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Monitoring changes in Thioredoxin and over-oxidised Peroxiredoxin in response to exercise in humans

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Abbreviations:

ANOVA: Analysis of Variance, CV: Coefficient of variance, DTNB: 5,5'-dithiobis (2-nitrobenzoic) acid, EDTA: ethylene diaminetetraacetic acid, FCS: Fetal Calf Serum, HIGH: high intensity steady state bout, HIIT: High intensity interval training, IL: Interleukin, LV-HIIT: Low volume high intensity interval training, MOD: moderate intensity steady state bout, NaCl: Sodium Chloride, NADPH: Nicotinamide adenine dinucleotide phosphate, PBMC: Peripheral Blood Mononuclear Cell, PRDX: Peroxiredoxin, RONS: Reactive oxygen and nitrogen species, RPM: repetitions per minute, RPMI: Roswell Park Memorial Institute SD: Standard deviation, -SH: Sulphur-hydryl, -SOH: Sulfenic acid, $-SO_2$: Sulfinic acid, $-SO_3$: Sulfonic acid, TNB: 5-thio-2-nitrobenzoic acid, TRX: Thioredoxin, TRX-R: Thioredoxin Reductase, VCO_2: Carbon dioxide consumption, $\dot{V}O_2$: Oxygen uptake, $\dot{V}O_{2\,MAX}$: Maximum oxygen uptake.

Abstract

Introduction: Peroxiredoxin (PRDX) and Thioredoxin (TRX) are antioxidant proteins that control cellular signalling and redox balance, although their response to exercise is unknown. This study aimed to assess key aspects of the PRDX-TRX redox cycle in response to three different modes of exercise.

Methods: Healthy males (n=10, mean ± SD: age 22 ± 3 yrs) undertook three exercise trials on separate days: two steady-state cycling trials at a moderate (60% $\dot{V}O_{2\,MAX}$; 27 min, MOD) and high (80% $\dot{V}O_{2\,MAX}$; 20 min, HIGH) intensity, and a low volume high intensity interval training trial (10×1 min 90% $\dot{V}O_{2\,MAX,}$ LV-HIIT). Peripheral blood mononuclear cells (PBMCs) were assessed for TRX-1 and over-oxidised PRDX (isoforms I-IV) protein expression before, during and 30 minutes following exercise (post+30). The activities of TRX reductase (TRX-R) and the NF-κB p65 subunit were also assessed.

Results: TRX-1 increased during exercise in all trials (MOD +84.5%; HIGH +64.1%; LV-HIIT +205.7%; $p<.05$), whereas over-oxidised PRDX increased during HIGH only (MOD -28.7%; HIGH +202.9%; LV-HIIT -22.7%; $p<.05$). TRX-R and NF-κB p65 activity increased during exercise in all trials, with the greatest response in TRX-R activity seen in HIGH ($p<.05$).

Discussion: All trials stimulated a transient increase in TRX-1 protein expression during exercise. Only HIGH induced a transient over-oxidation of PRDX, alongside the greatest change in TRX-R activity. Future studies are needed to clarify the significance of heightened peroxide exposure during continuous high intensity exercise and the mechanisms of PRDX-regulatory control.

Introduction

Exercise induces the production of reactive oxygen and nitrogen species (RONS), which act as important signalling molecules in the vast array of metabolic adaptations that take place in human tissues [1,2]. However, exercise of a certain intensity and duration can induce acute cellular oxidative stress, a state whereby RONS overwhelm endogenous antioxidant defence systems [3,4]. The exercise conditions required to achieve an optimal production of RONS in order to stimulate adaptive processes, *versus* RONS that may initiate damage, is currently unknown. Changes in markers of oxidative stress with exercise are commonly studied in cells of the immune system, such as peripheral blood mononuclear cells (PBMCs) [4,5] and recent work has focussed on the antioxidant proteins peroxiredoxin (PRDX) [6] and thioredoxin (TRX) [7]. PRDX and TRX are ubiquitous oxidoreductase proteins that contain thiol groups with a high capacity to control cellular levels of RONS and reduce oxidative stress [8,9]. PRDX can directly target and reduce biological peroxides such as hydrogen peroxide (H_2O_2), peroxynitrite and hydroperoxides [9]. TRX is central in maintaining the reduced state of various antioxidant peroxidase enzymes [10], including four isoforms (I-IV) of PRDX [11,12]. There is strong evidence that PRDX and TRX are central in modulating peroxide based signals within a variety of cell types [13]. Understanding how PRDX and TRX regulate the levels of RONS may be essential in aiding understanding of exercise-induced changes in RONS.

The oxidation states of TRX and PRDX have been studied extensively to facilitate the understanding of cellular signalling in health and disease. The catalytic cysteine of monomeric PRDX (20-30 kDa) can become oxidised by a peroxidase substrate to form sulfenic acid (-SOH), before rapidly reacting with an adjacent PRDX molecule to form a dimeric structure (*Figure 1*). TRX is the predominant antioxidant that reverses PRDX (isoforms I-IV) oxidation and dimer formation [11,14], by similarly utilising its catalytic

cysteine. TRX is subsequently maintained in a reduced state by the Nicotinamide adenine dinucleotide phosphate (NADPH) dependent enzyme TRX-reductase (TRX-R). Under conditions of high or prolonged peroxide exposure, PRDX has the capacity to become over–oxidised [15], and exceed the regulatory control of TRX [16]. Over-oxidation of PRDX forms sulfinic ($-SO_2$) and sulfonic acid ($-SO_3$) PRDX oxidation states (PRDX-SO_{2-3}) that have limited or no peroxidase activity respectively [17].

Previous work has highlighted that exercise can enhance the degree of PRDX over-oxidation (I-IV) in erythrocytes [18]. Only one study has previously addressed exercise-induced changes to PBMC PRDX oxidation states in humans. Turner et al, [6] observed an increase in PRDX over-oxidation following an ultra-endurance race (126.7 - 233.4km; 20.4 - 41.4 hours of continuous running) in middle aged men. The role of TRX in this redox cycle has not been previously monitored in response to exercise. The only study that has assessed TRX in humans reported an increase in plasma TRX in response to an ultra-endurance race [19]. The dissociation of TRX from TRX-interacting protein (TXNIP) and transcriptional activation of TRX via NF-κB are likely mechanisms governing this observed extracellular secretion. Indeed, RONS have been shown to upregulate the expression of a variety of antioxidant enzymes via increased NF-κB activity [20] and TRX can dissociate from TXNIP in a RONS-dependent manner [21].

To our knowledge no studies have monitored the over-oxidation of PBMC PRDX isoforms (I-IV) and their associations with TRX in response to modes of exercise that are more commonly undertaken (i.e. <30 minutes). Further, no studies have examined the impact of exercise intensity on these redox processes. The aim of the present study was to investigate perturbations to TRX-1 protein expression, TRX reductase activity and PRDX over-oxidation in PBMCs in response to three short duration exercise trials. To observe the mechanism of TRX-1 response, we also assessed changes in the activity of the p65 subunit of NF-κB.

Material and Methods

Participants

Ten healthy males (Table 1) undertook three exercise trials, each separated by at least seven days (*Supplementary Figure 1*). All participants gave their informed written consent and the study was approved by the Science, Technology, Engineering and Mathematics Ethical Review Committee at University of Birmingham (Approval number: ERN_12-0830). Participants were non-smokers and had not taken any vitamin supplements or anti-inflammatory drugs for fourteen days prior to the first laboratory visit. Participants were also required to refrain from any strenuous physical activity, consumption of alcoholic beverages or food or drink with high nitrate content (beetroot, lettuce, spinach and processed meats) for at least two days prior to each experimental session.

Preliminary Assessments

All experimental sessions took place within the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham. Participants visited the laboratory to complete questionnaires addressing health history and demographics, and to have height and weight assessed (*Seca Alpha, Hamburg, Germany*). Cardiorespiratory fitness ($\dot{V}O_{2\,MAX}$) was measured using an incremental test to exhaustion on an electromagnetically braked cycle ergometer (*Lode Excalibur Sport*, Groningen, *Netherlands*). Following a three-minute warm up at 30 watts, workload was increased by 30 watts every minute, until volitional exhaustion. Oxygen uptake was assessed continuously using a breath-by-breath system (*Oxygon Prx*, Jaeger, Wuerzberg, Germany) and heart rate monitored using a Polar Vantage heart rate monitor (Polar Vantage, Kempele, Finland). The following criteria were used to indicate that $\dot{V}O_{2\,MAX}$ had been reached: a fall in cadence below 60rpm, a respiratory exchange ratio

($\dot{V}CO_2/\dot{V}O_2$) >1.10-1.15, plateau in participant oxygen consumption or a maximal heart rate >220 beats min^{-1}- age [22]. $\dot{V}O_{2\,MAX}$ was expressed relative to body weight ($ml.kg^{-1}min^{-1}$).

Exercise Trials

Seven days after preliminary measurements, participants returned to the laboratory to undertake the first of three exercise trials. All trials were undertaken in the morning, following an overnight fast, and all participants performed the three trials in a randomised design. Each trial was separated by at least three days. Prior to each exercise trial, participants undertook a warm up (5 minutes) at a workload eliciting 40% $\dot{V}O_{2\,MAX}$. Exercise trials were: two workload matched steady-state cycling trials at moderate (60% $\dot{V}O_{2\,MAX}$; 27 minutes, MOD) and high (80% $\dot{V}O_{2\,MAX}$; 20 minutes, HIGH) intensity and a low volume high intensity interval training (LV-HIIT) trial. LV-HIIT consisted of ten 1 minute cycling intervals at 90% $\dot{V}O_{2\,MAX}$, with 1 minute low intensity cycling at 40% $\dot{V}O_{2\,MAX}$.

Blood sampling

Prior to exercise, a catheter (*Becton, Dickson & Company, Oxford, UK*) was inserted into the antecubital vein of the arm and a rested blood sample drawn after thirty minutes of supine rest (baseline). The catheter was kept patent with saline (0.9% NaCl). Subsequent blood samples were taken during the last minute of exercise (exercise) and then 30 minutes following the exercise trial (post+30). At each time point, 15 ml of blood was drawn into four separate vacutainer tubes containing potassium ethylene diaminetetraacetic acid (EDTA) (*Becton, Dickson & Company, Oxford, UK*).

169

170 **Blood Cell Isolation**

171 Three EDTA tubes (approximately 15 ml) from each time point were used to isolate
172 PBMCs from whole blood using density gradient centrifugation. Briefly, whole blood was
173 diluted 1:1 with Roswell Park Memorial Institute Media (RMPI), and then layered carefully
174 on top of Ficoll paque PLUS (GE Healthcare) (2:1), before centrifuging at 400*g* for 30
175 minutes at 21°C. The PBMC layer was aspirated and then washed three times with RPMI, by
176 centrifuging steps at 200*g* for 5 minutes. The final cell pellet was divided into two equal
177 aliquots. The first aliquot was lysed using RIPA buffer (1x, *Sigma Aldrich*) containing a
178 protease inhibitor cocktail (1μL/mL, *Sigma Aldrich*), vortexed thoroughly and lysate
179 collected. The second aliquot was resuspended in a freezing mixture (RPMI, fetal calf serum
180 (FCS) and dimethyl sulfoxide (DMSO); 7:2:1) and frozen at −1°C /min using a freezing
181 container (Nalgene "Mr Frosty" Thermoscientific). Both aliquots were stored at -80°C until
182 further analyses.

183

184 **Analytical Procedures**

185 Whole blood cell counts (i.e., total leukocyte differential) were assessed using the
186 coulter principle. In addition, haemoglobin (g/dL) and haematocrit (%) were assessed to
187 calculate blood volume changes as a result of exercise using the formulae shown in Bosch et
188 al, 2005 [23,24] (*Coulter Analyser, Beckman-Coulter, High Wycombe, UK*). Protein
189 concentration was determined using the bicinchoninic assay method [25].

190

191 *Western Blotting Protocol*

192 All reagent mixtures were sonicated thoroughly prior to use. PBMC protein lysates
193 (10 μg) were mixed 1:1 with laemmli sample buffer (10% 2-mercaptoethanol, *Sigma Aldrich,*

Dorset, UK) and separated on 15-18% polyacrylamide gels. Gels were electrophoresed at 115V for 105 minutes using electrophoresis buffer (25 mM Tris, 192 mM glycine, and 0.1% w/v SDS). Proteins were transferred onto Hybond-P® PVDF membrane (GE Healthcare, Amersham, UK) with transfer buffer (25 mM Tris, 192 mM glycine, and 20% w/v methanol) for 105 minutes at 170mA. Transfer was assessed by Ponceau S (*Sigma Aldrich, Dorset, UK*) before membranes were washed with sodium hydroxide (0.1 M) and then blocked overnight in non-fat milk (5%) in TBST blocking buffer (0.21 M NaCl, 0.05 M Tris Base, 0.1% w/v Tween). Membranes were washed 6 times (5 minutes) in TBST (0.21 M NaCl, 0.05 M Tris Base, 0.05% w/v tween-20) prior to incubation with rabbit polyclonal for anti-PRDX-SO_{2-3} (I-IV) (1:500, ab16830, *Abcam, Cambridge, UK*), mouse monoclonal anti-TRX-1 (1:1000, ab16965, *Abcam, Cambridge, UK)*, and mouse monoclonal anti-beta actin (1:10,000, A1978, *Sigma Aldrich, Dorset, UK*) antibodies for 2 hours at room temperature. Membranes were washed (6x5 min) before peroxidase conjugated goat anti-rabbit (for PRDX SO_{2-3} (I-IV) work, 1:10,000, A6154) or goat anti-mouse (for TRX-1 and beta actin work, 1:10,000, A0168) antibodies (*Sigma Aldrich, Dorset, UK*) were applied for 1 hour at room temperature. Following a further washing step (6 x 5 min), visualisation of proteins was undertaken using Amersham ECL Prime detection reagent (*GE Healthcare, Amersham, UK*). Imaging and band quantification was assessed using Syngene G:Box F3 (*Geneflow, Staffordshire, UK*) and Syngene tools software respectively, and expressed in arbitrary units.

Thioredoxin Reductase Activity

PBMCs were rapidly thawed in a water bath (37°C). Pellets were washed twice in RPMI and FCS (9:1) to discard excess DMSO. Approximately 2 million cells were counted using a haemocytometer and aliquoted for each time point (baseline and exercise). All samples were adjusted to the lowest sample protein concentration. The lysate was then

assessed for TRX reductase enzyme activity using a commercially available kit according to manufacturer instructions (ab83463, *Abcam, Cambridge, UK*). Briefly, assay buffer (10 µl) or TRX reductase inhibitor (10 µl) were added to two sets of identical samples (50 µl, diluted to 0.55mg/ml protein using assay buffer). All samples were then incubated with a reaction mix (30µl assay buffer + 8µl 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB) + 2µl nicotinamide adenine dinucleotide phosphate (NADH) per well) and absorbances (λ = 412 nm) determined immediately and at 25 minutes to monitor reaction kinetics (Labsystems Multiskan MS, Virginia, *USA*). Values were then obtained from a linear standard curve of known 5-thio-2-nitrobenzoic acid (TNB) concentrations (0-50 nmol/well).

NF- κB p65 subunit activation

The activity of the p65 subunit of NF-κB was assessed using a commercially available ELISA (*TransAM NF-kB p65 Chemi*, *Active Motif, La Hulpe, Belgium*). Briefly, PBMC lysates (6µg/well) were added to wells coated with an oligonucleotide, containing the NF-κB consensus site (5'-GGGACTTTCC-3'). An antibody specific to an epitope on the p65 subunit of NF-κB (50µl, 1:1000) was added to each sample, with binding occurring only when NF-kB was activated and bound to the target DNA sequence. Data are expressed as relative absorbance units (nm).

Statistical Analysis

The Kolmogorov Smirnov test was used to test for normally distributed data at all time points. Normally distributed variables (changes in lymphocytes, monocytes, lymphocyte: monocyte ratio and, NF-kB activity and TRX-R activity) in response to exercise were assessed by repeated measures ANOVA. All white blood cell values were adjusted for changes in blood volume in response to exercise. Post hoc analysis of any interaction effects

was performed by a test of simple effects by pairwise comparisons, with Bonferroni correction. Non-normally distributed data (TRX-1 and PRDX-SO_3 protein expression changes) in response to exercise were analysed using the Friedman's test, with Wilcoxon signed-ranked pairwise comparisons. Differences between variables (e.g., participant characteristics) at baseline were assessed using one-way analyses of variance (ANOVA). Values are presented as means ± standard deviation or error (indicated throughout manuscript). Statistical significance was accepted at the $p<.05$ level. Statistical analyses were performed using SPSS (PASW Statistics, release 21.0, SPSS Inc., Chicago, IL, USA).

Results

Participant characteristics

All participant characteristics are summarised in table 1. None of the participants were smokers, nor taking regular medications or antioxidant supplements.

Exercise Physiology Data

Table 2 reports the exercise physiology data during the three exercise trials. Total energy expenditure (Kcal/ kg) was significantly lower in LV-HIIT, compared to HIGH ($p<0.0001$) and MOD ($p<0.0001$) trials. There were no statistical differences in energy expenditure between MOD and HIGH. Peak heart rate and rate of perceived exertion was greatest during HIGH, relative to MOD ($p's<0.0001$) and LV-HIIT ($p's<0.05$). Peak heart rate was significantly greater in HIGH compared to LV-HIIT ($p=0.025$).

White Blood Cell Data

Table 3 reports the changes in lymphocyte number, monocyte number and lymphocyte: monocyte ratio, adjusted for changes in blood volume in response to the three

exercise trials. Lymphocyte number increased during exercise in all trials ($F_{2,18}$ = 54.3, p<0.0001) and returned to baseline post+30 ($F_{2,18}$ = 54.3, p<.0001). A significant time × condition interaction was observed ($F_{4,36}$ = 18.5, p<.0001) and pairwise comparisons revealed that the increase in lymphocyte number during HIGH was greater than MOD (p=0.002). Monocyte number increased during exercise in all trials (p=0.028) and returned to baseline levels post+30 (p=.010). No differences were observed in monocyte number between trials. No statistical differences were observed in lymphocyte: monocyte ratio in response to any of the exercise trials.

Thioredoxin-1 and Peroxiredoxin-SO_{2-3} (I-IV) protein expression changes

Figures 2 and 3 indicate changes in TRX-1 and PRDX SO_{2-3} (I-IV) expression in the three exercise trials following normalisation for beta-actin and quantification with Genetools (arbitrary values). Figures 2B and 3B are example western blots for one participant. TRX-1 increased in response to exercise in MOD (χ^2 (2) = 8.600, p=0.014), HIGH (χ^2 (2) = 6.200, p=0.045) and LV-HIIT (χ^2 (2) = 11.436, p=0.003). Post hoc analyses with Wilcoxon signed-rank tests revealed a significant increase in TRX-1 from baseline to during exercise in the LV-HIIT trial only (Z = -2.666, p=0.008). However, no differences were observed in TRX-1 protein expression during exercise between the three trials, i.e. increases in TRX-1 were not exercise intensity dependent. Total PRDX-SO_{2-3} increased in response to HIGH only (χ^2 (2) = 7.824, p=0.020). Post hoc analyses revealed that PRDX- SO_{2-3} increased during HIGH, relative to baseline (Z = -2.524, p=.012).

Thioredoxin Reductase Activity

Figure 4 indicates changes in TRX reductase activity from baseline to during exercise in the three exercise trials. TRX reductase activity increased during exercise, relative to

baseline in all trials ($F_{1,9}$ = 82.7, p=.002). A significant group x time interaction effect was found (p=0.010), and a test of simple effects analysis by pairwise comparisons indicated that the change in TRX reductase enzyme activity during exercise was greater in HIGH than MOD ($F_{2,18}$ = 3.1, p=.042). Differences between HIGH and LV-HIIT did not reach statistical significance (p=.123). TRX reductase activity values returned to baseline post+30 (data not shown).

NF- κB p65 subunit activation

Figure 5 indicates changes in NF-kB p65 subunit activation in response to exercise. NF-kB p65 activity increased during exercise in all trials, relative to baseline ($F_{2,18}$ = 4.0, p = 0.036). No differences were observed between trials.

Discussion

This study demonstrates that PBMC TRX-1 protein expression and NF-kB p65 activity increased during exercise in young healthy males, irrespective of exercise intensity. An increase in over-oxidised PRDX was shown during exercise in HIGH only. The activity of TRX-R increased during exercise in all trials, with the greatest response observed in HIGH.

To our knowledge this is the first study assessing changes in PBMC TRX-1 protein expression in response to single bouts of exercise in humans (*Figure 2*). Previously, TRX-1 protein expression in PBMCs of mice significantly increased 12 hours following a short bout of swimming exercise, with no changes observed between 30 minutes and 6 hours [7]. In response to heightened oxidative stress, TRX can scavenge RONS [12], reduce oxidised macromolecules [10] and regulate the expression/recycling of antioxidant proteins such as manganese superoxide dismutase [26] and reduced glutathione [27]. The transient changes in

both TRX-1 expression and NF-kB p65 activity (i.e. increase during exercise and return to baseline values within thirty minutes), suggests that cytosolic TRX-1 has been released in a RONS-dependent manner from its binding protein Thioredoxin-interacting protein (TXNIP) [21] to elicit an array of antioxidant actions during all exercise trials. NF-kB activity has been shown to be increased in response to various modes of exercise [28,29] and to elicit a variety of antioxidant actions [20,30]. Hollander et al, showed that superoxide dismutase expression increased in response to aerobic exercise in the vastus lateralis muscle of rats, with maximal NF-kB binding occurring at 2 and 10 hours post-exercise [30]. The transient activation of NF-kB in the current study could be, in part, due to direct TRX binding of NF-kB [8]. We suggest that thirty minutes following cessation of exercise, TRX and TXNIP have re-associated (i.e. reformation of disulphide bond), with transcriptionally regulated changes in TRX protein expression occurring later, supporting previous work by Sumida et al, [7]. These responses are in agreement with other data from our study confirming transient exercise-induced increases in whole-body oxidative stress (e.g., increased plasma lipid hydroperoxides and elevated plasma total antioxidant capacity) (data not shown; Wadley et al, 2014, manuscript under review). It is however surprising that PRDX over-oxidation returned to baseline values within thirty minutes of exercise in all trials (*Figure 3*). Recycling of over-oxidised PRDX is a process known to occur very slowly *in vitro* [31]. Despite there being limited data on this mechanism in exercising humans, this finding warrants further study.

Increases in PRDX over-oxidation in PBMCs during exercise may occur in response to higher levels of RONS such as H_2O_2 and peroxynitrite [32]. The catalytic cysteine residue of PRDX can utilise its peroxidase activity to regulate peroxide mediated cellular signalling [9,33]. In the current study, a significant increase in over-oxidised PRDX (isoforms I – III, *Figure 3*) was only observed during exercise in HIGH, suggesting that peroxide exposure was highest in this trial and/or that the reducing power of TRX was sufficient in the other trials to

limit PRDX peroxidase activity. Lower exercise-induced peroxide exposure in MOD and LV-HIIT may have limited PRDX cysteine oxidation to the initial oxidation state, -SOH. In this conformational change, a PRDX cysteine residue forms a disulphide bond with an adjacent PRDX molecule [14]. TRX-R, the reducing partner of TRX, showed an increase in activity during all exercise trials (*Figure 4*), with the greatest increase observed during HIGH. This too suggests higher peroxide exposure and greater formation of intra-molecular disulphides within the TRX protein in HIGH. Collectively, these results suggest that a threshold may exist, whereby exercise-induced RONS exposure can exceed TRX/TRX-R regulatory control and over-oxidise PRDX. This threshold may have been exceeded during short-duration, high intensity steady state exercise.

Both TRX [34] and PRDX [35] have been previously associated with heightened inflammation. Additional data (Wadley et al, 2014, manuscript under review) from this study demonstrated that plasma Interleukin (IL)-6 and IL-10 concentrations were significantly increased in response to exercise, with the greatest responses observed following HIGH and LV-HIIT. Recent evidence has demonstrated that following TRX-TXNIP dissociation, TXNIP can directly induce increases in IL-1β, an early inflammatory cascade cytokine [21] and signal for IL-6 production [36]. Similarly, PRDX has been linked with IL-23 [35] and IL-6 production in macrophages [37], primarily following oxidation-induced oligomerisation and switch from peroxidase to chaperone function [37]. Further research is necessary to understand the interactions and relationships between the redox cycle of PBMCs and the inflammatory response/immune cell function.

It must be noted that despite normalisation of PRDX and TRX data for total cell and protein content, shifts in the number and phenotype of lymphocytes and monocytes can occur during exercise [5,38]. Indeed, lymphocyte and monocyte number did increase in response to exercise in all trials (*Table* 3). However, previous work has indicated that shifts in the cellular

composition of peripheral blood do not influence biomarkers of oxidative stress [3]. A limitation to the current study is that PRDX (I-III) protein expression was not measured to quantify the relative oxidised to reduced ratio of PRDX proteins. With regards to exercise-induced changes in PBMC TRX protein expression, future work should expand upon the current findings, by distinguishing between TXNIP-mediated and transcriptional regulation of TRX by monitoring time-course responses to exercise of various intensities.

In conclusion, the present results show that both TRX-1 and PRDX are perturbed in response to exercise in PBMCs from untrained males. While similar increases in TRX-1 were observed in response to all exercise trials, only high intensity steady state exercise (i.e., HIGH) caused over-oxidation of PRDX. Future studies should expand upon this work to elucidate the implications of PRDX over-oxidation in response to high intensity steady state exercise.

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Conflict of Interest

None of the authors declare a conflict of interest.

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Tables

Table 1: **Characteristics of male subjects**.

	(N=10, male)
Age (yrs.)	22 ± 3
Weight (kg)	78.1 ± 11.0
Height (m)	1.8 ± 0.1
BMI ($kg.m^2$)	24.0 ± 3.1
VO_2max ($ml.kg.min^{-1}$)	42.7 ± 5.0

Table 1 Legend: Values are means ± standard deviation.

Table 2: **Exercise Physiology Data**.

	MOD	HIGH	LV-HIIT
Average Workload (Watts / kg)	1.40 ± 0.22 +++	2.14 ± 0.33 ***+	2.69 ± 0.54 ***
Peak Heart Rate (bpm)	146.70 ± 11.08 +++	185.00 ± 9.17 ***+	176.00 ± 13.80 ***
Total Energy Expenditure (Kcal / kg)	3.33 ± 0.43	3.39 ± 0.47 +++	2.63 ± 0.34 ***
Peak rate of Perceived Exertion (Borg Scale)	13.33 ± 1.42	17.90 ± 1.45 ***	16.20 ± 2.15 *

Table 2 Legend: Average workload (Watts/kg), peak heart rate (bpm), total energy expenditure (Kcal/kg) and peak rate of perceived exertion during the exercise trials. Values are means ± standard deviation. * indicates a significant difference in values, relative to MOD: * p<.05; ** p<.01; *** p<.0001). + indicates a significant difference in values, relative to LV-HIIT: + p<.05; ++ p<.01; +++ p<.0001).

Table 3: **Immune cell responses to the three exercise trials**.

	MOD			HIGH			LV-HIIT		
	Base	Exercise	Post+30	Base	Exercise	Post+30	Base	Exercise	Post+30
Lymphocytes ($\times 10^6$/ml)	1.89 ± 0.47	2.52 ± 0.73***	1.67 ± 0.39$^{\$\$\$}$	1.86 ± 0.42	4.27 ± 1.26***##	1.79 ± 0.40$^{\$\$\$}$	1.79 ± 0.47	3.28 ± 1.31***	1.61 ± 0.40$^{\$\$\$}$
Monocytes ($\times 10^6$/ml)	0.29 ± 0.15	0.41 ± 0.21*	0.22 ± 0.08$^{\$\$}$	0.32 ± 0.18	0.56 ± 0.39*	0.31 ± 0.14$^{\$\$}$	0.23 ± 0.11	0.37 ± 0.10*	0.33 ± 0.16$^{\$\$}$
Lymphocyte: Monocyte Ratio	7.60 ± 3.04	7.19 ± 4.71	8.54 ± 3.45	7.44 ± 3.58	8.86 ± 4.55	6.79 ± 3.12	10.02 ± 6.50	9.47 ± 5.74	5.64 ± 2.09

Table 3 Legend: Lymphocyte, Monocyte and Lymphocyte: Monocyte Ratio data, adjusted for changes in blood volume in response to exercise in the MOD, HIGH and LV-HIIT trials. Values are means ± standard deviation. * indicates a significant difference relative to baseline: * p<.05; ** p<.01; *** p<.0001). $ indicates a significant difference relative to during exercise: $ p<.05; $$ p<.01; $$$ p<.0001). # indicates a significantly greater response during HIGH relative to MOD (p<.002).

568 **Figure Legends**

569 *Figure 1:* **A schematic representation of the oxidation states of PRDX and TRX**
570 **following peroxide exposure.** R = R-group -SH = Sulfhydryl group SRX = Sulfiredoxin S-
571 S = Disulphide Bond

572

573 *Figure 2:* **Total TRX-1 levels in response to the three exercise trials**. A) Bars represent
574 total TRX-1 in PBMC lysates before (Baseline), during (Exercise) and thirty minutes
575 following exercise (Post+30). Values are means ± standard error. * indicates significant
576 differences during exercise, relative to baseline: * p<.05; ** p<.01; *** p<.0001). B)
577 Example western blot of a single subject. The image represents PBMC lysates under reducing
578 conditions.

579

580 *Figure 3:* **Total PRDX-SO_{2-3} (I-IV) levels in response to the three exercise trials**. A) Bars
581 represent total PRDX-SO_{2-3} (I-IV) in PBMC lysates before (Baseline), during (Exercise) and
582 thirty minutes following exercise (Post+30). Values are means ± standard error. * indicates
583 significant differences during exercise, relative to baseline: * p<.05; ** p<.01; *** p<.0001).
584 B) Example western blot of a single subject. The image represents PBMC lysates under
585 reducing conditions. The two distinct bands in are representative of the different isoforms of
586 PRDX. Over-oxidised PRDX isoforms 1 and 2 resolve at 20-22kDa, PRDX-3 at 26kDa and
587 PRDX-4 at 31kDa (no band).

588

589 *Figure 4:* **Changes in Thioredoxin Reductase Activity during exercise, relative to**
590 **baseline in response to the three exercise trials**. Bars represent changes in Thioredoxin
591 Reductase Activity in PBMC lysates from baseline to during exercise. Values are means ±

standard error. * indicates significant differences in response to exercise, relative to baseline in all trials: * p<.05; ** p<.01; *** p<.0001). # indicates a significant difference in the response between HIGH and MOD during exercise (p<0.042). Enzyme activity was also assessed 30 minutes post-exercise and values returned to baseline levels (data not shown).

Figure 5: **Changes in NF-κB p65 subunit activation in response to the three exercise trials**. Bars represent relative absorbance values in PBMC lysates before (Baseline), during (Exercise) and thirty minutes following exercise (Post+30). Values are means ± standard error. * indicates significant differences during exercise, relative to baseline: * p<.05; ** p<.01; *** p<.0001).

Supplementary Figure 1: **Experimental protocol**. Participants completed 3 randomised exercise trials in a fasted state. LV-HIIT consisted of ten 1 minute stages at 90% $\dot{V}O_{2\,MAX}$, interspersed with nine 1 minute intervals at 40% $\dot{V}O_{2\,MAX}$.

↓ Blood samples (baseline, exercise & post+30); W, Warm up

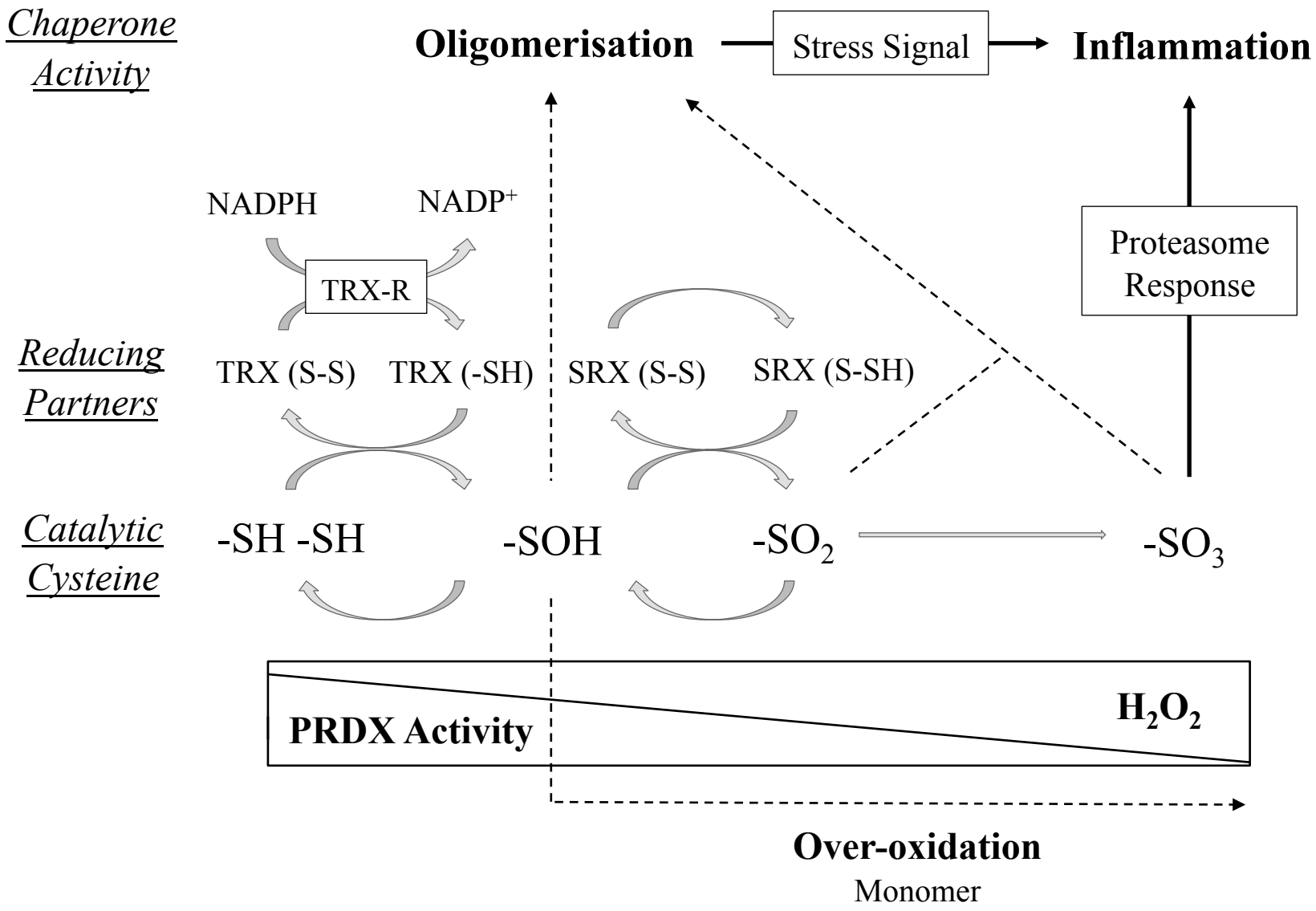
Supplementary Table

Supplementary Table 1: **Changes in blood and plasma volume in response to exercise**.

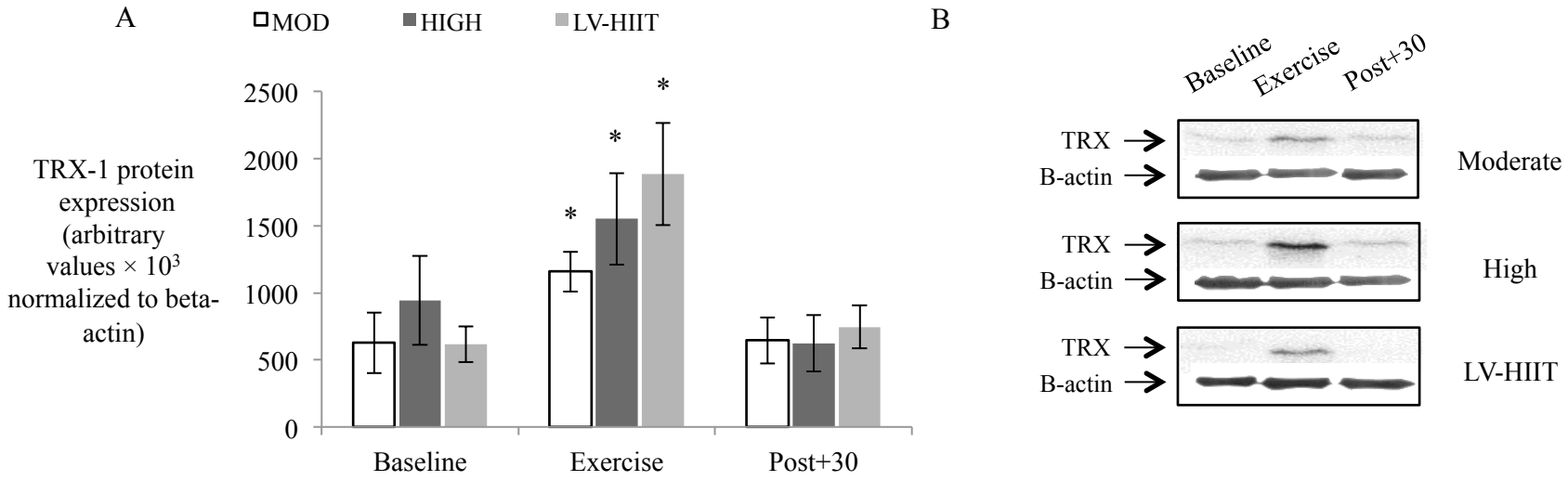
	MOD			HIGH			LV-HIIT		
	Base	Exercise	Post+30	Base	Exercise	Post+30	Base	Exercise	Post+30
Blood Volume (%)	100	94.2 ± 4.7***	99.9 ± 5.0$^{\$\$\$}$	100	92.3 ± 2.9 ***	100.7 ± 4.5$^{\$\$\$}$	100	91.8 ± 4.2***	99.3 ± 4.2$^{\$\$\$}$
Plasma Volume (%)	57.6± 2.7	55.8 ± 12.7**	57.5 ± 3.8$^{\$\$}$	56.4 ± 3.7	48.2 ± 3.5**	56.9 ± 3.8$^{\$\$}$	57.7 ± 2.3	49.3 ± 4.2**	56.7 ± 4.4$^{\$\$}$

Supplementary Table 1 Legend: Changes in blood volume, relative to baseline (100) are indicated. Plasma volume was calculated by monitoring changes in blood haematocrit. Values are means ± standard error. * indicates significant differences in response to exercise, relative to baseline in all trials: * p<.05; ** p<.01; *** p<.0001). $^{\$}$ indicates a significant difference relative to during exercise: $^{\$}$ p<.05; $^{\$\$}$ p<.01; $^{\$\$\$}$ p<.0001).

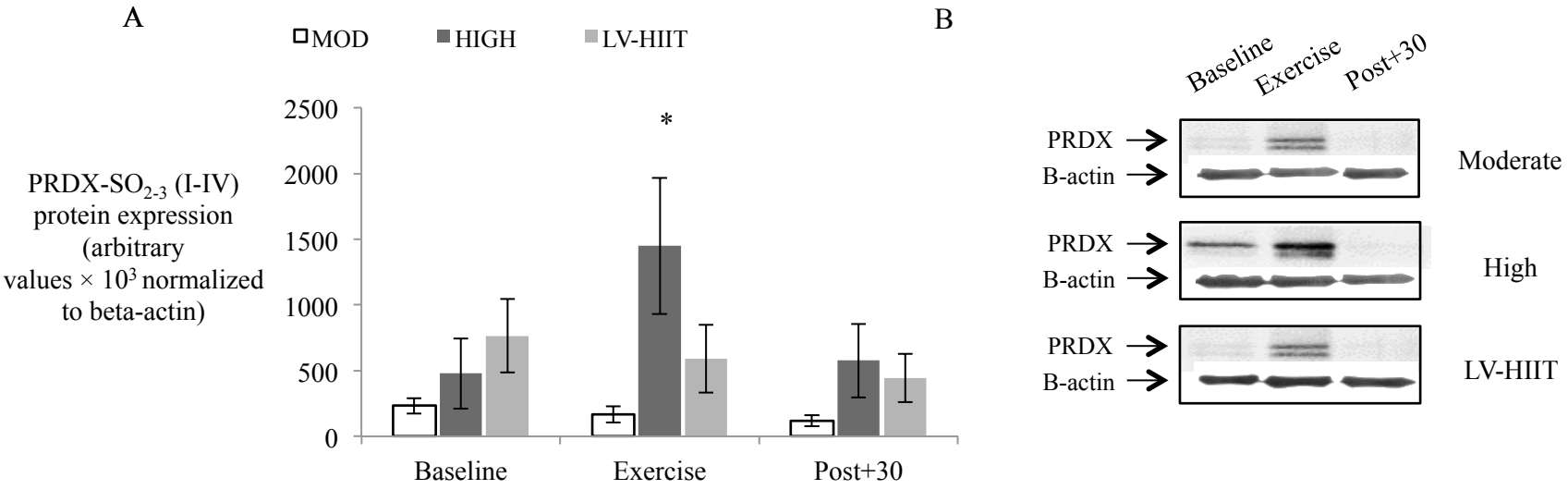
Wadley (2014): Effects of exercise on redox protein expression
Figure 1



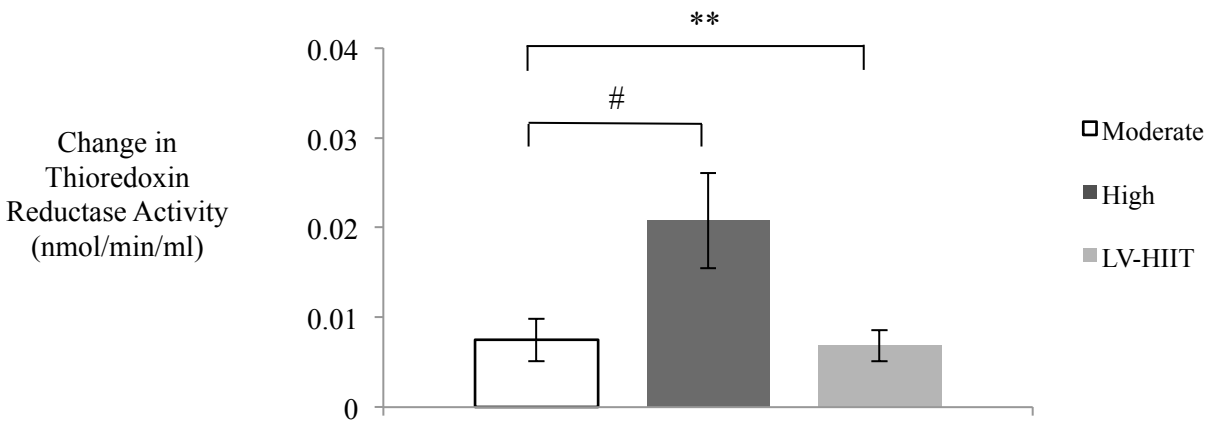
Wadley (2014): Effects of exercise on redox protein expression
Figure 2



Wadley (2014): Effects of exercise on redox protein expression
Figure 3



Wadley (2014): Effects of exercise on redox protein expression
Figure 4



Wadley (2014): Effects of exercise on redox protein expression
Figure 5