

**Acute HIIIE elicits similar changes in human skeletal muscle mitochondrial H<sub>2</sub>O<sub>2</sub> release, respiration and cell signaling as endurance exercise even with less work**

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**Running Head:** Acute exercise intensity and muscle mitochondria

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## Abstract

It remains unclear whether high-intensity interval exercise (HIIE) elicits distinct molecular responses to traditional endurance exercise relative to the total work performed. We aimed to investigate the influence of exercise intensity on acute perturbations to skeletal muscle mitochondrial function (respiration and reactive oxygen species), metabolic and redox signaling responses. In a randomized, repeated measures crossover design, eight recreationally active individuals ( $24 \pm 5$  years;  $\text{VO}_{2\text{peak}} 48 \pm 11 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) undertook continuous moderate-intensity (CMIE: 30 min, 50% peak power output [PPO]), high-intensity interval (HIIE: 5x4 min, 75% PPO, work-matched to CMIE), and low-volume sprint interval (SIE: 4x30 s) exercise,  $\geq 7$  days apart. Each session included muscle biopsies at baseline, immediately and 3 h post-exercise for high-resolution mitochondrial respirometry ( $\text{JO}_2$ ) and  $\text{H}_2\text{O}_2$  emission ( $\text{JH}_2\text{O}_2$ ), gene and protein expression analysis. Immediately post-exercise and irrespective of protocol,  $\text{JO}_2$  increased during complex I+II leak/state-4 respiration but  $\text{JH}_2\text{O}_2$  decreased ( $p < 0.05$ ). AMP-activated protein kinase (AMPK) and acetyl co-A carboxylase (ACC) phosphorylation increased  $\sim 1.5$  and  $2.5$ -fold respectively, while thioredoxin-reductase-1 protein abundance was  $\sim 35\%$  lower after CMIE vs. SIE ( $p < 0.05$ ). At 3 hours post-exercise, regardless of protocol,  $\text{JO}_2$  was lower during both ADP-stimulated state-3 OXPHOS and uncoupled respiration ( $p < 0.05$ ) but  $\text{JH}_2\text{O}_2$  trended higher ( $p < 0.08$ ); *PPARGC1A* mRNA increased  $\sim 13$ -fold, and peroxiredoxin-1 protein decreased  $\sim 35\%$ . In conclusion, intermittent exercise performed at high intensities has similar dynamic effects on muscle mitochondrial function compared with endurance exercise, irrespective of whether total workload is matched. This suggests exercise prescription can accommodate individual preferences while generating comparable molecular signals known to promote beneficial metabolic adaptations.

250 words

## 49 INTRODUCTION

50 Exercise is a front line strategy for the improvement of metabolic health and the  
51 prevention of numerous chronic diseases (21). Therefore, it is of clinical and public health  
52 relevance to understand the efficacy of various exercise modalities. Whether high intensity  
53 interval exercise (HIIE) elicits similar or even greater beneficial metabolic adaptations than  
54 traditional endurance type exercise remains unclear. In particular, there are conflicting reports  
55 regarding whether skeletal muscle metabolic perturbations and consequent adaptive  
56 responses are proportional to the intensity of an exercise bout when total work performed is  
57 controlled (3, 12, 18, 40). Moreover, the precise mechanisms that underlie these adaptive  
58 responses remain incompletely understood.

59 Increases in content and/or respiratory function of skeletal muscle mitochondria  
60 represent an important adaptive response to regular aerobic exercise training (16). Despite  
61 this, the acute effects of a single bout of exercise on mitochondrial function remain relatively  
62 less studied. Mitochondrial bioenergetics (i.e. rates of ATP synthesis via oxidative  
63 phosphorylation) are regulated in response to exercise induced perturbations (i.e.  $pO_2$ , pH,  
64  $Ca^{2+}$ , ATP and NADH status). This can occur via complex cellular signaling events, activity  
65 of rate-limiting enzymes such as pyruvate dehydrogenase upstream of the mitochondrial  
66 electron transport system (ETS), and conceivably also by post-translational modifications to  
67 ETS proteins, although the latter has not specifically been demonstrated under exercise  
68 conditions (7, 28, 33). The mitochondrial ETS also intrinsically generates reactive oxygen  
69 species (ROS) in the form of the superoxide anion ( $O_2^{\cdot-}$ ), which is dismutated spontaneously  
70 or more rapidly by superoxide dismutase (SOD) to hydrogen peroxide ( $H_2O_2$ ) (46). The main  
71 sites of  $O_2^{\cdot-}/H_2O_2$  generation during exercise are considered to be of non-mitochondrial origin  
72 such as NADPH oxidase and xanthine oxidase (49, 55, 71), yet under basal conditions

mitochondria are a primary source of  $O_2^{\cdot-}/H_2O_2$  (24, 55). Therefore, as the skeletal muscle cellular environment returns towards basal conditions during recovery from acute exercise, mitochondria may not only rapidly revert back to being the primary source of  $O_2^{\cdot-}/H_2O_2$ , but additionally have altered rates of  $O_2^{\cdot-}/H_2O_2$  generation. We recently reported altered skeletal muscle mitochondrial respiratory function immediately post-exercise in well trained humans (37) as well as altered post-exercise mitochondrial  $H_2O_2$  emission in humans who are obese (66). However, to our knowledge, no study has investigated the acute effects of exercise intensity on mitochondrial function in human skeletal muscle. It is conceivable that differential mitochondrial responses to acute exercise may occur depending on intensity, since higher exercise intensity requires recruitment of a greater proportion of fast-twitch muscle fibers, whose mitochondria have been shown to have distinct functional characteristics (1).

The physiological implications of altered mitochondrial respiration and ROS emission in the hours post-exercise, are that numerous exercise-mediated adaptive responses in muscle are known to be redox-sensitive (31, 53, 57, 67). Redox sensitive signal transducers include p38 mitogen activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK) (19, 29, 30). These can promote mitochondrial biogenesis signaling via peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC1 $\alpha$ ; encoded by the gene *PPARGC1A*) (4, 60) as well as the upregulation of antioxidant capacity via transcription factors such as nuclear factor erythroid 2-related factor 2 (NRF2, encoded by the gene *NFE2L2*) (15). Downstream transcriptional targets of NRF2 include genes that encode enzymes critical for cellular redox homeostasis including SOD, glutathione peroxidase (GPX), thioredoxin (TRX), peroxiredoxin (PRDX) and thioredoxin reductase (TXNRD) (15). Additional regulation of post-exercise mitochondrial  $O_2^{\cdot-}/H_2O_2$  generation may occur via



mitochondrial membrane remodelling processes (fission and fusion) via dynamin-related protein 1 (DRP1) and mitofusin (MFN2), respectively (2, 52); along with uncoupling protein-3 (UCP3), which, upon activation can dissipate inner mitochondrial membrane potential to mitigate  $\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$  generation (41). Taken together, altered patterns of mitochondrial  $\text{O}_2^{\cdot-}/\text{H}_2\text{O}_2$  emission may have important downstream effects on a range of redox-sensitive adaptive processes in the hours following exercise (31).

Therefore, the aim of this study was to test the hypothesis that when total work performed is accounted for, higher exercise intensity leads to greater post-exercise perturbations to skeletal muscle mitochondrial function (respiration and  $\text{H}_2\text{O}_2$  emission), along with gene and protein responses related to key metabolic adaptations and redox homeostasis in young, healthy humans.

## **METHODS**

### **Participants**

Eight young, healthy and recreationally active individuals (six males, two females) participated in this study (mean  $\pm$  SD: age  $24.5 \pm 5.5$  yrs.; height  $179 \pm 8$  cm; weight  $79.4 \pm 6.0$  kg; BMI  $24.8 \pm 2.7$   $\text{kg}\cdot\text{m}^{-2}$ ;  $\text{VO}_{2\text{peak}}$   $48.4 \pm 11.2$   $\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) as reported recently (50, 51). All volunteers provided written informed consent after screening for contraindications to exercise via a health assessment questionnaire. Potential participants for this study were excluded if they were currently smoking, had musculoskeletal or other conditions that prevented daily activity, symptomatic or uncontrolled metabolic or cardiovascular disease, or (females) taking oral contraception. This study was approved by and conducted in accordance with the Victoria University Human Research Ethics Committee.

## Experimental Design

Participants visited the Victoria University exercise physiology laboratory on four occasions. An initial visit involved screening and a graded cycling exercise test to determine  $\dot{V}O_{2\text{peak}}$  and subsequent exercise workloads to which participants were then familiarized. Three experimental trials were then conducted using a crossover study design. Trial order was randomized using the Microsoft Excel list randomize function. Trials were conducted 7-14 d apart for males, and 28 d apart for females during the early follicular phase of the menstrual cycle to control for ovarian hormone fluctuations. In each of the three experimental trials, muscle biopsy samples were collected at baseline (BASE), immediately post exercise (EX); and 3 h post-exercise (3HR).

## Dietary and exercise control

Participants reported to the laboratory in an overnight-fasted state. Participants recorded all food consumed in that 24 h period in a food diary and abstained from alcohol and caffeine for 48 h and structured exercise for 24 h before each experimental trial. Photocopies of the food diary were returned to participants who were instructed to replicate this diet for the second and third visits. One litre of drinking water was provided *ad libitum* to be consumed during and after exercise, but matched between trials.

## Exercise protocols

All exercise sessions were performed on an electrically braked Velotron cycle ergometer (Racermate, Seattle, WA). Participants initially performed a graded exercise test (GXT) protocol to determine peak power output (PPO) and peak oxygen uptake ( $\dot{V}O_{2\text{peak}}$ ). Briefly, the test started at 50 W and increased by 25 W each minute until perceived exhaustion was achieved as indicated by volitional cessation of cycling, or a pedalling

cadence decreasing to below 60 rpm despite strong verbal encouragement. PPO was defined as the final complete stage, plus the fraction of the incomplete stage (26). Expired gases were collected throughout the test, and  $\text{VO}_{2\text{peak}}$  was determined with an on-line gas collection system (Moxus Modular  $\text{VO}_2$  System, AEI Technologies, Pittsburgh, PA) calibrated as per the manufacturer's instructions. Heart rate was measured using a Polar heart rate monitor (Polar Electro, Finland). In the same visit after adequate recovery, participants were then familiarized with the experimental trial exercise protocols and workloads. One of the three exercise sessions were performed in each experimental trial. The continuous moderate-intensity exercise (CMIE) was performed at 50% of PPO for 30 min. The high-intensity interval exercise (HIIE) protocol consisted of 5 x 4 min intervals at 75% PPO interspersed with 1 min passive recovery and was matched for the total kilojoules of work performed in the CMIE protocol. The sprint exercise session (SIE) consisted of 4 x 30 s maximal sprint cycling efforts, with 4.5 min passive recovery intervals. The SIE session was not matched to CMIE/HIIE because it would be unrealistic for participants to perform an equal volume of sprint exercise given its physical demand. For the SIE exercise session, pedalling resistance was determined as a torque factor relative to body mass, optimized during the familiarisation session to achieve a pedalling cadence throughout each interval of approximately 100-120 rpm at the beginning of the 30 s bout without decreasing below ~ 40 - 50 rpm at the end. Verbal encouragement was given throughout.

### **Muscle biopsy sampling**

Muscle samples were obtained from the middle third of the *vastus lateralis* muscle using the percutaneous needle biopsy technique as previously described (51). Briefly, after injection of a local anaesthetic into the skin and fascia (1% Xylocaine, Astra Zeneca, Australia), a small incision was made and a muscle sample taken (~120 mg) using a

Bergström biopsy needle with suction. Each biopsy was taken from a separate incision ~1 cm proximal from the previous biopsy. Muscle samples were dissected free of any visible connective tissue then one portion frozen in liquid nitrogen and stored at -80 °C, and another placed in ice-cold BIOPS preserving solution for mitochondrial functional analyses (see below).

### **Preparation of permeabilized muscle fibers**

To ‘capture’ the acute regulatory effects of exercise on mitochondrial function, immediately after the biopsy, muscle fiber bundles were placed into ice-cold preserving solution (BIOPS; containing in mM: 7.23 K<sub>2</sub>EGTA, 2.77 CaK<sub>2</sub>EGTA, 5.77 Na<sub>2</sub>ATP, 6.56 MgCl<sub>2</sub>·6H<sub>2</sub>O, 20 taurine, 15 phosphocreatine, 20 imidazole, 0.5 dithiothreitol, 50 K<sup>+</sup>-MES; pH 7.1) then prepared as per our previous work (66). Briefly, a small portion of muscle fibers were mechanically separated then transferred to ice-cold BIOPS supplemented with saponin (50 µg/mL) for 30 min with agitation. This was followed by agitation in ice-cold respiration buffer (MiR05; in mM: 0.5 EGTA, 10 KH<sub>2</sub>PO<sub>4</sub>, 3 MgCl<sub>2</sub>·6H<sub>2</sub>O, 60 lactobionic acid, 20 taurine, 20 HEPES, 110 D-sucrose, 1 mg/mL bovine serum albumin; pH 7.1). Two portions of the fiber bundles were blotted on filter paper for 5 s, and wet-weight (ww) mass was recorded using a microbalance (3 – 4 mg-ww per replicate).

### **Mitochondrial respiration and hydrogen peroxide emission assay**

To determine mitochondrial function and concomitant ROS emitting potential in the form of H<sub>2</sub>O<sub>2</sub> (JH<sub>2</sub>O<sub>2</sub>), permeabilized muscle fiber bundles were assessed in duplicate using a high resolution respirometer (Oxygraph O2k, Oroboros Instruments, Innsbruck, Austria) in respiration buffer MiR05 as per our previous work (62, 66). Briefly, a substrate, uncoupler, inhibitor titration (SUIT) protocol was performed at 37°C with O<sub>2</sub> concentration maintained

196 between 300-500  $\text{nmol.ml}^{-1}$ . Specifically, sequential titrations of substrates were added firstly  
 197 to assess mitochondrial Complex I leak ( $LEAK_{CI}$ ) with malate (2 mM) and pyruvate (10 mM),  
 198 followed by succinate (10 mM) to assess Complex II ( $LEAK_{CI+II}$ ) state 4 respiration.  
 199 Oxidative phosphorylation (state 3 respiration) supported by CI+II substrates ( $OXPHOS_{CI+II}$ )  
 200 was then determined with titrations of adenosine diphosphate (ADP) at 0.25, 1 and 5 mM; the  
 201 latter being considered a saturating concentration since it did not lead to significantly greater  
 202  $JO_2$  rates compared with 1 mM. Cytochrome *c* (10  $\mu\text{M}$ ) was added to confirm membrane  
 203 integrity (< 15% increase in  $O_2$  flux), then peak uncoupled respiratory flux was measured  
 204 after 2-4 titrations of 25 nM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to  
 205 assess maximal capacity of the electron transfer system supported by convergent CI and CII  
 206 substrate input ( $ETS_{CI+II}$ ). Inhibitors of specific complexes were then applied: rotenone (1  
 207  $\mu\text{M}$ ) to inhibit CI resulting in ETS supported only by CII substrate flux ( $ETS_{CII}$ ), followed by  
 208 the CIII inhibitor antimycin A (5  $\mu\text{M}$ ) to determine background  $O_2$  flux. These  $JO_2$  values  
 209 were subtracted from all prior measures to account for any artefactual non-ETS  $O_2$   
 210 consumption (mean  $\pm$  SD across all experiments:  $3.86 \pm 1.39 \text{ pmol.s}^{-1}.\text{mg-ww}^{-1}$ ).  
 211 Measurements of oxygen fluxes were averaged from both chambers during steady-state for  
 212 each respiratory state. If one of the chambers did not reach steady state flux, that value was  
 213 excluded from the analysis of that respiratory state. Throughout the respiration protocol, rates  
 214 of  $H_2O_2$  emission were simultaneously assessed via the Amplex UltraRed (25  $\mu\text{M}$ ; Molecular  
 215 Probes, Invitrogen) and horseradish peroxidase (2.5  $\text{U.mL}^{-1}$ ) reaction with  $H_2O_2$  in the  
 216 presence of added superoxide dismutase (SOD; 2.5  $\text{U.mL}^{-1}$ ). The formation of the fluorescent  
 217 reaction product (resorufin) was measured via excitation/emission at 525/600  $\text{nm}$  (Oroboros  
 218 O2k-Fluorescence LED-2 Module, Anton Paar, Graz, Austria) (27, 34). Signal was calibrated  
 219 at the beginning of each experiment with 40 nM titrations of  $H_2O_2$  and expressed relative to  
 220 sample mass ( $\text{mg.wet-weight}^{-1}$ ).

## **Real-time quantitative polymerase chain reaction**

RNA was isolated from BASE and 3HR muscle samples by mechanical homogenization (Tissuelyser, Qiagen, Australia) with Tri reagent, followed by 1-bromo-3-chloropropane and isopropanol precipitation (Sigma Aldrich, Castle Hill, NSW, Australia), which was then dissolved in DNase and RNase free water. RNA samples were tested spectrophotometrically (Bio-Photometer, Eppendorf, Germany) for concentration at 260 nm and quality, indicated by the 260:288 nm absorbance ratio (mean  $\pm$  SD:  $2.15 \pm 0.18$ ). One microgram of RNA was then reverse transcribed to cDNA (iScript kit, BioRad, Gladesville NSW, Australia). Real-time qPCR reactions were carried out in a Mastercycler RealPlex 2 (Eppendorf, Hamburg, Germany) with Taq enzyme reagent (iTaq SYBR Green, BioRad, Gladesville NSW, Australia) and forward and reverse primers (Sigma Aldrich, Castle Hill, NSW, Australia) for target mRNAs, which were generated from the NCBI Primer-BLAST database as shown in Table 1. The conditions for RT-qPCR were an initial 3 min annealing phase at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C. Following this, a 20 min melting curve (60°C to 95°C) was performed to confirm the amplification of a single product. Cycle thresholds ( $C_T$ ) were calculated using software (RealPlex, Eppendorf) and used to quantify mRNA expression via the  $-2\Delta\Delta C_T$  method (39) normalized to a housekeeping gene,  $\beta 2$  microglobulin ( $\beta$ -2M).

## **Muscle protein extraction and western blotting**

Frozen muscle was processed for western blotting as per our previous work (66). Protein (6-8  $\mu$ g per lane) was then loaded into precast 26 well stain-free 4-20% gradient gels (Criterion™ TGX Stain-Free™ Precast, BioRad, Gladesville NSW, Australia) along with molecular weight ladder (PageRuler® Plus, Thermo Scientific, Australia) and pooled sample.

246 The pooled sample was made by combining small volumes of all samples into a single pooled  
247 sample and used to construct a five-point standard curve (2 to 16  $\mu$ g protein) on all gels to  
248 allow direct comparison of blot intensities via linear regression, as described in detail  
249 elsewhere (47). Stain-free gels were activated by UV light (ChemiDoc™ MP, BioRad,  
250 Gladesville NSW, Australia) and imaged to visualize the total protein of each lane. Proteins  
251 were then transferred to PVDF membranes (Trans-Blot® Turbo™, BioRad, Gladesville  
252 NSW, Australia), blocked, and then incubated overnight at 4°C with the following primary  
253 antibodies diluted 1:1000 in TBST containing 5% BSA and 0.1% sodium azide: anti-  
254 phospho-Acetyl Co-A carboxylase (p-ACC $\beta$ <sup>Ser221</sup>, Cell Signaling #11818), anti-phospho  
255 AMP-activated protein kinase (p-AMPK<sup>Thr172</sup>, Cell Signaling #2531), anti-Dynamin Related  
256 Protein 1 (DRP1, Cell Signaling #5391), anti-Glutathione (Abcam #19534), anti-Glutathione  
257 Peroxidase 1 (GPX1, Cell Signaling #3286), anti-phospho heat shock protein 27 (p-  
258 HSP27<sup>ser82</sup>, Enzo ADI-SPA-524), Anti-Heat shock protein 72 (HSP72; Enzo ADI-SPA-810),  
259 Mitoprofile cocktail (MitoSciences #MS601, consisting of anti-complex I subunit NDUF8  
260 [complex-I, Abcam #110242], anti-complex II subunit 30kDa [complex-II, Abcam #14714],  
261 anti-complex III subunit core 2 [complex-III, Abcam #14745], anti-complex IV subunit II  
262 [complex-IVs2, Abcam #110258], and anti-ATP synthase subunit alpha [complex-V, Abcam  
263 #14748]), anti-complex IV subunit IV (complex-IVs4, Mito Sciences #MS407), anti-  
264 Mitofusin 2 (MFN2; Cell Signaling #9482), PRDX pathway cocktail (Abcam #184868,  
265 consisting of anti-Peroxiredoxin 1 [PRDX1], anti-Thioredoxin [TRX], and anti-Thioredoxin  
266 reductase-1 [TXNRD1]), anti-PPAR $\gamma$  coactivator-1 $\alpha$  (PGC1 $\alpha$ , Cell Signaling #2178), anti-  
267 Mitochondrial Transcription Factor 1 (TFAM, Abcam #475017), anti-uncoupling protein 3  
268 (UCP3, Abcam #10985). Membranes were then probed with appropriate horseradish  
269 peroxidase-conjugated secondary antibody (PerkinElmer, Glen Waverly, Victoria, Australia),  
270 at a dilution of 1:50,000 – 100,000 in 5% non-fat milk TBST for 1 hr at room temperature.

ECL (SuperSignal® West Femto, Thermo Scientific, Australia) was applied for imaging with a high sensitivity CCD camera (ChemiDoc™ MP, BioRad, Gladesville NSW, Australia) and subsequent analysis was performed (ImageLab v 5.1, BioRad, Gladesville NSW, Australia). Total protein loading of each sample was determined from stain-free images of each gel and these values were then used to normalize each protein of interest after normalization to its standard curve.

### **Co-immunoprecipitation of glutathionylated proteins**

Frozen muscle was sectioned and homogenized as described above, but with 200 µL non-denaturing lysis buffer (20 mM Tris HCl pH 8; 137 mM NaCl; 1% Triton X-100; 2 mM EDTA; 1% v/v protease inhibitor cocktail [Sigma Aldrich]; and 25 mM N-ethylmaleimide). Muscle lysate (50 µg protein) was added to 100 µL washed protein-A sepharose beads (GE Health/Amersham Biosciences), with 1 µL anti-glutathione primary antibody (Abcam #19534) which was incubated for 4 h at 4°C with rotation. Antigen-antibody-bead conjugates were centrifuged and supernatant discarded (supernatant was tested for efficacy of IP pull-down during optimisation), followed by a further 3 washes. Subsequently, 50 µL denaturing lysis buffer (125 mM Tris-HCl, 4% SDS, 10% Glycerol, 10 mM EGTA, 100 mM DTT) was added to the bead-protein-antibody conjugate, then heated at 95°C for 5 min to elute proteins from the bead-antibody conjugate. Bromophenol blue dye (1% v/v) was added before performing SDS-PAGE and immunoblotting as described above with the Mitoprofile cocktail (MitoSciences #MS601) and UCP3 (Abcam #10985) antibodies.

### **Statistical analysis**

Data were analysed by one-way (exercise-intensity) or two-way (exercise-intensity x time) ANOVA with repeated measures where appropriate (SPSS Statistics, IBM v1.0.0.642).



Mauchly's test of sphericity was performed and Greenhouse-Geisser correction applied where non-sphericity was detected. Where significant main interaction, time, or exercise-intensity effects were detected, post hoc analyses were conducted with Bonferroni correction for multiple comparisons. Statistical significance was accepted at  $p < 0.05$ , and trends indicated at  $p \leq 0.10$ . Where significant main effects were detected, effect sizes (ES) were calculated on data pooled from all three exercise protocols for pairwise comparison between time points using Cohen's  $d$  with 95% lower to upper confidence intervals (CI), without correction for multiple comparisons. Data are reported as mean  $\pm$  SD for  $n=8$  unless otherwise stated.

## RESULTS

### Physiological responses to exercise

Total external work of work-matched CMIE and HIIE was 4-fold greater than SIE (Table 2). Greater exercise intensity increased rating of perceived exertion ( $SIE > HIIE > CMIE$ ,  $p < 0.001$ ), despite HIIE leading to the highest  $HR_{peak}$  ( $p < 0.001$ , Table 2).

### Mitochondrial respiration

State-4 leak respiration supported by complex-I ( $LEAK_{CI}$ ) or I+II ( $LEAK_{CI+II}$ ) substrates was not differentially affected by exercise intensity, but increased with time by 65% ( $p=0.003$ ; ES 1.34, 95% CI 1.03 to 1.65; Figure 1B) and 40% ( $p < 0.001$ ; ES 0.77, 95% CI 0.59 to 0.95; Figure 1C) respectively, in a BASE vs. EX comparison across all exercise protocols. At 3HR,  $LEAK_{CI}$  remained ~30% elevated above BASE ( $p < 0.047$ ; ES 0.57, 95% CI 0.33 to 0.82). State-3 oxidative phosphorylation (5 mM ADP) supported by complex I+II ( $OXPHOS_{CI+II}$ ) was not affected by exercise intensity (*exercise-intensity  $\times$  time*,  $p=0.154$ ), but

was ~15% lower at 3HR compared with EX across all exercise protocols ( $p=0.003$ ; ES -0.27, 95% CI -0.44 to -0.10; Figure 1D). Uncoupled respiration (with FCCP) supported by complex I+II ( $ETS_{CI+II}$ ), or II only (plus rotenone) was not affected by exercise intensity, but was 15% ( $p=0.009$ ; ES -0.35, 95% CI -0.53 to -0.16; Figure 1E) and 30% ( $p=0.024$ ; ES -0.52, 95% CI -0.81 to -0.24; Figure 1F) lower, respectively, at 3HR compared with EX across all exercise protocols. The  $JO_2$  ratio between 0.25 v.s. 5 mM ADP under  $OXPHOS_{CI+II}$  conditions was not significantly affected by exercise protocol or time (data not shown).

### Mitochondrial $H_2O_2$ emission

During both complex-I ( $LEAK_{CI}$ ) and I+II ( $LEAK_{CI+II}$ ) supported leak respiration,  $JH_2O_2$  was not affected by exercise intensity, but was elevated ~55% during  $LEAK_{CI}$  at 3HR relative to EX across all exercise protocols ( $p=0.018$ ; ES 0.94, 95% CI 0.55 to 1.33; Figure 2B). In contrast, during  $LEAK_{CI+II}$ ,  $JH_2O_2$  was ~30% lower at EX ( $p=0.008$ ; ES -0.83, 95% CI -1.08 to -0.57), and ~40% lower at 3HR ( $p<0.001$ ; ES -0.97, 95% CI -1.22 to -0.72; Figure 2C) relative to BASE across all exercise protocols. During complex I+II supported state-3 oxidative phosphorylation respiration ( $OXPHOS_{CI+II}$ ),  $JH_2O_2$  was unaffected by exercise intensity, but tended to be elevated ~65% at 3HR relative to EX across all protocols ( $p=0.057$ ; ES 0.71, 95% CI 0.32 to 1.10; Figure 2D). Similarly, during uncoupled respiration (plus FCCP) supported by complex I+II substrates ( $ETS_{CI+II}$ ) there was no effect of exercise intensity on  $JH_2O_2$  but this tended to be increased by ~95% ( $p=0.072$ ; ES 0.63, 95% CI 0.26 to 0.99; Figure 2E) at 3HR compared to EX across all protocols. However,  $JH_2O_2$  was unaffected by exercise during uncoupled respiration supported by complex-II only (plus rotenone), (Figure 2F). Expressed as a ratio relative to  $JO_2$ ,  $JH_2O_2$  was lower in  $LEAK_{CI}$  at EX vs. BASE ( $p=0.030$ ; ES 1.01, 95% CI -1.47 to -0.54), but during all other respiratory states the overall effects of exercise on this ratio closely reflected absolute  $JH_2O_2$  rates.

## Protein phosphorylation responses to exercise

There were no effects of exercise intensity on phosphorylation of AMPK<sup>Thr172</sup> ( $p=0.197$ ), ACC $\beta$ <sup>Ser221</sup> ( $p=0.490$ ), or HSP27<sup>Ser82</sup> ( $p=0.568$ ); however, each of these increased by ~1.5 fold ( $p=0.001$ ; ES 0.86, 95% CI 0.52 to 1.19; Figure 3B), ~2.5 fold ( $p<0.001$ ; ES 2.15, 95% CI 1.68 to 2.62; Figure 3C) ~2.5 fold ( $p=0.051$ ; ES 1.09, 95% CI 0.62 to 1.57; Figure 3D) across all exercise protocols at EX relative to BASE, respectively.

## Exercise and redox sensitive gene expression

There were no significant effects of exercise intensity, however there were main effects for increased skeletal muscle mRNA levels of *PPARGC1A* ( $p=0.027$ ; ES 0.86, 95% CI 0.47 to 1.25), *UCP3* ( $p=0.027$ ; ES 0.70, 95% CI 0.31 to 1.09), *BNIP3* ( $p=0.010$ ; ES 0.48, 95% CI 0.09 to 0.88) and *PRDX1* ( $p=0.034$ ; ES 0.36, 95% CI -0.12 to 0.83) at 3HR relative to BASE across all exercise protocols (Figure 4 A and B). There were trends for increases in *MFN2* ( $p=0.057$ ; ES 0.37, 95% CI -0.03 to 0.76), *DRP1* ( $p=0.091$ ; ES 0.45, 95% CI 0.06 to 0.85), *GPX1* ( $p=0.092$ ; ES 0.58, 95% CI 0.19 to 0.98) and *TXNRD1* ( $p=0.069$ ; ES 0.43, 95% CI 0.04 to 0.82) mRNA levels at 3HR compared with BASE across all protocols, while there were no main effects of time on *NFE2L2* ( $p=0.427$ ) or *SOD1* ( $p=0.282$ ) or *SOD2* mRNA ( $p=0.186$ ; Figure 4B).

## Skeletal muscle antioxidant and mitochondrial protein abundance

Overall, we found no effect of time nor exercise intensity in the expression levels of key antioxidant enzymes TRX and GPX1, the chaperone HSP72, mitochondria related proteins MFN2, DRP1, PGC1 $\alpha$  (Figure 5) or complexes I-V (Figure 6). However, there was significantly decreased abundance of PRDX1 by ~35% ( $p=0.033$ ; ES -0.69, 95% CI -0.95 to

-0.44; Figure 5B), TFAM by ~15% ( $p=0.007$ ; ES -0.34, 95% CI -0.54 to -0.14; Figure 5J) and a tendency for lower UCP3 by ~20% ( $p=0.078$ ; ES -0.56, 95% CI -0.82 to -0.30; Figure 5I) at 3HR compared with BASE across all exercise protocols. There was a significant main interaction effect of *exercise intensity x time* on thioredoxin reductase (TXNRD1) protein abundance ( $p=0.032$ ). Specifically, at EX, there was ~35% less TXNRD1 protein detected following the CMIE protocol compared with the SIE protocol ( $p=0.007$ ; ES -0.72, 95% CI -1.33 to -0.10; Figure 5E).

### Mitochondrial protein S-glutathionylation

No significant effects of exercise were found for S-glutathionylation of mitochondrial proteins ATP-synthase- $\alpha$  ( $p=0.975$ ), Complex-IV<sup>subunit2</sup> ( $p=0.931$ ), or UCP3 ( $p=0.668$ ; Figure 7).

## DISCUSSION

The present study design allowed for a direct within-subject comparison of mitochondrial responses to acute bouts of CMIE and HIIE on a work-matched basis, as well as comparisons of these with low-volume sprint interval exercise (~25% of the total work volume of CMIE/HIIE) in young healthy humans. The main findings were that regardless of the exercise protocol performed, peak mitochondrial H<sub>2</sub>O<sub>2</sub> emission (during non-phosphorylating complex-I+II supported respiration) was lower immediately post-exercise, yet rates of mitochondrial H<sub>2</sub>O<sub>2</sub> emission tended to be elevated three hours later during complex-I+II supported ADP-stimulated oxidative phosphorylation and also uncoupled respiratory states. These acute changes in mitochondrial oxidant emission occurred concomitantly with increases in mitochondrial oxygen consumption rates during non-

phosphorylating respiratory states immediately post-exercise, yet three hours post-exercise oxygen consumption was lower during oxidative phosphorylation and uncoupled respiratory rates. Consistent with these acute mitochondrial responses to exercise, a range of key muscle metabolism-related protein phosphorylation events, as well as gene and protein expression of putative redox-sensitive targets generally increased to equivalent levels in the early post-exercise period regardless of protocol.

In the present study, higher exercise intensity had more pronounced effects on systemic physiologic responses as indicated via session  $HR_{peak}$  and RPE, in addition to blood lactate, glucose, and activity of specific stress-activated protein kinases, recently reported elsewhere (50, 51). Despite this, we found no exercise protocol-dependent effects on mitochondrial parameters. To establish where these divergent responses to different exercise protocols occurred (i.e. only at the mitochondria or also at the whole muscle level), we assessed key molecular markers of skeletal muscle energy metabolism and overall stress induced by the exercise protocols. Intriguingly, phosphorylation of AMPK<sup>Thr172</sup> (indicative of cellular bioenergetic perturbation) increased to a similar degree regardless of exercise protocol, as did its downstream substrate ACC $\beta$ <sup>Ser221</sup>. Furthermore, mRNA expression of *PPARGC1A*, the gene encoding PGC1 $\alpha$ , and a target of AMPK signaling, increased ~13 fold at 3HR, regardless of exercise protocol. Previously, Egan *et al.* (18) reported that high intensity continuous exercise generated greater mitochondrial biogenesis signaling than work-matched moderate intensity continuous exercise. A possible explanation for this is the longer exercise duration and that the lowest exercise intensity protocol employed in that study (18) was 40%  $\dot{V}O_{2peak}$ , whereas the lowest in the present study was ~55%  $\dot{V}O_{2peak}$ . Indeed, the 3 h post-exercise increases in *PPARGC1A* mRNA expression, p-AMPK and p-ACC in all intensities of the present study is similar to what was reported for their HIIE (80%

$\dot{V}O_{2peak}$ ) exercise. In support of this notion, Chen *et al.* (8) demonstrated that AMPK phosphorylation only occurred following exercise at 60%  $\dot{V}O_{2peak}$  and above, but not at 40%  $\dot{V}O_{2peak}$ . Recently, a well-designed study by MacInnis *et al.* (40) compared training responses to single-leg cycling CMIE in one leg and HIIE matched for both work and duration in the opposite leg. They reported superior mitochondrial adaptations (assessed via increased citrate synthase activity and mitochondrial respiratory rates) following HIIE training, although this occurred in a fiber-type dependent manner (40), the effects of which are likely an important factor in the long-term response to training (38). It is possible that there were additional factors not controlled for in the present study that may contribute to a greater response to repeated bouts (i.e. training) of HIIE compared to CMIE, such as number of transitions between work and rest (10). Nevertheless, our findings are consistent with other studies comparing HIIE to CMIE, that have reported equivalent, but not greater adaptive responses when matched for total work performed (3, 72). Interestingly, we also found that SIE led to equivalent mitochondrial and signaling responses as the CMIE and HIIE, despite consisting of considerably less total work. This suggests that the stimulus provided by each of the exercise protocols in the present study reached a threshold at least sufficient for activation of the assessed signaling pathways in muscle. Indeed, this is in line with earlier findings demonstrating the efficacy of low volume SIE (5, 22, 25). However, the complexity of the molecular signals generated and transduced in response to exercise should not be understated, since other signaling pathways can be differentially activated to ultimately elicit specific training adaptations to distinct exercise modalities (51). Taken together, our findings nevertheless support the notion that similarly robust molecular signals can be generated in skeletal muscle in response to a broad range of exercise stimuli.

Relatively few studies have investigated acute post-exercise changes in mitochondrial function. Given that  $O_2^{\cdot-}/H_2O_2$  generation is intrinsically linked to the respiratory state (11), the present findings of altered mitochondrial  $H_2O_2$  emission in the hours post-exercise in a respiratory state-dependent manner is consistent with previous reports from our group and others showing that exercise acutely alters mitochondrial respiration (37, 63, 64, 66). Despite the lack of effect of exercise intensity, there was a robust and dynamic effect of acute exercise on mitochondrial function, such as decreasing post-exercise  $JH_2O_2$  during the succinate driven  $LEAK_{CI+II}$  respiration state. Succinate-driven  $JH_2O_2$  formation occurs primarily via superoxide generation due to reverse electron flow through the flavin mononucleotide site in complex-I under experimental conditions of high inner mitochondrial membrane potential in the absence of ADP (46). Conceivably, high membrane potential could occur during situations of prolonged low ATP demand (24) such as with physical inactivity and sedentary lifestyle. Our findings therefore suggest a mechanism by which exercise may decrease  $JH_2O_2$  in the post-exercise ‘basal’ respiratory state. This may be pertinent for attenuation of oxidative stress that has been associated with various pathophysiologic states including insulin resistance (32).

The decreased post-exercise  $H_2O_2$  emission under reverse electron flow mitochondrial respiratory conditions may be attributed to increased proton leak (i.e. uncoupling) at the inner mitochondrial membrane in response to exercise. This is supported by our finding of simultaneously increased post-exercise  $O_2$  flux under the same  $LEAK_{CI+II}$  respiratory state, and is consistent with findings from an earlier study using permeabilized muscle mitochondria in young, healthy humans (63). The increased post exercise mitochondrial membrane proton leak would also decrease proton-motive force available to drive ATP synthesis. This supports our observation of lower state-3 ADP stimulated oxidative-

phosphorylation (*OXPHOS*) respiration at three hours post-exercise, and is comparable to the changes observed in permeabilized muscle mitochondria following high-intensity running in horses (69). One previous study in humans reported no change in *OXPHOS*  $JO_2$  following exhaustive human exercise (64). However, their measurements were made under conditions where the flux of substrates through the ETS would be submaximal, since only complex-I substrates were used with no convergent electron input from the complex-II substrate succinate, likely masking any effect of exercise on maximal *OXPHOS* activity. It should be noted, however, that we cannot exclude the potential contribution of pyruvate dehydrogenase activity, which is well known to be regulated by exercise (54). Given that we used pyruvate as the sole complex-I substrate, this may affect substrate availability for complex-I in our experimental system. Nevertheless, during this *OXPHOS*<sub>CI+II</sub> respiratory state, we found a trend for elevated  $JH_2O_2$  at 3 h post exercise and a significant elevation with uncoupled *ETS*<sub>CI+II</sub>. This is despite the ETS functioning in the ‘normal’ forwards direction under these respiratory states (i.e. any  $O_2^{\cdot-}/H_2O_2$  formed not via reverse electron flow). This suggests that the elevated  $O_2^{\cdot-}/H_2O_2$  formed specifically in this ADP stimulated state-3 respiratory state may be attributed to altered ETS respiratory complex activity, potentially via exercise-induced post-translational modifications. Intriguingly, the trend for elevated  $JH_2O_2$  at 3HR during uncoupled respiration was absent after the addition of rotenone (*ETS*<sub>CII</sub>), suggesting an effect of exercise directly or indirectly at the complex-I<sub>Q</sub> site (74). While it should be noted that the use of inhibitors and saturating substrate concentrations used in our *ex vivo* preparation may not recapitulate the native *in vivo* cellular environment and rates of superoxide formation, these findings nonetheless highlight that acute exercise can modify a) mitochondrial inner membrane proton leak and b) ETS derived  $O_2^{\cdot-}/H_2O_2$  emission characteristics in the hours post-exercise.



Mitochondrial function may be regulated by redox-mediated post translational modifications such as S-glutathionylation of cysteine residues within ETS proteins (42). To investigate this possibility in the context of exercise, we probed S-glutathionylation of mitochondrial ETS subunits including ATP-synthase subunit- $\alpha$  (complex-V) and cytochrome c oxidase subunit 2 (complex-IV) using the mitochondrial cocktail antibody following immunoprecipitation with anti-GSH (Figure 7). We were unable to observe any significant effects of exercise on these, possibly as a result of limited sample material only allowing for  $n=4$ . While the NDUF8 complex-I subunit detected by the commonly used mitochondrial cocktail antibody did not display detectable levels of S-glutathionylation, other subunits of complex-I such as NDUF7 and NDUFV1 contain iron-sulphur clusters susceptible to oxidation and are known regulators of complex-I  $O_2^{\cdot-}/H_2O_2$  generation (17, 23). We also probed for S-glutathionylation of UCP3, which is known to regulate inner mitochondrial membrane potential and thereby modulate respiratory function and rates of superoxide formation (43). Although we also did not detect significant effects of exercise on this, future studies may utilize mass spectrometry to investigate these and other redox mediated post-translational protein modifications in further detail (33).

We observed a decrease in PRDX1 protein abundance in muscle at 3HR, a cytosolic protein with low  $K_M$  for  $H_2O_2$  (i.e. scavenges low levels of  $H_2O_2$ ) (6). The decreased PRDX1 abundance at 3HR could impair the scavenging of mitochondrial  $H_2O_2$ , allowing localized ROS accumulation for the induction of redox signaling. This decrease is consistent with a recent report demonstrating that peroxiredoxins are rapidly degraded by ubiquitin mediated processes after being oxidized (59). Potentially in response to this, there was a small yet significant increase in *PRDX1* mRNA levels 3 hours post-exercise, supporting the notion that the PRDX/TRX antioxidant pathway plays an important role in exercise-induced redox

signaling (70). TXNRD1 is a cytosolic protein that reduces TRX using NADPH, to in turn reduce PRDX. Interestingly, TXNRD1 protein content was lower after CMIE compared to SIE. This was the sole indication of a significant exercise protocol-dependent effect on muscle redox homeostasis in the present study. It is possible that similar mechanisms exist for the degradation of TXNRD1 similar to that of peroxiredoxins (59). We recently reported elsewhere that specific components of muscle and plasma redox homeostasis pathways were affected by exercise intensity (50). Therefore, it is tempting to speculate that different exercise protocols may exert subtle, yet important effects in fine-tuning specific aspects of muscle redox homeostasis, which warrants further investigation.

The *NFE2L2* gene encodes NRF2, a redox-sensitive transcription factor and master-regulator of the antioxidant transcriptional response (15). Although *NFE2L2* mRNA was unchanged 3 hours after exercise, it is likely that this would have peaked and returned to baseline levels before the 3HR time point (15, 48). Interestingly, downstream gene targets of NRF2 were not significantly affected by exercise: *SOD1*, *SOD2* and *GPX1* mRNA expression, although there was a small yet significant increase in *PRDX1* mRNA at 3 h post-exercise. It is possible that the exercise protocols in the present study, irrespective of work, were not sufficient for full activation of the NRF2 transcriptional response, or at least at the time points assessed. Among other putative redox and exercise sensitive responses assessed, cytosolic heat-shock protein HSP27 phosphorylation increased post-exercise as expected, while total abundance of the higher molecular weight HSP72 protein was unaffected. Previously, exercise intensity-dependent increases of HSP72 were shown in rat skeletal muscle (44) as well as 3 days after HIIE in humans (9), and this has also been shown to occur via an exercise-induced  $O_2^{\cdot-}/H_2O_2$  pathway (58). However, numerous environmental factors and molecular signals can also induce HSP72 expression in addition to  $O_2^{\cdot-}/H_2O_2$  (14, 56).

We observed an increase in *BNIP3* mRNA expression 3 hours post-exercise which is involved in the mitochondrial quality control processes via mitophagy (75). Mitophagy has previously been shown to be affected by exercise via both PGC1 $\alpha$  (68) and O<sub>2</sub><sup>·-</sup>/H<sub>2</sub>O<sub>2</sub> (36). Our data suggest this response is not differentially affected by exercise intensity in human muscle, consistent with many of the other mitochondrial parameters measured in this study. Also unaffected by the present exercise protocols was abundance of DRP1 and MFN2, which regulate outer mitochondrial membrane fission and fusion, respectively. MFN2 protein levels did however show a tendency to increase to a greater extent at 3 hours post-exercise with SIE compared with CMIE. This is in line with previous literature showing that MFN2 protein was unchanged after 3 h of ‘low’ intensity voluntary wheel running in mice (52) yet increased 3 h after 60 min of exercise at 70%  $\dot{V}O_{2peak}$  in healthy middle aged humans (35). Nevertheless, it should be noted that post-exercise mitochondrial dynamics are most likely determined by acute post-translational modifications to fission/fusion proteins rather than their total abundance alone (65).

While this randomized crossover study design provided a number of novel findings, some potential limitations should also be considered. The small sample size and sex-distribution (male, *n*=6, female, *n*=2) in the present study precludes the detection of potentially subtle sex-dependent differences in responses to exercise (20). Although we tested females during the early follicular phase of the menstrual cycle to minimize the impact of ovarian hormone fluctuations, and that skeletal muscle mitochondrial respiratory function was previously shown to be equivalent between men and women (61), sex-specific effects should nevertheless be considered in future investigations. Higher intensity exercise involves the recruitment of a greater proportion of fast twitch fibers (73), however, in the present study fiber-type specific responses were not assessed which could potentially mask some exercise-

intensity specific effects as recently reported (40). In our mitochondrial respiration experiment, addition of succinate prior to ADP in the absence of rotenone stimulates maximal levels of superoxide production due to reverse electron transfer, and the effect of exercise on this was a key outcome measure in the present study. However, it should be acknowledged that the ROS generated in this respiratory state could influence subsequent respiratory measures by altering redox sensitive components of the ETS or other proteins such as the adenine nucleotide translocase (ANT). Due to limited sample material it was not possible to measure cellular glutathione levels (GSH and the oxidized form, GSSG) which would have been informative to understand whether the observed post exercise changes in  $\text{JH}_2\text{O}_2$  were primarily due to altered rates of ROS generation alone or whether changes in GSH mediated oxidant scavenging also contributed to this effect. It is also possible that other non-ETS mitochondrial enzymes not assessed in the present study could contribute to the background net emission of mitochondrial  $\text{H}_2\text{O}_2$ . Lastly, it should be noted that while the Amplex UltraRed assay is intended to report mitochondrial  $\text{H}_2\text{O}_2$  emission towards the cytosol, it may not be exclusive to this since the fluorescent reaction product, resorufin, has been shown to interact with intracellular sources of peroxides and/or peroxynitrites (13) or carboxylesterases (45). Thus, it is conceivable that this could confound absolute quantification of  $\text{JH}_2\text{O}_2$  in the present study. However, the relative changes in  $\text{JH}_2\text{O}_2$  observed can be attributed to specific ETS sites, since these effects were observed only with their respective site-specific substrate and/or inhibitor combination.

## **Perspectives and significance**

In conclusion, we provide novel evidence that mitochondrial function (respiration and  $\text{H}_2\text{O}_2$  emission) in human skeletal muscle are transiently altered in a respiratory state-dependent manner in the hours following continuous moderate and high intensity interval

exercise irrespective of whether these exercise modes are matched for total work. Moreover, regardless of exercise protocol, there were comparable responses across a range of known and putative redox and exercise-sensitive transcriptional and protein responses. Importantly, a total of only two minutes of sprint interval exercise was sufficient to elicit similar responses as 30 minutes of continuous moderate intensity aerobic exercise. This suggests that exercise may be prescribed according to individual preferences while still generating similar signals known to confer beneficial metabolic adaptations. These findings have important implications for improving our understanding of how exercise can be used to enhance metabolic health in the general population.

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**Table 1: List of primer sequences for real-time PCR**

Gene	NCBI RefSeq	Forward primer 5' - 3'	Reverse primer 5' - 3'
<i>β-2M</i>	NM_004048.2	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
<i>BNIP3</i>	NM_004052.3	TGGACGGAGTAGCTCCAAGA	AAAGAGGAACTCCTTGGGGG
<i>DRP1</i>	NM_012062.4	CACCCGGAGACCTCTCATTC	CCCCATTCTTCTGCTTCCAC
<i>GPX1</i>	NM_000581.2	CGCCACCGCGCTTATGACCG	GCAGCACTGCAACTGCCAAGCAG
<i>MFN2</i>	NM_014874.3	CCCCCTTGTCTTTATGCTGATGTT	TTTTGGGAGAGGTGTTGCTTATTTC
<i>NFE2L2</i>	NM_006164.4	AAGTGACAAGATGGGCTGCT	TGGACCACTGTATGGGATCA
<i>PPARGC1A</i>	NM_013261.3	GGCAGAAGGCAATTGAAGAG	TCAAAACGGTCCCTCAGTTC
<i>PRDX1</i>	NM_001202431.1	CCCAACTTCAAAGCCACAGC	AAAGGCCCTGAACGAGATG
<i>SOD1</i>	NM_000454.4	GGTCCTCACTTTAATCCTCTAT	CATCTTTGTCAGCAGTCACATT
<i>SOD2</i>	NM_001024465.1	CTGGACAAACCTCAGCCCTA	TGATGGCTTCCAGCAACTC
<i>TXNRD1</i>	NM_003330.3	AGCATGTGATGTGAGGACGG	AGAGTCTTGCAGGGCTTGTC
<i>UCP3</i>	NM_003356.3	CCACAGCCTTCTACAAGGGATTTA	ACGAACATCACCACGTTCCA

Abbreviations: *β-2M*,  $\beta_2$  microglobulin; *BNIP3*, BCL2/adenovirus E1B 19kDa interacting protein 3; *DRP1*, Dynamin 1-like protein; *GPX1*, Glutathione peroxidase-1; *MFN2*, Mitofusin-2; *NFE2L2*, Nuclear factor erythroid 2-related factor 2; *PPARGC1A*, Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; *PRDX1*, Peroxiredoxin-1; *SOD1*, Cytosolic superoxide dismutase 1; *SOD2*, Mitochondrial manganese superoxide dismutase; *TXNRD1*, Thioredoxin reductase 1; *UCP3*, Uncoupling protein-3.

853 **Table 2: Physiological demands and responses to acute exercise protocols**

	CMIE	HIIE	SIE
Total exercise session time incl. rest periods (min)	30 ± 0 <sup>bc</sup>	24 ± 0 <sup>ac</sup>	15 ± 0 <sup>ab</sup>
Exercise time (min)	30 ± 0 <sup>bc</sup>	20 ± 0 <sup>ac</sup>	2 ± 0 <sup>ab</sup>
Mean power output (Watts)	163 ± 36 <sup>bc</sup>	245 ± 54 <sup>ac</sup>	645 ± 71 <sup>ab</sup>
Total mechanical work (kJ)	294 ± 65 <sup>c</sup>	294 ± 65 <sup>c</sup>	76 ± 14 <sup>ab</sup>
Intensity (% PPO)	50 ± 0 <sup>bc</sup>	75 ± 0 <sup>ac</sup>	198 ± 25 <sup>ab</sup>
Intensity (% $\dot{V}O_{2peak}$ )	54 ± 3 <sup>bc</sup>	77 ± 1 <sup>ac</sup>	-
HR, session peak (bpm)	158 ± 15 <sup>b</sup>	182 ± 11 <sup>ac</sup>	168 ± 9 <sup>b</sup>
RPE, session peak (AU)	13 ± 2 <sup>bc</sup>	18 ± 1 <sup>ac</sup>	20 ± 0 <sup>ab</sup>

854 CMIE, continuous moderate intensity exercise; HIIE, high intensity interval exercise; SIE, sprint interval  
855 exercise; RPE, rating of perceived exertion (6-20). Data are mean ± SD, n=8. <sup>a</sup>*p*<0.05, compared to CMIE;  
856 <sup>b</sup>*p*<0.05, compared to HIIE; <sup>c</sup>*p*<0.05, compared to SIE.  
857

## Figure legends

### Figure 1. Mitochondrial respiration from permeabilized human skeletal muscle fibers.

(A) Representative mitochondrial oxygen flux ( $JO_2$ ) trace depicts one sample in a single chamber from a baseline condition. Light gray line (left y-axis) is chamber  $O_2$  concentration, dark line (right y-axis) is mitochondrial  $O_2$  consumption ( $JO_2$ ) rate throughout the substrate inhibitor uncoupled titration (SUIT) protocol. Various respiratory states were induced as follows: (B) state-4 leak (*LEAK*) supported by complex-I substrates malate and pyruvate, (C) addition of succinate for complex-II, (D) ADP (5 mM) stimulated state-3 (*OXPHOS*) and (E) uncoupled (*ETS*) states with complexes I+II substrate input or (F) complex-II only after rotenone complex-I inhibition. Muscle samples were taken at baseline (BASE), immediately after exercise (EX) and after 3 hours recovery (3HR). CMIE, continuous moderate intensity exercise; HIIE, high intensity interval exercise; SIE, sprint interval exercise; ww, wet-weight muscle. Data are mean  $\pm$  SD, for  $n=8$ . Main time effect  $p<0.05$ : #compared to BASE, †compared to EX.

### Figure 2. Mitochondrial $H_2O_2$ emission from permeabilized human muscle fibers.

(A) Representative mitochondrial hydrogen peroxide trace depicts one sample in a single chamber from a baseline condition. Light gray line (left y-axis) is cumulative Amplex UltraRed fluorescent reaction product in the chamber proportional to  $H_2O_2$  formation, dark line (right y-axis) is  $H_2O_2$  emission rate ( $JH_2O_2$ ) throughout the substrate inhibitor uncoupled titration (SUIT) protocol used to induce various respiratory states: (B) state-4 leak (*LEAK*) supported by complex-I substrates malate and pyruvate, (C) addition of succinate for complex-II. (D) ADP (5 mM) stimulated state-3 (*OXPHOS*) and (E) uncoupled (*ETS*) states with complexes I+II substrate input or (F) complex-II only after rotenone complex-I inhibition. Muscle samples were taken at baseline (BASE), immediately after exercise (EX) and after 3 hours recovery (3HR). CMIE, continuous moderate intensity exercise; HIIE, high intensity interval exercise; SIE, sprint interval exercise; ww, wet-weight muscle. Data are mean  $\pm$  SD, for  $n=8$ . Main time effect  $p<0.05$  unless otherwise stated: #compared to BASE, †compared to EX.

### Figure 3. Muscle protein phosphorylation responses to exercise.

Representative western blots (A) of phosphorylated acetyl-coA carboxylase at serine 221 (B), AMP activated protein kinase at threonine 172 (C) and heat shock protein of 27 kDa serine 82 (D); before (BASE), immediately (EX) and 3 hours (3HR) after continuous moderate intensity (CMIE), high intensity interval (HIIE) and sprint interval exercise (SIE). Representative blots are from a single participants' samples. Blot densitometry was normalized to stain-free total protein, and quantified relative to standard curves generated on each membrane (not shown). Data are mean  $\pm$  SD,  $n=8$ . Main time effect  $p<0.05$  unless otherwise stated: #compared to BASE, †compared to EX.

### Figure 4. mRNA expression of genes associated with mitochondrial biogenesis, morphology and mitophagy (A), and redox homeostasis (B) 3h after the different exercise bouts.

Muscle mRNA expression analysed by qPCR is fold-change normalized to a housekeeping gene,  $\beta 2$ -microglobulin ( $\beta 2M$ ) at 3 hours post exercise relative to respective baseline (depicted by dashed line). CMIE, continuous moderate intensity exercise; HIIE, high intensity interval exercise; SIE, sprint interval exercise. Data are mean  $\pm$  SD,  $n=8$ . Main time effect across all exercise protocols: #  $p<0.05$  compared to baseline.

**Figure 5. Proteins involved in redox homeostasis and mitochondrial function.**

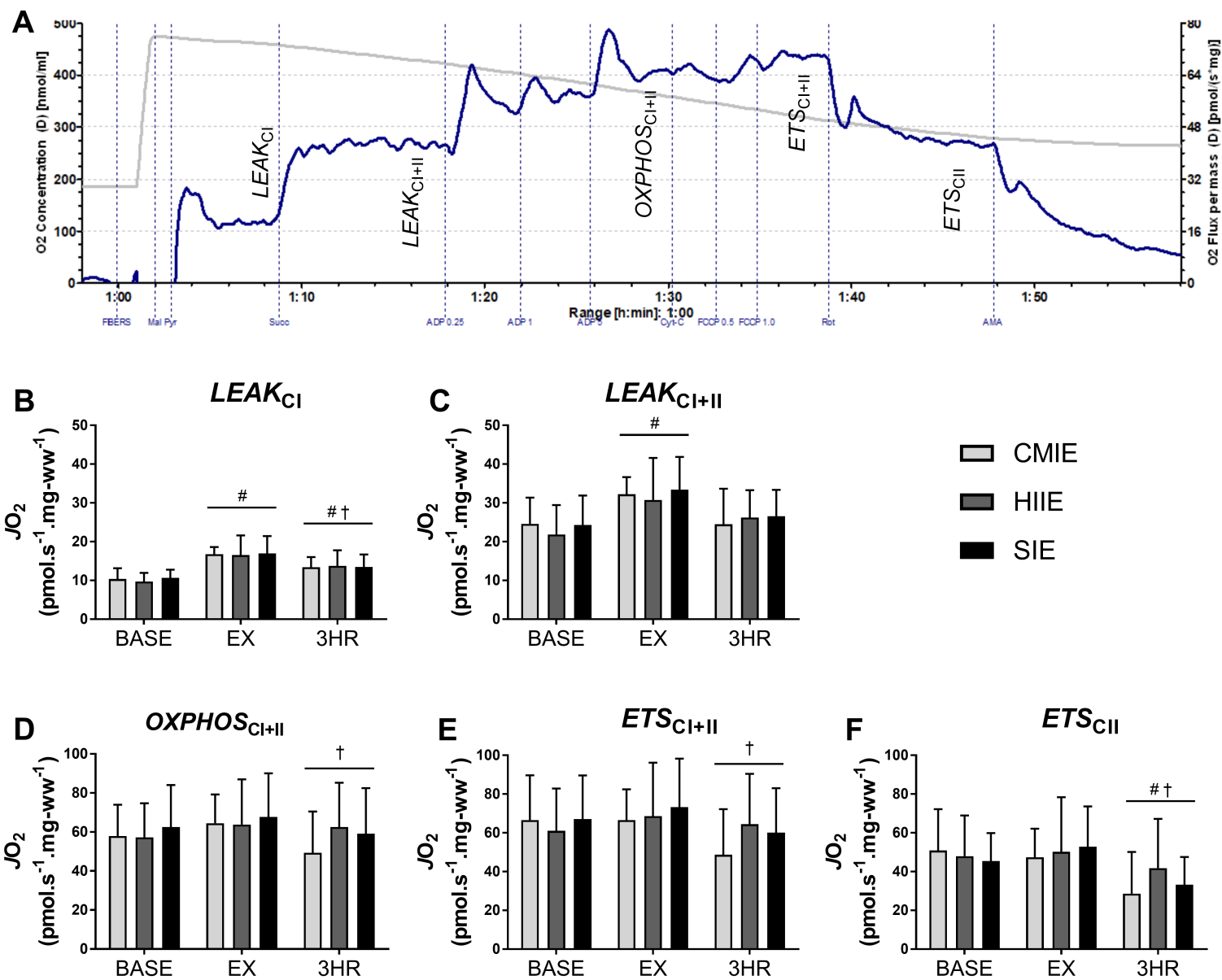
(A) Representative western blots. Blots were quantified for antioxidant proteins (B) peroxiredoxin, (C) thioredoxin, (D) glutathione peroxidase-1, and (E) thioredoxin reductase-1; mitochondrial morphology proteins (F) mitofusin-2 and (G) dynamin-related protein-1; mitochondrial proteins (H) peroxisome proliferator-activated receptor gamma coactivator 1-alpha, (I) uncoupling protein 3 and (J) mitochondrial transcription factor A; and (K) heat-shock protein of 72 kDa. Blot densitometry was normalized to stain-free total protein, and quantified relative to internal calibration curves on each membrane. Exercise was continuous moderate intensity (CMIE), high intensity interval (HIIE) and sprint interval (SIE) exercise, samples obtained at baseline (BASE), immediately post exercise (EX) and after 3 h recovery (3HR). Representative blots are shown from one subject. Data are mean  $\pm$  SD,  $n=8$ . Main time effect  $p<0.05$  unless otherwise stated: #compared to BASE, †compared to EX. \* $p<0.05$  exercise intensity effect.

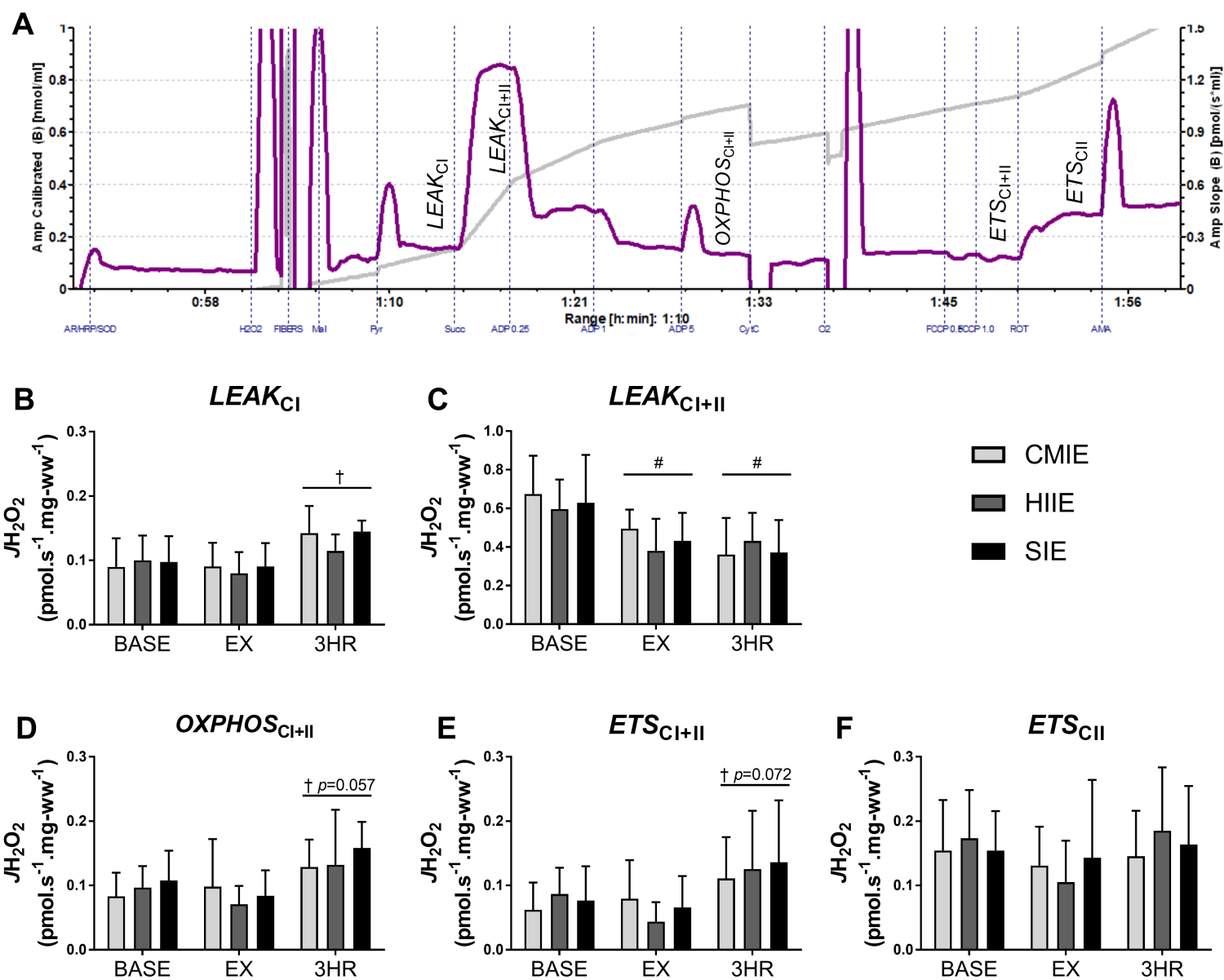
**Figure 6: Mitochondrial complex I – V protein abundance.**

Subunits of complex I (NDUFB8), II (SDHB), III (UQRCR2), IV subunit 2 (MTCO2), IV subunit 4 (COX-IV) and V (ATP5A) were assessed by Western blot (A-F, respectively). Exercise was continuous moderate intensity (CMIE), high intensity interval (HIIE) and sprint interval (SIE) exercise, samples obtained at baseline (BASE), immediately post exercise (EX) and after 3 h recovery (3HR). Blot densitometry was normalized to stain-free total protein, and quantified relative to internal calibration curves on each membrane. Representative blots are shown from one subject. Data are mean  $\pm$  SD,  $n=8$ .

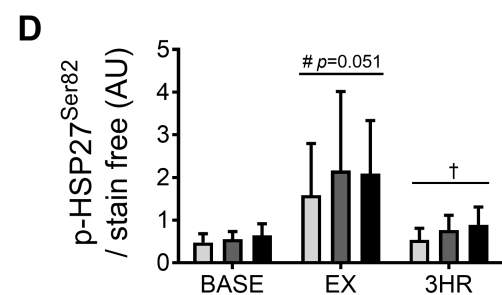
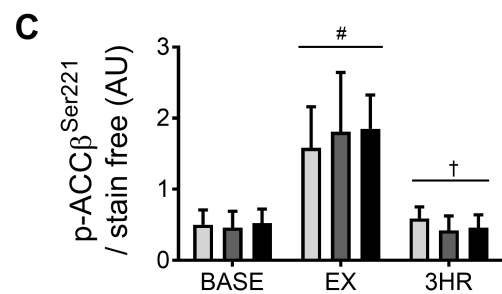
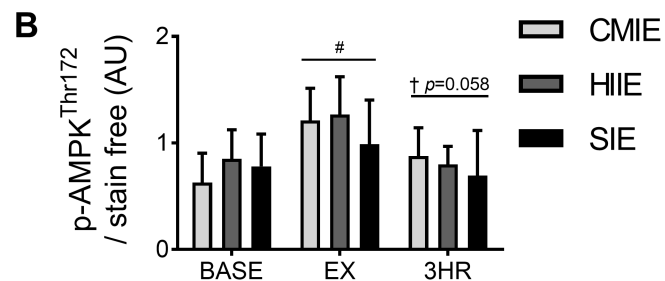
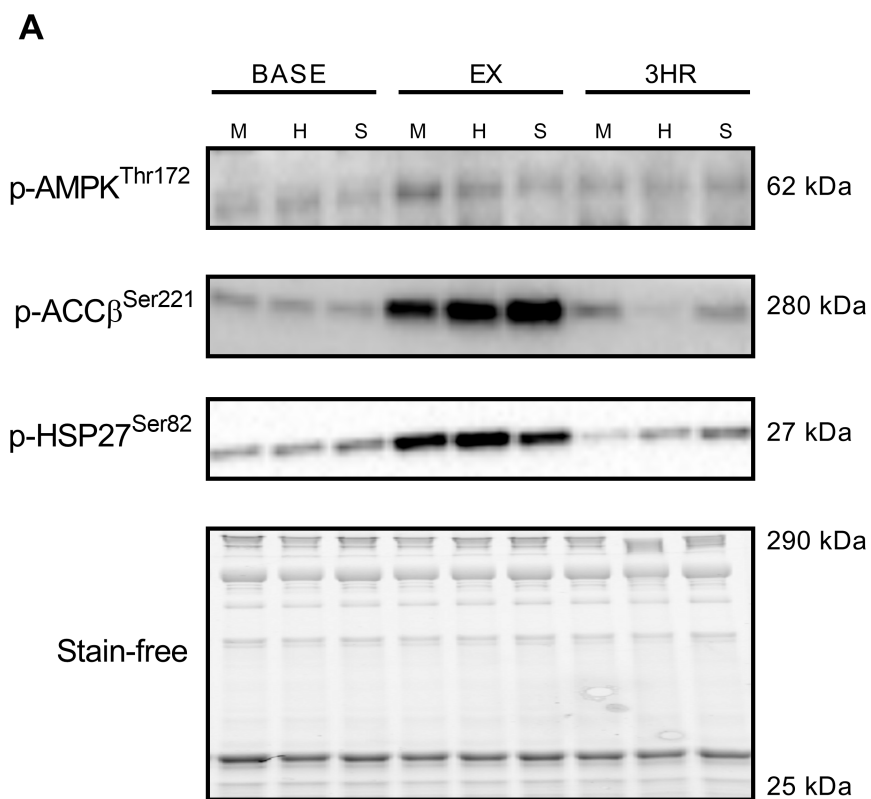
**Figure 7: Mitochondrial protein S-glutathionylation.**

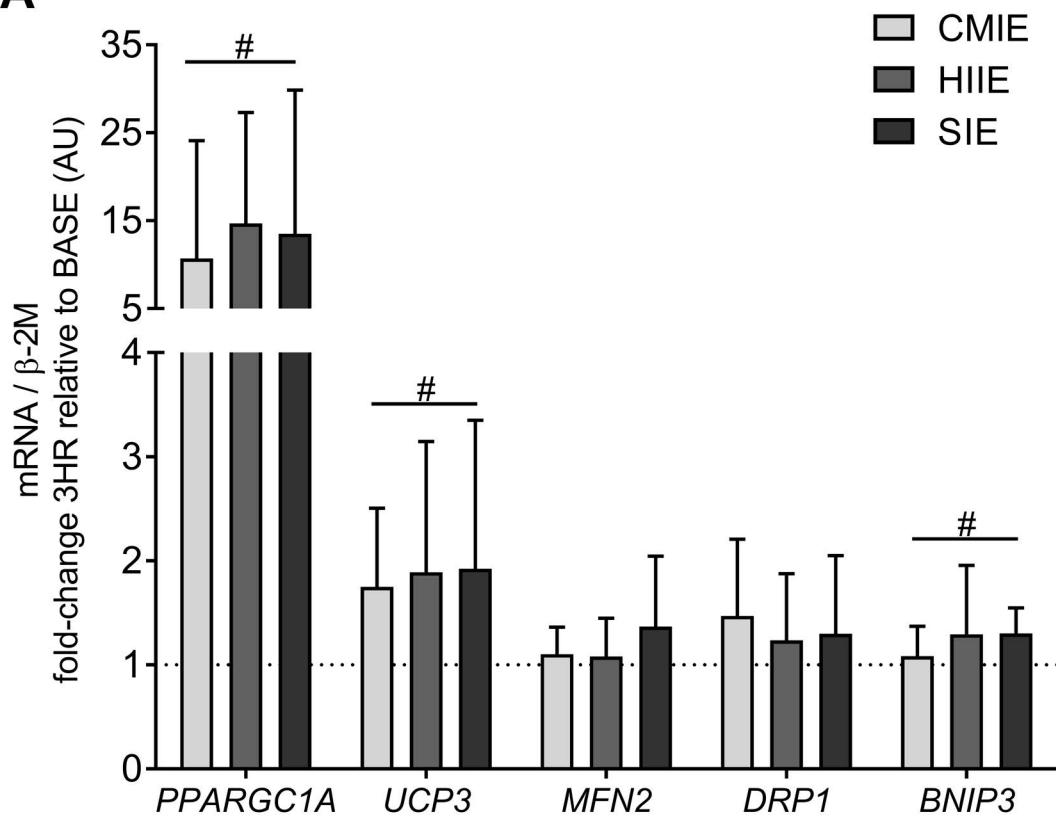
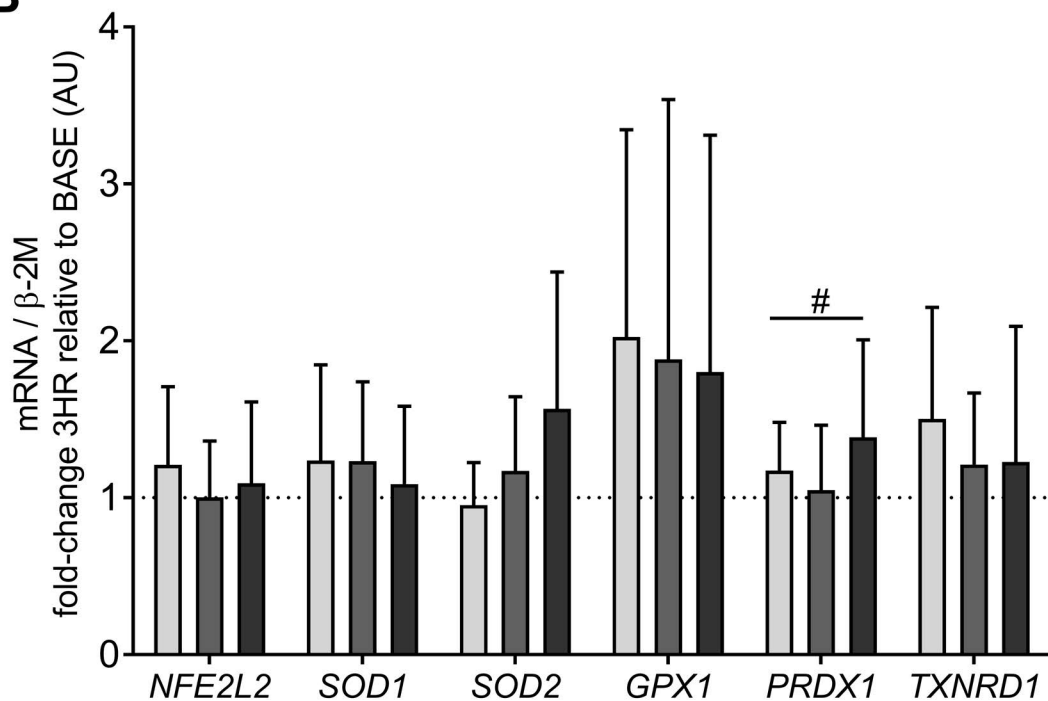
Protein (50  $\mu$ g) from whole muscle lysate was co-immunoprecipitated with anti-GSH antibody on sepharose beads, then detected via Western blot, as per *methods*. Representative images show mitochondrial complexes V (ATP5A), IV subunit 2 (MTCO2), and UCP-3 from one subject at each time point (A) and co-IP pull-down was confirmed on the same membrane using a negative control in the absence of anti-GSH antibody in the pull-down (No-IP), along with whole muscle lysate as positive control (lysate) (B). Protein S-glutathionylation was expressed relative to each respective baseline level of protein glutathionylation (C-E). Exercise was continuous moderate intensity (CMIE), high intensity interval (HIIE) and sprint interval (SIE) exercise, samples obtained at baseline (BASE), immediately post exercise (EX) and after 3 h recovery (3HR). Data are mean  $\pm$  SD for  $n=3-4$ .

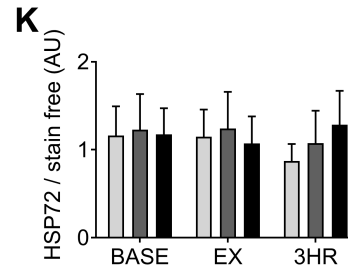
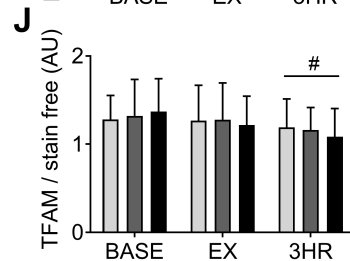
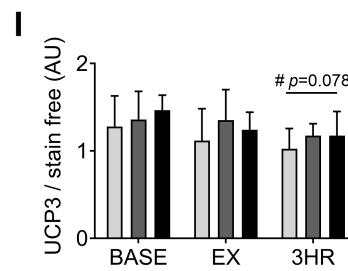
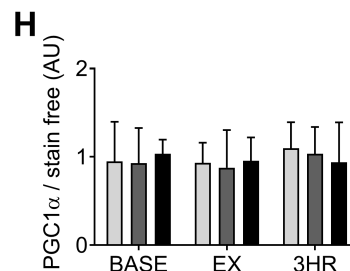
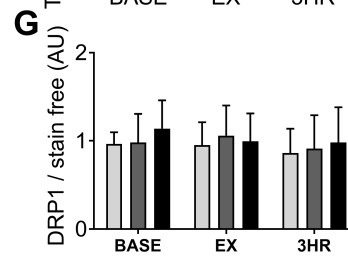
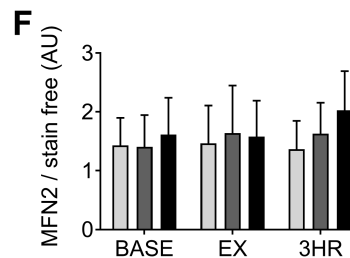
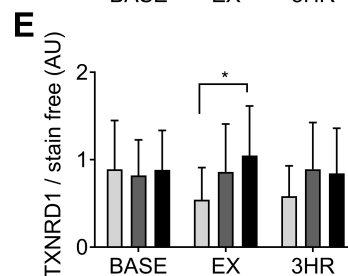
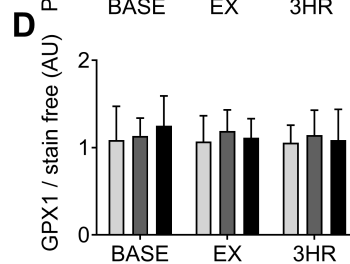
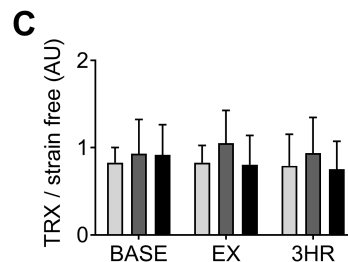
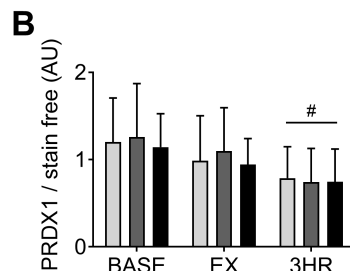
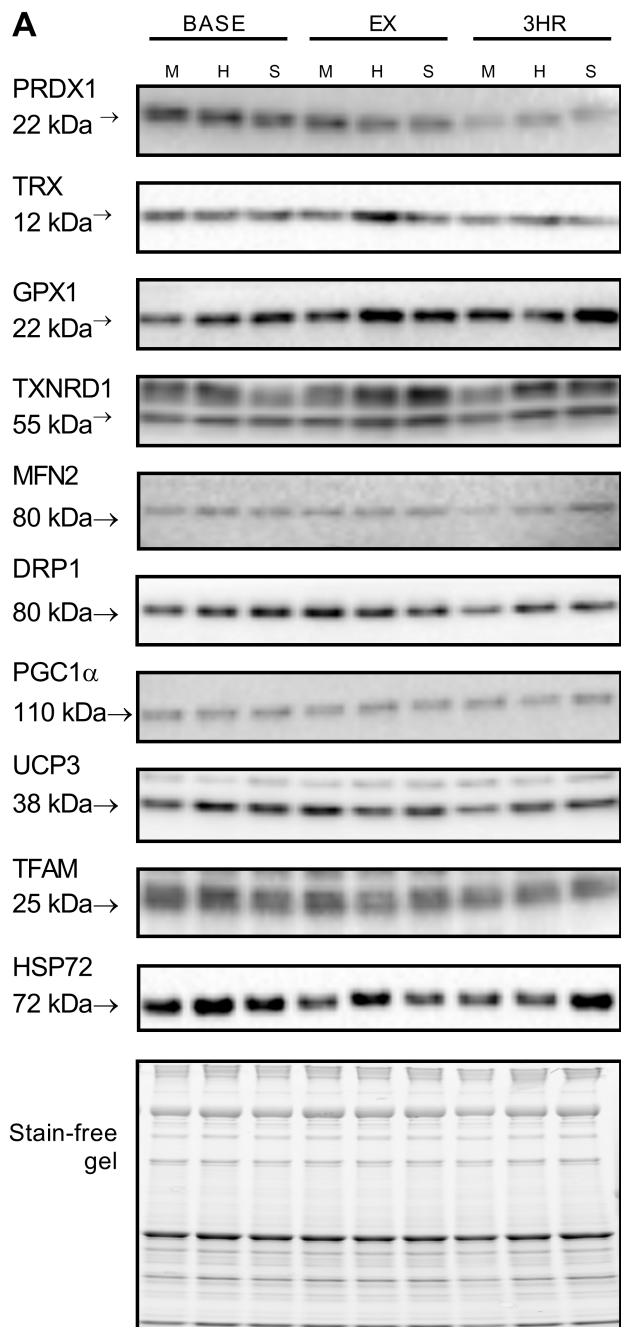




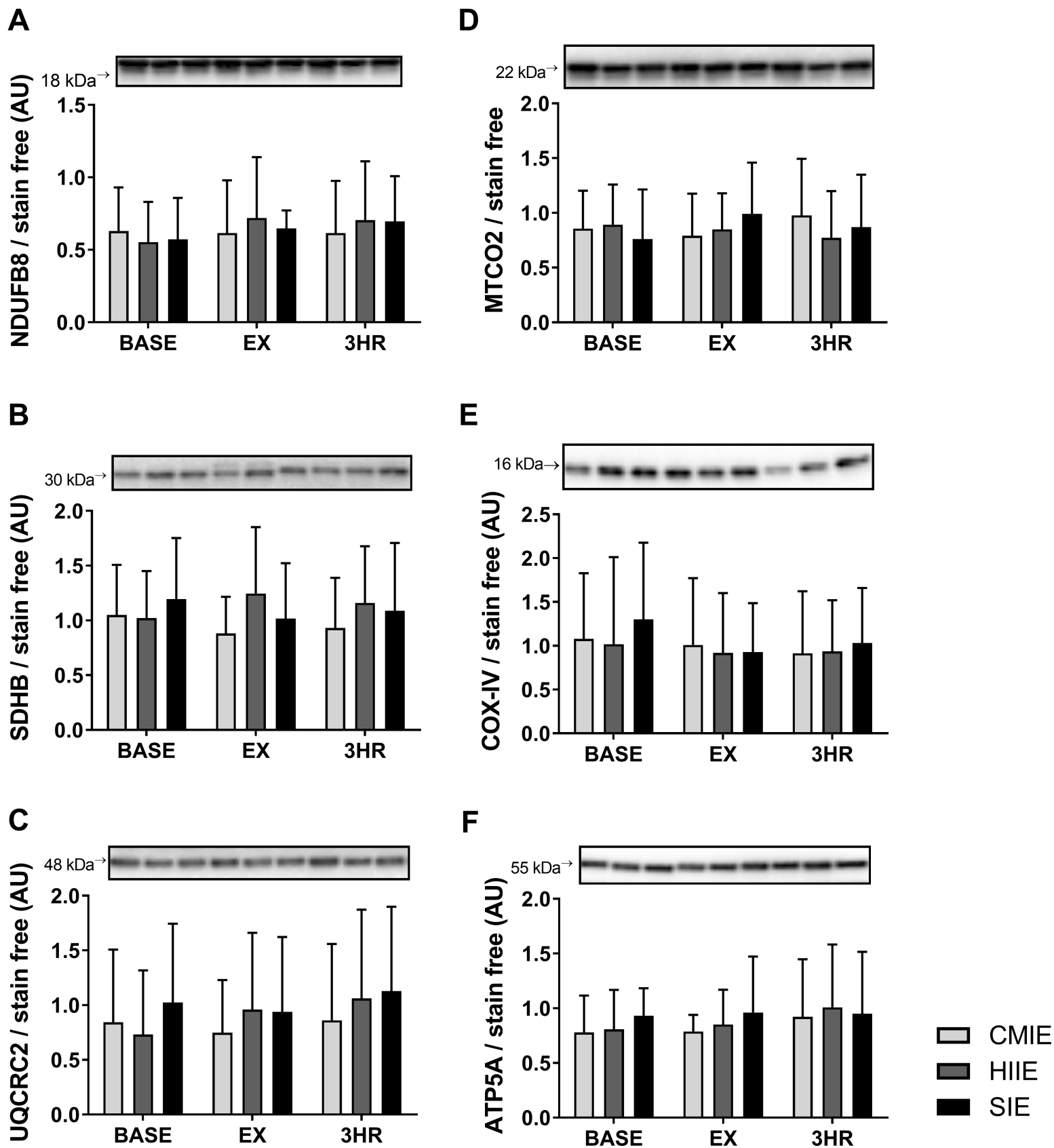




**A****B**



CMIE  
 HIIE  
 SIE



**A**

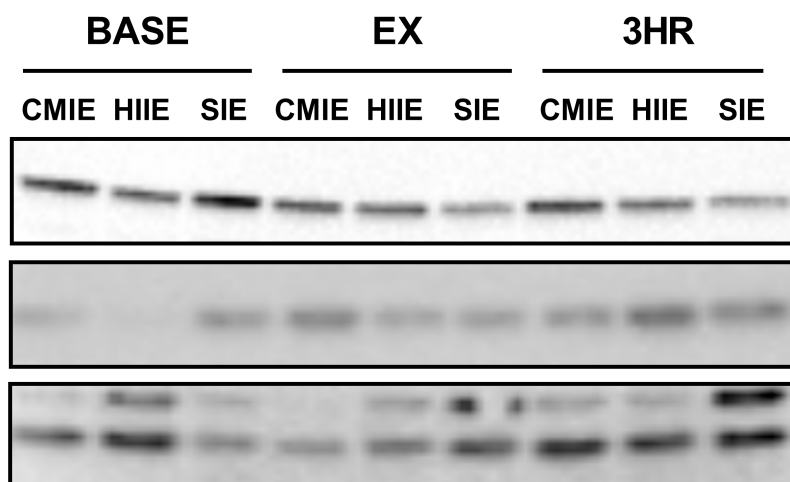
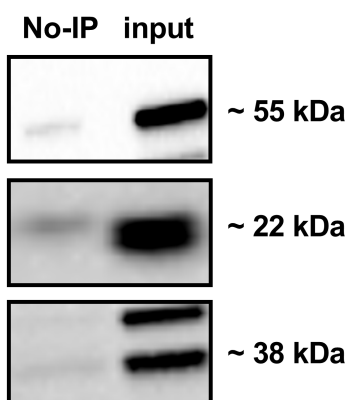
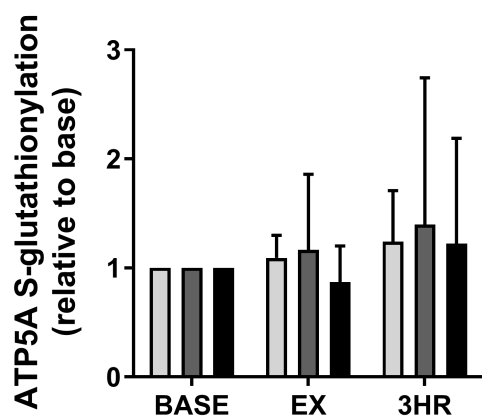
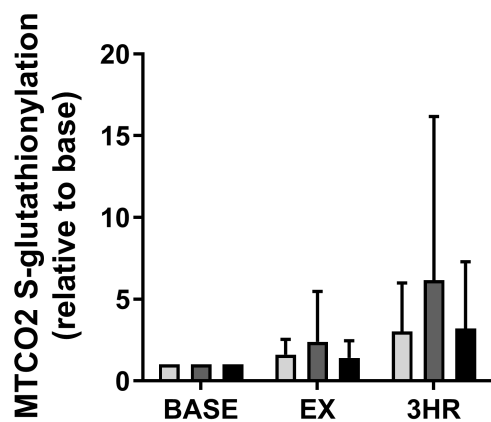
IP:  
Glutathione

WB:

ATP5A  
(Complex-V)

MTCO2  
(Complex-IVs2)

UCP3

**B****C****D****E**