2O_2 emission rates). H_2O_2 was not detectable in the presence of 10mM ADP (data not shown). The ability of 100μM ADP to suppress H_2O_2 is reported in (C). Simultaneous measurements enabled the fraction of electron leak to H_2O_2 to be determined in the absence (Succinate) and presence of 100μM ADP (+100μM D) (D). D, adenosine diphosphate (ADP); P+M+S, pyruvate+malate+succinate; RCR, respiratory control ratio. *n*=6, values are reported as mean ± SEM. * p<0.05 versus room air.