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# The effect of prior exercise on *ex vivo* induction of heme oxygenase-1 in human lymphocytes

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**Abstract**
It was postulated that prior demanding exercise would suppress the induction of the oxidant-responsive protein Heme Oxygenase-1 (HO-1) in mononuclear cells following subsequent *ex vivo* $H_2O_2$ treatment. Eight male subjects completed two trials in a randomized order (one rest and one exercise) and *ex vivo* HO-1 protein induction was determined following $H_2O_2$ treatment in lymphocytes and monocytes before and after each trial using a newly developed and reproducible assay. Lymphocytes obtained 2 h post-exercise showed a modest reduction in HO-1 protein expression in response to *ex vivo* treatment with $H_2O_2$ ($p < 0.05$). The plasma concentration of the HO-1 suppressor $\alpha$1-antitrypsin increased immediately post-exercise ($p < 0.05$) and it is tentatively suggested that this may explain the modest transient reduction in *ex vivo* HO-1 protein induction in lymphocytes in response to an independent oxidant challenge following a prior bout of demanding exercise.

**Keywords:** *Free radicals, oxidative stress, hydrogen peroxide, physical activity, mononuclear cells, inflammation*

## Introduction

Heme oxygenase is the rate-limiting enzyme in heme degradation and produces bilirubin, carbon monoxide and free iron [1]. The induction of the isoform heme oxygenase-1 (HO-1) is a general response to oxidative stress in mammalian cells [2]. Early investigations showed that HO-1 was induced in fibroblasts following UVA irradiation [3] and it is now accepted that many factors that alter redox state induce HO-1 [4]. Importantly, HO-1 plays a critical role in cellular homeostasis and is an anti-inflammatory, anti-apoptotic and protective enzyme [5,6]. Immune cells such as lymphocytes are continually exposed to oxidants such as hydrogen peroxide ($H_2O_2$) in both the circulation and during their recirculation through tissues and the manner in which they respond to reactive intermediates may influence their ability to function. Several studies have shown that lymphocytes can up-regulate various protective proteins, including HO-1. For example, $H_2O_2$ treatment [7], hyperbaric oxygen treatment [8] and heavy metals [9] all induce lymphocyte HO-1.

Exercise leads to oxidative stress and consequently, as would be predicted, acute demanding exercise has the potential to increase HO-1 expression in lymphocytes [10,11]. In spite of the ostensible benefits associated with the exercise-induced up-regulation of genes such as HO-1, there is considerable evidence that certain forms of demanding exercise can have a profound detrimental impact on lymphocyte function and the cellular immune system. For example, lymphocyte proliferation [12–14] and the ability to secrete cytokines when stimulated [14] is impaired

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for several hours after acute demanding exercise. Furthermore, experiments in rodents show that the ability to survive a viral challenge is impaired following participation in strenuous exercise [15]. Collectively, these results suggest that the stress associated with participation in demanding exercise leads to a transient inability of the immune system (including cells such as lymphocytes) to respond when required. Lymphocytes and monocytes are required to operate in the face of acute oxidative stress (e.g. at sites of free-radical production associated with inflammation), and it is possible that one aspect of compromised immunity post-exercise includes a reduced ability of lymphocytes and monocytes to maintain the normal response to an oxidant challenge following demanding exercise.

In order to test this hypothesis, the first step was to examine whether the lymphocyte and monocyte response to an external oxidant challenge is impaired or unaffected by participation in a bout of demanding exercise. In the present investigation, we developed a reproducible *ex vivo* assay using $H_2O_2$ to induce HO-1 protein in human mononuclear cells and we used this assay to examine whether prior intense exercise affects *ex vivo* lymphocyte and monocyte HO-1 protein induction. We show that a single bout of prior demanding exercise leads to a modest suppression in *ex vivo* HO-1 protein induction in lymphocytes in response to an independent oxidant challenge ($H_2O_2$).



## Materials and methods

### *Subjects*

Healthy male volunteers were recruited for the two phases of this investigation. In the preliminary study to develop the *ex vivo* $H_2O_2$ assay 10 donors were recruited (mean age, height and body mass were $26 \pm 4$ years, $177 \pm 2$ cm, $80.0 \pm 3.2$ kg). Subsequently, eight males volunteered to take part in the exercise study (mean age, height and body mass were $25 \pm 4$ years, $180 \pm 6$ cm, $78.8 \pm 8.9$ kg). These volunteers were familiar with intermittent activities such as football, squash, and rugby, and completed on average $3 \pm 1$ h of activity per week. Subjects provided written informed consent, and each study was approved by the local ethics committee. Individuals taking prescribed medication or food supplements and subjects who smoked were excluded from participation.

### *Experimental design and procedures*

*Preliminary study.* The aim of the preliminary study was to examine the time course and reproducibility of *ex vivo* HO-1 protein induction in human lymphocytes and monocytes following $H_2O_2$ treatment. Subjects reported to the laboratory on two occasions (trial 1 and trial 2) 2 weeks apart for blood sampling after an overnight fast. During the 72 h period prior to each visit, subjects maintained a record of food and fluid intake which was reproduced as closely as possible in the 72 h before the second visit. Food and fluid records were analysed using the software COMP-EAT 4.0 (Nutrition Systems, UK). Subjects refrained from physical activities that elicited a heart rate (HR) of 100 beats per minute or more for 72 h prior to each sample. Additionally, individuals were asked to refrain from consuming alcohol in the 72 h prior to each visit. Isolated mononuclear cells (MNCs) were exposed to $H_2O_2$ treatment and left to recover for 4, 6, 24 and 48 h prior to determination of HO-1 protein by flow cytometry.

*Exercise study.* Subjects completed two preliminary exercise tests on a treadmill to determine the relationship between running speed and oxygen uptake ($\dot{V}O_2$), and maximal oxygen consumption ($\dot{V}O_2$ max) as described elsewhere [11]. Oxygen uptake was used to calculate relative exercise intensity. Mean $\dot{V}O_2$ max was $57.7 \pm 5.7$ ml/kg/min.

Subjects completed one exercise trial and one rest trial (60-min quiet sitting) in a randomized order $\sim 2$ weeks apart. During the 72 h period prior to each main trial, subjects' food and fluid intake and physical activity were controlled as described in the preliminary study above. Subject compliance to physical activity instructions was monitored with a HR monitor (Vantage NV, Polar Electro OY, Finland). Additionally, individuals were asked to refrain from consuming alcohol in the 72 h prior to each main trial and for 48 h following the main trial.

On the main trial day subjects arrived in the laboratory following an overnight fast. A resting venous sample was taken, and in the exercise trial subjects completed a 5 min warm-up on the treadmill at a speed equivalent to 50% $\dot{V}O_2$ max prior to starting the intermittent running protocol. The intermittent exercise protocol consisted of a 60-min run divided into four 15 min blocks, each separated by a 2-min rest period, comprised of repeated cycles of activity equivalent to 65, 85 and 100% $\dot{V}O_2$ max. This protocol alternated between 65% (3 min) and 85% (2 min) $\dot{V}O_2$ max with the last 2 min of each 15 min block being performed at 100% $\dot{V}O_2$ max. This protocol was developed because intermittent exercise performed at the same average work rate as continuous exercise has been associated with increased physiological strain [16] and we hypothesized that this model would elicit greater changes in HO-1 in response to acute exercise. The overall intensity of this protocol was estimated to be 75% $\dot{V}O_2$ max for 60 min with a total rest time of 6 min. HR was measured every 15 s during exercise using short range telemetry (Vantage NV, Polar Electro OY, Finland) and expired gas samples were collected during the

12th min of each block of activity. Upon completion of each 15-min bout of exercise or rest, a finger prick capillary sample was taken for determination of blood lactate concentration (2300 STAT Plus, YSI Incorporated, Yellow Springs, USA). Subjects consumed water *ad libitum* and the volume consumed was recorded. Further venous blood samples were collected immediately after exercise or rest (0 h), 2, 4 and 6 h post-exercise or rest, with two additional samples at 24 and 48 h post-exercise or rest. Subjects were given a standardized meal following the 0 h sample and again between the 2 and 4 h samples (combined total: 1771 kcal, 62% carbohydrate, 12% protein, and 26% fat).

Lymphocyte HO-1 mRNA and protein were analysed prior to and 0, 2, 4, 6, 24, and 48 h following 60 min exercise or rest. Furthermore, at each time point, isolated MNCs were exposed to $H_2O_2$ treatment and left to recover for 48 h prior to determination of HO-1 protein.

*Reagents: Preliminary and exercise studies*

All reagents were from Sigma-Aldrich (Poole, UK) unless otherwise specified. Routine tissue culture reagents were purchased from Gibco Invitrogen Ltd. (Paisley, UK). Foetal calf serum (FCS) was purchased from PAA laboratories (Somerset, UK). LightCycler DNA Master SYBR Green I and bovine serum albumin (BSA) standard solution were purchased from Roche Molecular Biochemicals (Lewes, UK). TRIZOL reagent and Superscript first-strand synthesis kit were purchased from Invitrogen Life Technologies (Paisley, UK). The mouse anti-HO-1 monoclonal antibody (Stressgen, Victoria, Canada) was purchased from Bioquote Ltd (York, UK). Ethanol was purchased from Fisher Scientific (Loughborough, UK). Phosphate-buffered saline (PBS) was purchased from Oxoid Ltd. (Basingstoke, UK). Hydrogen peroxide ($H_2O_2$) was purchased from Calbiochem (Poole, UK).

*Blood collection and processing: Preliminary and exercise studies*

Venous blood samples were collected after subjects had been in a supine position for 15 min. Twenty-five millilitres of whole blood was collected by venepuncture in the ante-cubital vein and dispensed into EDTA-coated tubes (Sarstedt Ltd., Leicester, UK). Whole blood differential leukocyte counts and plasma were obtained as described previously [11]. Peripheral blood mononuclear cells were isolated using a one-step centrifugation technique (Lymphoprep, Nycomed, Norway). Harvested cells were re-suspended in 20 ml PBS and counted using an automated haematology analyser (SF-3000, Sysmex UK Ltd., UK). For subsequent lymphocyte HO-1 mRNA analysis, 10–15 million MNCs in 20 ml RPMI (containing 1% FCS) were transferred into a 150 $cm^2$ culture flask and placed in a humidified atmosphere at 37°C with 5% $CO_2$ for 45 min and lymphocytes obtained by allowing monocytes to adhere to plastic. Lymphocytes were lysed in TRIZOL, with the volume added dependent on cell concentration (1 ml per $5 \times 10^6$ cells) and then frozen at −70°C until subsequent analysis. Plasma was analysed for interleukin-6 (IL-6) using a commercially available solid phase high-sensitivity ELISA (Quantikine, R and D Systems Inc., Abingdon, UK). Plasma IL-6 was only measured at baseline and immediately post-exercise as plasma IL-6 levels have been shown to peak at the cessation of exercise before progressively declining into recovery [17]. Plasma $\alpha$1-antitrypsin (AAT) concentration was measured by turbidimetric immunoassay (Randox Laboratories, Co. Antrim, UK) using an automated spectrophotometer (Cobas-Mira N, Roche Diagnostics, UK).

*$H_2O_2$ treatment: Preliminary and exercise studies*

Isolated human MNC's ($\sim 3 \times 10^6$ cells) were treated in 15 ml centrifuge tubes and exposed to a final concentration of 50 μm $H_2O_2$ for 30 min at 37°C. Following treatment, cells were washed once in PBS and then re-suspended in conditioned medium taken from TK6 cells, a B cell line [2]. In the preliminary study the cells were replaced at 37°C for 4, 6, 24 or 48 h recovery. The findings from this investigation showed HO-1 protein induction to be reproducible at the 48 h time point, hence, in the exercise study, the cells were left at 37°C for 48 h following $H_2O_2$ treatment. TK6 cells were cultured routinely at 37°C in 5% $CO_2$ in RPMI-1640 medium (including glutamine), supplemented with 50 μ/ml penicillin, 50 μg/ml streptomycin, 0.2% sodium bicarbonate and 10% horse serum. Conditioned medium was used for the treatment in all experiments because the addition of fresh medium instead of conditioned medium has been shown to induce HO-1 [18]. Cell viability was assessed at each time point using the trypan blue exclusion assay. The cell suspension was centrifuged (170 *g*, 5 min at room temperature) and placed in aliquots for measurement of total protein as described previously [11] with HO-1 protein determined by flow cytometry.

*Flow cytometry: Preliminary and exercise studies*

Approximately $1 \times 10^6$ MNCs were fixed and permeabilized in 2 ml 70% ice-cold ethanol and samples were kept at 4°C until subsequent analysis. Lymphocyte and monocyte HO-1 protein was analysed by indirect immunofluorescence using a monoclonal mouse anti-HO-1 antibody IgG1 and fluorescein isothiocyanate (FITC) conjugated goat anti-mouse polyvalent immunoglobulins antibody. An isotype

control was performed with FITC mouse IgG1 antibody (DAKO, Glostrup, Denmark) and the same anti-mouse IgG-FITC secondary to determine background fluorescence. Cell analysis was performed in a Becton Dickinson FACScan (Cellquest version 3.3 software, Belgium) using 488 nm excitation and detection in the green fluorescence channel (FL1-530 ± 30 nm bandpass filter). The lymphocyte and monocyte populations were differentiated by size and granularity in the forward vs side scatter gram and gated. Background dead cells/debris was excluded by electronic threshold and data for 10 000 events of the lymphocyte population acquired. HO-1 protein was expressed as the fold change in median fluorescence intensity (MFI) of the treated vs sham-treated controls. In the exercise and rest trial, the MFI of the post-test samples were normalized relative to the baseline pre-test sample.

### *Real-time RT-PCR: Exercise study*

A two-step RT-PCR approach using SuperScript first-strand synthesis kit was used to analyse lymphocyte HO-1 mRNA accumulation as described previously [19]. Briefly, 2 μg of total RNA was reverse transcribed. A 1:10 dilution of each cDNA sample was freshly prepared in PCR-grade water and 2 μl were used in each PCR reaction. Real-time PCR reactions were carried out in the LightCycler (Roche Molecular Biochemicals, UK) using the fluorescent dye SYBR Green I. HO-1 and GAPDH were quantified independently using an external standard curve. The normalized HO-1 levels were expressed relative to baseline samples in rest and exercise trials, respectively.

### *Immunophenotyping (CD3/4 and CD3/8): Exercise study*

Dual-stained cell suspensions (FITC and R-phycoerythrin (PE)) were analysed in a FACScan (Becton Dickinson, Cellquest version 3.3 software, Belgium) using 488 nm excitation and detection in the green fluorescence channel for FITC (FL1: 530 ± 30 nm bandpass filter) and orange fluorescence channel for PE (FL2: 585 ± 42 nm bandpass filter). Electronic compensation was set to correct for spectral overlap in both FITC and PE emissions. An isotype control was performed with FITC mouse IgG1 and PE mouse IgG2a monoclonal antibodies to determine background fluorescence. Gates based on cell size and granularity were used to distinguish between lymphocyte and monocyte populations for fluorescence analysis. The data was analysed using two-dimensional dot plots of FL2 vs FL1 fluorescent profiles.

### *Statistics*

*Preliminary study.* In order to examine whether differences between mean values existed between trials and over the time course, a two-way ANOVA was performed. Where a significant time effect was observed, paired *t*-tests were performed to determine which time points were different. Reproducibility of the response was assessed using re-test correlation (Pearson's Product Moment) for each pair of observations at each time point. Significance was accepted at the 5% level. Values are presented as mean ± SD.

*Exercise study.* The difference between pre- and post-testing values was calculated for each individual (change score) at each time point post-testing and the mean change was analysed by *t*-test. Non-normally distributed variables were transformed logarithmically before statistical analysis. Pearson's Product Moment correlations were calculated comparing the peak fold increase in IL-6 concentration with the peak fold increase in HO-1 mRNA accumulation in the exercise trial. The statistical calculations described were performed using a pre-formatted spreadsheet [20] in addition to SPSS version 11 (SPSS Inc., Chicago, USA). Significance was accepted at the 5% level. Values are presented as mean ± SD.

## Results

### *Preliminary study:* Ex vivo *induction of HO-1 protein following $H_2O_2$ treatment*

HO-1 protein was induced 48 h after $H_2O_2$ treatment in both lymphocytes and monocytes (Table I). This was evident in both trials with a mean fold change in lymphocyte HO-1 protein of 2.1 ± 0.9 and 1.9 ± 1.0 in trials 1 and 2, respectively ($p < 0.01$). In monocytes, the mean fold change in HO-1 protein was 1.3 ± 0.4 and 1.5 ± 0.6 ($p < 0.01$) in trials 1 and 2, respectively (Table I). The results of the *ex vivo* assays were highly reproducible. At 48 h following recovery from $H_2O_2$ treatment, HO-1 protein induction in both trials correlated highly in lymphocytes ($r = 0.84$, $p < 0.01$, $n = 10$) but was not significant in monocytes ($r = 0.46$, $p > 0.05$, $n = 10$) (Table I). Although HO-1 expression was reproducible within-subjects, there was a large degree of inter-individual variation in the magnitude of HO-1 protein induction at 48 h. In lymphocytes (Figure 1), this ranged from a 1.4-fold change to a 4.6-fold change from sham-treated controls with the differences in monocytes being less pronounced (1.2-fold change to 2.6-fold change, data not shown).

Table I. Reproducibility data for lymphocyte and monocyte HO-1 protein expression at basal levels, and 4, 6, 24, and 48 h following $H_2O_2$ treatment. Values are the fold change in HO-1 protein of the median fluorescence intensity (MFI) of $H_2O_2$-treated samples vs the MFI of the respective sham-treated control. Using two-way ANOVA there was an effect of time for HO-1 protein inductionin lymphocytes and monocytes ($p < 0.001$ and $p < 0.05$ in lymphocytes and monocytes, respectively). Significant differences in HO-1 protein induction were found 48 h following recovery from treatment in lymphocytes ($p < 0.01$) and monocytes ($p < 0.01$), respectively.

| Parameter | Trial 1 | Trial 2 | Correlation coefficient ($r$) |
|---|---|---|---|
| Lymphocytes | | | |
| 48 h | 2.1 ± 0.9 | 1.9 ± 1.0 | 0.84** |
| 24 h | 1.1 ± 0.3 | 1.3 ± 0.3 | 0.38 |
| 6 h | 1.0 ± 0.3 | 1.1 ± 0.3 | −0.06 |
| 4 h | 0.9 ± 0.2 | 1.1 ± 0.2 | −0.02 |
| 0 h | 1.0 ± 0.0 | 1.0 ± 0.0 | — |
| Basal | 1.0 ± 0.2 | 0.9 ± 0.1 | 0.62 |
| Monocytes | | | |
| 48 h | 1.3 ± 0.4 | 1.5 ± 0.6 | 0.46 |
| 24 h | 1.0 ± 0.3 | 1.1 ± 0.3 | 0.24 |
| 6 h | 0.9 ± 0.4 | 0.9 ± 0.3 | 0.22 |
| 4 h | 0.8 ± 0.2 | 1.0 ± 0.2 | 0.02 |
| 0 h | 1.0 ± 0.0 | 1.0 ± 0.0 | — |
| Basal | 1.1 ± 0.2 | 1.1 ± 0.1 | 0.57 |

** denotes a significant correlation coefficient ($p < 0.01$). Values are mean ± SD ($n = 10$ for all time points other than basal, where $n = 6$).

### *Exercise study: Effect of prior exercise on* ex vivo *lymphocyte and monocyte HO-1 protein induction*

The intensity of the intermittent exercise protocol varied between 65–100% $\dot{V}O_2$ max. Measurement of oxygen uptake was only possible during the 65% $\dot{V}O_2$ max stage and was 68 ± 5% across the 60 min protocol. Mean values for blood lactate and heart rate were 0.8 ± 0.4 mmol/l and 61 ± 4 beats/min during the rest trial and 4.9 ± 1.6 mmol/l and 166 ± 13 beats/min during the exercise trial. Ambient temperature and humidity were similar in exercise and resting trials, being 19.6 ± 0.5°C vs 19.8 ± 0.9°C and 41.1 ± 12.3% vs 43.3 ± 8.6%, respectively.

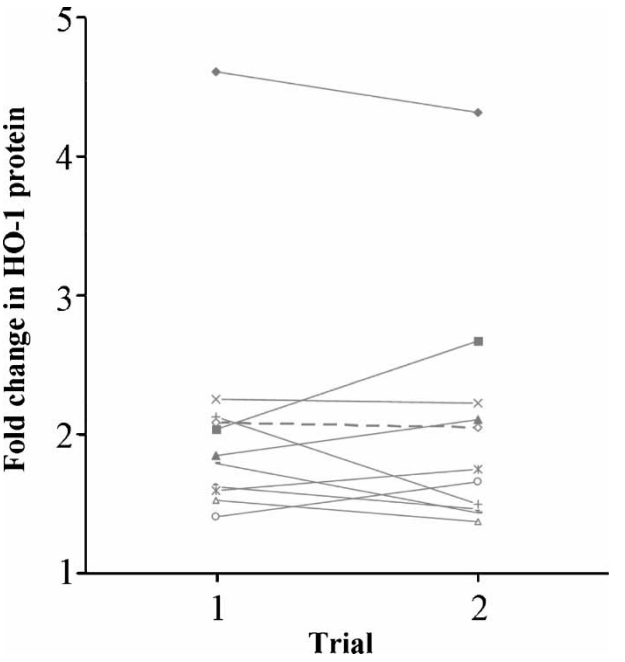


Figure 1. Preliminary study: HO-1 protein induction in human lymphocytes following $H_2O_2$ treatment in trial 1 and trial 2. Individual (—) and Mean (---) values for the fold change in HO-1 protein are presented ($n = 10$).

### *Leukocyte numbers*

Leukocyte count increased up to 2 h after exercise and remained significantly elevated 6 h post-exercise when compared to the same time point in the resting trial ($p < 0.01$) with the largest change occurring in the neutrophil population ($p < 0.01$) (Table II). An increase in the monocyte population 2 h post-exercise ($p < 0.01$) was accompanied by a decline in lymphocyte count ($p < 0.05$) when compared to the corresponding time point in the rest trial (Table II). Cell count had returned to resting values by 24 h post-exercise. Immunophenotyping of lymphocyte sub-populations CD3/4 and CD3/8 revealed no differences in the distribution of these sub-populations following exercise (data not shown).

### *Lymphocyte HO-1 mRNA and lymphocyte and monocyte HO-1 protein*

Lymphocyte HO-1 mRNA accumulation in untreated cells did not increase significantly at any time point post-exercise (Figure 2). Furthermore, lymphocyte and monocyte HO-1 protein expression in untreated freshly-harvested samples did not change with exercise or rest (Table III).

### *Lymphocyte and monocyte HO-1 protein induction following $H_2O_2$ treatment*

The main aim of this investigation was to examine the effect of prior intense exercise on *ex vivo* lymphocyte and monocyte HO-1 protein induction. Prior to and after the exercise or rest trial, lymphocytes and monocytes were isolated and left to recover for 48 h following $H_2O_2$ treatment and *ex vivo* lymphocyte and monocyte HO-1 protein induction was determined by flow cytometry. Lymphocytes obtained 2 h after exercise and treated with $H_2O_2$ demonstrated a reduction in the subsequent induction of HO-1 protein 48 h later (Figure 3). In six out of seven subjects lymphocyte HO-1 protein induction following $H_2O_2$ treatment was reduced 2 h post-exercise when compared to the rest trial (Figure 4; $p < 0.05$). In monocytes, a moderate effect was seen at the same time point, although this was not statistically significant ($p > 0.05$, data not shown).

Table II. Leukocyte count prior to and following 60 min exercise and rest. Values are mean ± SD ($n = 8$). Time point 0 h represents the sample taken immediately following the 60 min period of exercise or rest.

| | | Time (h) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Cell type ($10^9$/l) | Trial | Baseline | 0 | 2 | 4 | 6 | 24 | 48 |
| Total leukocytes | Rest | 4.8 ± 1.0 | 4.9 ± 1.2 | 4.7 ± 1.2 | 4.7 ± 1.1 | 4.9 ± 1.0 | 4.7 ± 1.0 | 4.8 ± 0.8 |
| | Exercise | 4.6 ± 1.0 | 5.9 ± 1.8⋆ | 9.2 ± 2.0† | 8.5 ± 1.6† | 7.6 ± 1.6† | 4.6 ± 0.9 | 4.2 ± 0.6 |
| Neutrophils | Rest | 2.5 ± 0.9 | 2.8 ± 1.0 | 2.8 ± 1.0 | 2.7 ± 1.0 | 2.7 ± 0.8 | 2.6 ± 0.9 | 2.7 ± 0.9 |
| | Exercise | 2.3 ± 1.0 | 3.5 ± 1.7⋆ | 7.5 ± 2.0† | 6.4 ± 1.7† | 5.2 ± 1.4† | 2.5 ± 1.0 | 2.2 ± 0.7 |
| Monocytes | Rest | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 |
| | Exercise | 0.4 ± 0.1 | 0.4 ± 0.2 | 0.5 ± 0.1† | 0.4 ± 0.1 | 0.5 ± 0.2 | 0.3 ± 0.1 | 0.3 ± 0.1 |
| Lymphocytes | Rest | 1.7 ± 0.4 | 1.5 ± 0.2 | 1.4 ± 0.1 | 1.5 ± 0.1 | 1.7 ± 0.1 | 1.5 ± 0.3 | 1.5 ± 0.3 |
| | Exercise | 1.7 ± 0.4 | 1.7 ± 0.4 | 1.1 ± 0.2⋆ | 1.5 ± 0.2 | 1.8 ± 0.3 | 1.6 ± 0.3 | 1.5 ± 0.3 |

† denotes a significant difference between the change score of leukocyte cell counts in the exercise trial compared to the change score at the same time point in the rest trial [Change = (2 h post-exercise − baseline) − (2 h post-rest − baseline)] ($p < 0.01$).
⋆$p < 0.05$.



*Cell viability*

Cell viability immediately following the isolation procedure was 97 ± 2% in both the rest and exercise trials (mean ± SD, $n = 8$). Exercise or rest did not affect cell viability following $H_2O_2$ treatment. In the rest trial this was 82 ± 7% and 58 ± 4% in the sham and $H_2O_2$-treated samples 48 h after treatment, respectively, compared to 80 ± 5% and 57 ± 6% in the exercise trial.

*Plasma interleukin-6 and α1-antitrypsin*

Plasma IL-6 concentration (Figure 5, $p < 0.01$) and plasma α1-antitrypsin (Figure 6, $p < 0.05$) increased immediately following the exercise trial. There was a positive correlation between the change in plasma IL-6 and the change in plasma AAT ($r = 0.71$, $p = 0.05$).

## Discussion

The primary novel finding of the present investigation is that lymphocytes harvested 2 h after an acute bout of demanding exercise demonstrated a reduced up-regulation of HO-1 protein expression in response to an oxidant challenge ($H_2O_2$). This is the first investigation to demonstrate altered *ex vivo* lymphocyte HO-1 protein induction in response to an independent exogenous oxidant challenge following prior acute exercise. The same effect was observed in monocytes, although this was not statistically significant.

In the hours following demanding exercise there is a well-characterized decline in the number of lymphocytes in the circulation [21]. In the present study, the lymphocyte count was lowest 2 h post-exercise and at this time point there was a reduced induction of lymphocyte HO-1 protein in response to $H_2O_2$ treatment. We did not observe any changes in the distribution of lymphocyte sub-populations post-exercise (immunophenotyping of lymphocyte sub-populations CD3/4 and CD3/8); therefore, the reduced induction of lymphocyte HO-1 protein in response to $H_2O_2$ treatment was not a result of functionally different cells. Importantly, it has also been reported that the number of apoptotic lymphocytes increases 2 h after demanding exercise [22] and the ability of lymphocytes to proliferate is depressed 2 h post-exercise [13]. Therefore, although speculative, it is possible that the reduced HO-1 induction in lymphocytes could either be symptomatic of, or implicated in, post-exercise immuno-suppression following demanding exercise.

One explanation for the reduced ability to up-regulate HO-1 in response to $H_2O_2$ treatment is some form of exercise-induced suppression of the normal HO-1 response. Recently, Maes et al. [23] showed that the hepatic protein α1-antitrypsin (AAT) acts as a suppressor of HO-1 induction in response to an oxidative challenge and they showed that plasma AAT was directly involved in HO-1 suppression in patients with Alzheimer's Disease. In the present study, we have demonstrated that the concentration of plasma AAT is transiently increased immediately

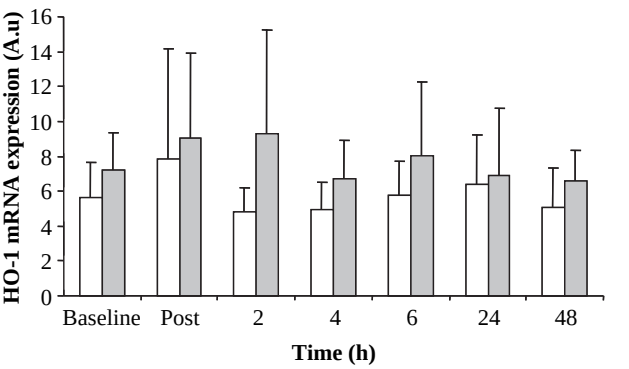


Figure 2. Lymphocyte HO-1 mRNA expression in the rest (open bars) and exercise (closed bars) trials. Venous samples were collected at baseline and immediately following the 60 min period of exercise or rest (post-0 h), with further samples collected at 2, 4, 6, 24, and 48 h following exercise or rest. HO-1 mRNA is expressed relative to GAPDH in arbitrary units (A.u). Values are mean ± SD ($n = 8$).

Table III. Basal HO-1 protein expression 0, 2, 4, 6, 24, and 48 h following 60 min exercise or rest expressed as a fold change from baseline. Values are mean ± SD ($n = 8$). Time point 0 h represents the sample taken immediately following the 60 min period of exercise or rest.

| | Time (h) | | | | | | |
|---|---|---|---|---|---|---|---|
| Trial | Baseline | 0 | 2 | 4 | 6 | 24 | 48 |
| Lymphocytes | | | | | | | |
| Rest | 1.00 ± 0.00 | 0.94 ± 0.13 | 0.98 ± 0.14 | 0.97 ± 0.09 | 0.99 ± 0.20 | 0.95 ± 0.10 | 1.01 ± 0.25 |
| Exercise | 1.00 ± 0.00 | 1.01 ± 0.19 | 0.97 ± 0.25 | 0.95 ± 0.24 | 1.02 ± 0.23 | 1.01 ± 0.21 | 1.00 ± 0.15 |
| Monocytes | | | | | | | |
| Rest | 1.00 ± 0.00 | 1.16 ± 0.40 | 1.18 ± 0.33 | 1.14 ± 0.45 | 1.17 ± 0.67 | 1.14 ± 0.32 | 1.28 ± 0.74 |
| Exercise | 1.00 ± 0.00 | 1.05 ± 0.30 | 1.03 ± 0.38 | 1.02 ± 0.29 | 1.09 ± 0.41 | 1.18 ± 0.35 | 1.16 ± 0.28 |

after exercise. To our knowledge this is the first study to show an increase in plasma AAT concentration following a single bout of demanding exercise. Furthermore, we also report that there was a positive correlation between the increase in plasma IL-6, a primary mediator of hepatocyte AAT production [24], and the increase in plasma AAT. Although further work is required to establish whether this association is causal, it is possible that the increased release of IL-6 from exercising skeletal muscle [25] causes an increase in hepatic production of AAT and that one consequence of increased plasma AAT is a transient suppression of the ability of lymphocytes to induce HO-1 in response to an independent oxidative challenge. The temporal discrepancy between an increase in AAT and the suppression of HO-1 induction probably indicates that AAT does not work directly but through one or more intermediate(s). Maes et al. [23] showed that the HO-1 suppressive effect of AAT was able to act quickly (~2 h) and was probably mediated by transcriptional repression of the HO-1 gene. One putative mediator for this response could be the heme-binding protein Bach-1, a transcriptional repressor for HO-1 [26]. We tentatively suggest that the lack of HO-1 induction 2 h following exercise may reflect some form of transient suppression of HO-1 and a failure to induce HO-1 when required. This may reflect a novel role for a muscle-derived protein (i.e. IL-6) in changing the hepatic production of an HO-1 suppressor (AAT) and ultimately down-regulating the inducibility of HO-1 in lymphocytes.

An alternative explanation for the present findings is that the reduced induction of lymphocyte HO-1 protein in response to $H_2O_2$ treatment following exercise reflects a positive adaptive response to the exercise itself. Acute exercise is known to increase the expression of heat shock proteins (HSP) and antioxidant enzymes in lymphocytes [27,28] as well as antioxidant nutrients in plasma and lymphocytes [29,30]. Therefore, one explanation for the reduced increase in HO-1 protein following *ex vivo* treatment may potentially be a consequence of increases in HSP and endogenous antioxidants and indicate an adaptive response to exercise. According to this interpretation, the decreased induction of HO-1 by $H_2O_2$ 2 h after exercise may be indicative of a reduced need to up-regulate this cyto-protective and anti-inflammatory protein perhaps as a consequence of increased protection against oxidants such as $H_2O_2$. Several investigations have shown an adaptive response to subsequent oxidative stress using a variety of initial stressors [31–34]. The time for this adaptive response has varied from 4 h [34] to 48 h following the initial exposure [31], whereas in the present investigation the decrease in *ex vivo* lymphocyte HO-1 protein induction following treatment was observed 2 h post-exercise. It should be noted that although the blood sample was taken 2 h after exercise the blood processing time (~2.5 h) should be taken into account as these cells would be metabolically active during this time. Interestingly, this was a transient effect because the induction of HO-1 protein in lymphocytes in response to $H_2O_2$ treatment had returned to resting levels 4 h following exercise. As a result, we feel that it is unlikely that the transient reduction in the ability to induce HO-1 reflects an up-regulation of protective proteins (e.g. HSPs or antioxidant enzymes) because these effects would remain for longer than just a few hours [35].

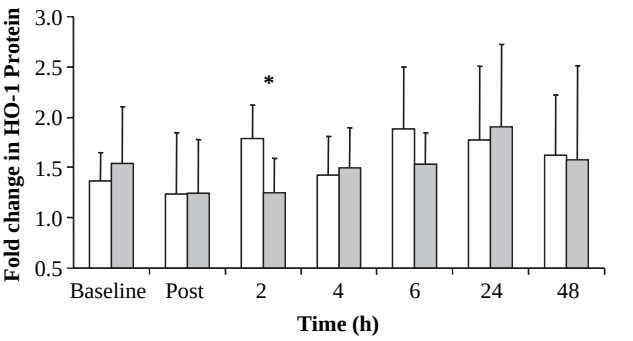


Figure 3. The effect of prior rest (open bars) or exercise (closed bars) on *ex vivo* lymphocyte HO-1 protein induction following 48 h recovery from $H_2O_2$ treatment. Data represents the mean ± SD of the change in lymphocyte HO-1 protein following $H_2O_2$ treatment at each time point from the baseline. Samples were collected at baseline, immediately following the exercise or rest (post), and at 2, 4, 6, 24, and 48 h following exercise or rest. * denotes a significant difference between the change in lymphocyte HO-1 protein induction from baseline 48 h following $H_2O_2$ treatment 2 h post-exercise and 2 h post-rest ($p < 0.05$). $n = 8$ for the exercise and rest trials, except at 2 h in the rest trial where $n = 7$.

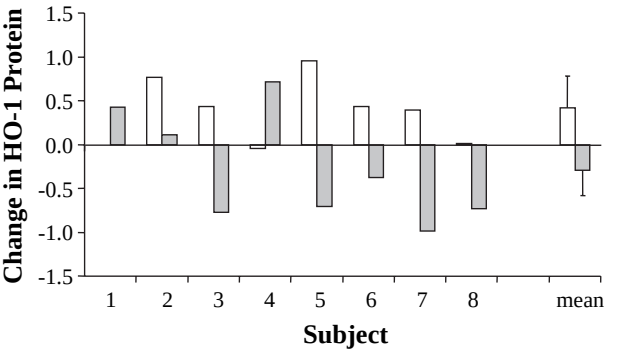


Figure 4. The individual change in lymphocyte HO-1 protein 48 h after $H_2O_2$ treatment 2 h post-exercise (closed bars) or 2 h post-rest (open bars). The change at 2 h represents the difference between HO-1 induction at 2 h post-exercise and baseline in the exercise trial with the difference between HO-1 induction at 2 h post-rest and baseline in the rest trial [Change = (2 h post-exercise − baseline) − (2 h post-rest − baseline)]. The mean ± SD is included (exercise trial, $n = 8$; rest trial, $n = 7$). There was a missing value for subject 1's rest trial as a result of a problem with the pre-rest treatment.

Importantly, the reduced level of HO-1 protein induction in lymphocytes in response to $H_2O_2$ treatment 2 h following exercise was not mediated by HO-1 itself as the acute exercise employed in the present investigation did not increase lymphocyte HO-1 protein.

In the current investigation, mean lymphocyte HO-1 protein induction increased ~1.5-fold 48 h following $H_2O_2$ treatment prior to exercising or resting for 60 min. Previous studies have shown that HO-1 protein is induced in lymphocytes as early as 6 h following $H_2O_2$ treatment with HO-1 expression maintained at least 18 h following treatment [7]. Other investigators have reported a time-dependent increase in lymphocyte HO-1 protein following exposure to a non-genotoxic HBO treatment with maximal HO-1 protein induced at 24 h [33]. In osteoarthritic chondrocytes [36] and peritoneal

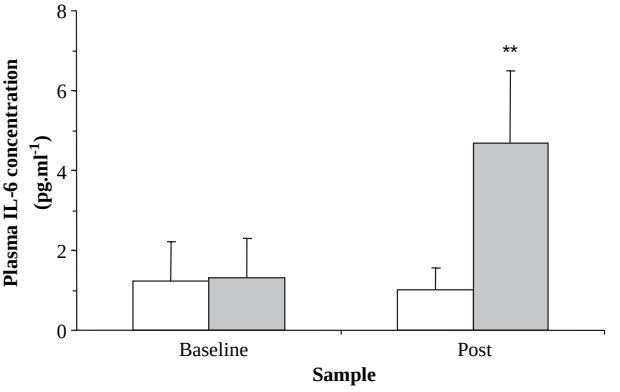


Figure 5. Plasma IL-6 concentration in rest (open bars) and exercise (closed bars) trials. ** denotes a significant difference between pre and post values in the exercise trial only ($p < 0.01$). Values are mean ± SD ($n = 8$). Post represents the 0 h post-exercise/rest time point.

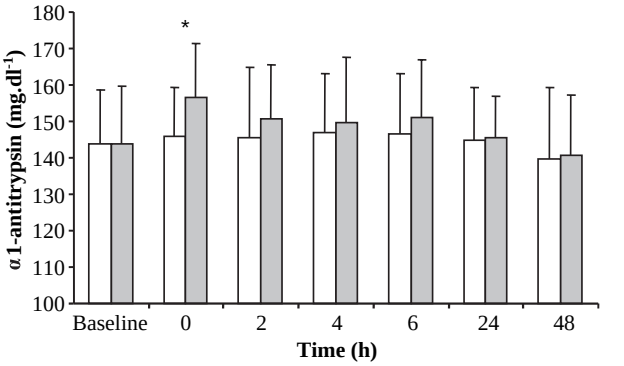


Figure 6. Plasma α1-antitrypsin concentration in rest (open bars) and exercise (closed bars) trials. Values are mean ± SD ($n = 8$). * denotes a significant difference between the change in AAT concentration from baseline immediately post-exercise (0 h) and immediately post-rest (0 h) ($p < 0.05$).

macrophages [37] maximal HO-1 protein induction has been reported 48 h after stimulation. Therefore, it appears that the induction of HO-1 protein is often delayed and it has been suggested that this reflects an autocrine mechanism [36,37]. It is also noteworthy that there is a large inter-individual variation in the ability of lymphocytes and monocytes to induce HO-1. Importantly, we have shown that these inter-individual differences are maintained (i.e. that they are not random) and that HO-1 protein induction in human lymphocytes and monocytes is a reproducible response. The variability between subjects is presumably explained by either differences in environment (e.g. dietary intake of antioxidants or levels of physical activity) or genotype [38].

Previous studies have shown that both a competitive half marathon run [10] and prolonged treadmill running [11] increase basal HO-1 expression in lymphocytes (i.e. endogenous HO-1 in freshly-harvested and unstimulated lymphocytes as opposed to induction *ex vivo*). In the present study, the reduced inducibility of HO-1 to $H_2O_2$ took place without a change in basal expression of HO-1 in unstimulated lymphocytes. The exercise protocol used in the current study was intermittent in nature to achieve a high overall intensity of exercise (~75% $\dot{V}O_2$ max). Importantly, subjects completed four bouts of sustained work at 100% maximal $O_2$ consumption in 60 min. Nevertheless, in contrast to our earlier investigation of continuous running at a lower intensity but for a longer period of time [11], the intermittent exercise used in the current study did not increase basal HO-1 mRNA or protein in lymphocytes. For HO-1 protein, this discrepancy may be partly explained by the use of flow cytometry in the present investigation whereas we used Western blotting in our earlier work [11], although this would not explain the discrepancy for HO-1 mRNA. We believe that the disparity between these studies is more likely to be due to differences in subject fitness

or familiarity with particular forms of exercise or it may suggest that the induction of HO-1 in lymphocytes depends on the exercise duration and intensity; with prolonged running leading to a more pronounced change in HO-1 expression.

In summary, there was a modest and transient decrease in HO-1 induction following $H_2O_2$ treatment in lymphocytes obtained 2 h following demanding intermittent exercise. We propose that this may be symptomatic of a short-term maladaptive response whereby demanding exercise transiently suppresses the ability of lymphocytes to up-regulate this cytoprotective and anti-inflammatory protein. One candidate for this exercise-induced suppression is the hepatic protein AAT that we have shown for the first time is increased immediately after demanding exercise. It remains unclear whether more moderate intensity exercise or regular exercise elicits the same response. Because of the critical role for HO-1 in health and disease, an exercise-induced suppression of HO-1 induction provides a useful experimental tool to better understand the regulation of this enzyme. Future work should seek to identify whether AAT is critical for an exercise-induced suppression in the ability of lymphocytes to up-regulate HO-1 in response to oxidative stress and the mechanisms behind this effect.



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