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Exercise-induced expression of heme oxygenase-1 in human lymphocytes

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Abstract

The aim of the present investigation was to determine whether an acute bout of exercise increases heme oxygenase-1 (HO-1) mRNA accumulation in human lymphocytes. Eight male subjects performed separate exercise and rest trials in a randomised order at least 10 days apart. In the exercise trial subjects ran for 75-min at a speed corresponding to 70% maximal oxygen uptake, and in the resting trial subjects sat calmly in the laboratory for an equivalent period of time. Lymphocytes were harvested from blood samples taken before and after each trial. Total RNA was isolated and used to determine the fold-change in HO-1 mRNA accumulation relative to baseline values using real time reverse transcription-polymerase chain reaction. HO-1 protein was determined by Western blotting. Six of the eight subjects showed an increase in HO-1 mRNA greater than two-fold after exercise. The median peak fold-change was 2.7 fold with one subject showing a particularly pronounced response (20-fold) 24 h post-exercise. In the rest trial the level of HO-1 mRNA did not change over the period of investigation. There was also an increase in HO-1 protein 2 h after exercise. These results complement an earlier study showing that acute exercise of a different type (half marathon) leads to an increase in HO-1 expression in lymphocytes.

Keywords: *Physical activity, oxidative stress, free radicals, leukocytes, gene expression, inflammation*

Introduction

There is considerable evidence demonstrating that the induction of Heme Oxygenase-1 (HO-1) is cytoprotective. For example, increased HO-1 protein expression following UVA-induced oxidative stress in human fibroblasts reduces membrane damage upon subsequent exposure to UVA.[1] Furthermore, Poss and Tonegawa[2] showed that fibroblasts taken from HO-1 deficient mice had decreased levels of survival in comparison to control fibroblasts after exposure to oxidants. In an alternative experimental model, the induction of HO-1 has been shown to protect human lymphocytes against oxidative stress due to hyperbaric oxygen treatment.[3] Interestingly, Poss and Tonegawa[2] reported that whilst HO-1 deficient mice died as a result of endotoxin injection, mice with normal HO-1 activity survived.[2] This finding points towards a role for HO-1 in immune function, and it appears that HO-1 is both immuno-protective[4] and has anti-inflammatory properties.[5,6]

A common characteristic of HO-1 inducers is their ability to cause oxidative stress.[7,8] Given the well-documented observation that exercise leads to oxidative stress,[9–12] an acute bout of exercise would also be expected to lead to an increased expression of HO-1. Interestingly, Niess and co-workers reported that an acute bout of exercise leads to an increase in leukocyte HO-1 protein.[13] Using flow cytometry, these investigators demonstrated that a half marathon run

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provoked an increase in the percentage of monocytes, lymphocytes and granulocytes expressing HO-1. Given the cytoprotective and anti-inflammatory property of this enzyme, such a response may have important implications for leukocyte function. However, the initial observations by Niess and co-workers[13] were limited by the fact that samples were taken before and after a competitive event—with each subject working at a different and unknown exercise intensity. There was also no corresponding rest trial carried out at a similar time of day on a separate occasion in order to account for any potential diurnal variation in HO-1. In order to further develop exercise as a model to examine HO-1 up-regulation *in vivo*, there is a need to examine the responses to a carefully prescribed bout of exercise performed at the same relative exercise intensity for each individual. It is also important to determine whether there are any changes throughout the day in the absence of exercise (i.e. during a day of rest).

The aim of the present investigation was to examine changes in HO-1 expression following an exercise trial where subjects completed a controlled bout of treadmill running and a corresponding resting trial over the same period of time on a different day.

Materials and methods

Subjects

Eight male students volunteered to take part in this investigation, which had approval from the local Ethics Committee. All subjects were informed verbally and in writing about the nature and demands of the study, and subsequently completed a health history questionnaire and gave their written informed consent. Subjects who smoked or took vitamin supplements were excluded from the investigation. Mean age, height and body mass were 21 ± 1 years, 179 ± 1 cm and 73.9 ± 2.4 kg, respectively. The sum of skinfold measurements taken at four sites (biceps, triceps, subscapular and suprailiac) was 26 ± 2 mm. Subjects were all habitually active and took part in 5 ± 1 exercise sessions per week (comprising both endurance and resistance exercise).

Preliminary measurements

Subjects performed two preliminary treadmill-based tests at least seven days prior to the main trial using methods described previously.[14] Briefly, subjects performed an incremental submaximal running test to determine the relationship between running speed and oxygen uptake, and also an incremental running test to determine maximal oxygen uptake ($\dot{V}O_2$ max). Mean $\dot{V}O_2$ max was 64.4 ± 2.4 ml kg^{-1} min^{-1}.

Experimental design and procedures

Subjects performed an exercise and rest trial in a randomised order at least 10 days apart. In the exercise trial subjects ran on a level treadmill for 75 min at a speed corresponding to 70% $\dot{V}O_2$ max, and in the resting trial subjects sat calmly in the laboratory for the same period of time. Subjects weighed and recorded their food and fluid intake for two days before the first trial and on the day of this trial, and they were instructed to repeat this diet as closely as possible during the second trial. Food and fluid records were analysed using the software COMP-EAT 4.0 (Nutrition Systems, UK). Subjects were asked to refrain from exercise for 48 h prior to each trial and on the day of a trial.

On the morning of a trial subjects arrived at the laboratory after an overnight fast of approximately 10 h. A resting venous blood sample was taken after subjects had been standing for at least 15 min. During the exercise trial, subjects performed a standardised warm up consisting of running at 50% $\dot{V}O_2$ max (5 min) and gentle stretching (5 min) prior to beginning the 75 min run. One-minute expired air samples, ratings of perceived exertion[15] and heart rate (Sports Tester, Polar Electro, Finland) were taken every 15 min throughout exercise. Subjects were allowed to consume water *ad libitum* during both exercise and rest trials, although the volume of water was monitored and used in the subsequent calculation of sweat loss. Nude body mass was determined before and after exercise and rest trials. Blood samples were taken immediately after the 75-min exercise or rest trial and then again 1 and 2 h later. Subjects returned to the laboratory the following morning after an overnight fast and a final venous blood sample was taken.

Blood processing and analysis

Whole blood (~16 ml) was placed into EDTA coated tubes and analysed for the various leukocyte populations using an automated haematology analyser (SF-3000, Sysmex UK Ltd., UK). Plasma was subsequently removed following centrifugation for 10 min at 1000*g*. The cell pellet was made up to the original volume using RPMI media supplemented with 10% foetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mmol l^{-1} glutamine and 0.2% w/v sodium carbonate. Samples were then immediately used to separate mononuclear cells and polymorphonuclear cells in a single centrifugation using two layers of separation media with different densities (Histopaque 1077 and 1119, Sigma-Aldrich Ltd., UK). Harvested cells were re-suspended in the same RPMI media described above prior to being counted. Lymphocytes were obtained by incubating the mononuclear cells in 150 cm^2 culture flasks for 60 min and allowing monocytes to adhere to plastic (37°C in 5% CO_2). Two aliquots of cell suspension containing approximately 3 × 10^6 cells were centrifuged to obtain cell pellets, which were frozen on dry ice for 10 min and stored at −80°C for subsequent analysis of HO-1 protein. From the remaining cell suspension

approximately 10 or 15 × 10^6 cells were pelleted, lysed immediately in 2 or 3 ml TRIZOL reagent and stored at − 80°C. Total cellular RNA was extracted according to the manufacturer's protocol (Invitrogen Life Technologies, UK). The total RNA yield from lymphocyte samples was approximately 1 μg/million cells.

Serum was obtained by allowing whole blood (~4 ml) to clot for 20 min, followed by centrifugation at 1000*g*. Serum was analysed for interleukin-6 (IL-6) using a commercially available solid-phase high-sensitivity ELISA (Quantikine, R & D Systems Inc., UK).

Real-time RT-PCR

HO-1 mRNA was quantified by two-step RT-PCR as described previously.[16] Briefly, 1 μg of total RNA was reverse transcribed using SuperScript first-strand synthesis kit (Invitrogen Life Technologies, UK) according to the manufacturer's protocol. A 1:10 dilution of each cDNA sample was freshly prepared in PCR-grade water and 2 μl were used in each PCR reaction. The primer pair used for the human HO-1 cDNA was 5′-AAG AGG CCA AGA CTG CGT TC-3′ (forward) and 5′-GGT GTC ATG GGT CAG CAG C-3′ (reverse) and that for the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was 5′-GAC ATC AAG AAG GTG GTG AA-3′ (forward) and 5′-TGT CAT ACC AGG AAA TGA GC-3′ (reverse). 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 normalised HO-1 levels were expressed relative to baseline samples in rest and exercise trials, respectively.

Western blot analysis

Chemicals were purchased from Sigma-Aldrich Ltd. (UK) unless otherwise stated. Total cell extracts from cell pellets containing approximately 3 × 10^6 cells were prepared in Munroe buffer (1 × protease inhibitor cocktail, Roche Molecular Biochemicals, UK; 0.3% Nonidet P40, BDH, UK) and the protein concentration determined as previously described.[17] Lymphocyte protein yields were approximately 30–35 μg/million cells. Samples (40 μg/lane) were electrophoresed in a 12.5% polyacrylamide gel (containing 0.1% sodium dodecyl sulfate; SDS) in Tris-glycine buffer (Fisher, UK). Electrophoresis was carried out at 80 V for 2 h (Mini-protean 3, BioRad, UK). Samples were transferred onto Hybond ECL nitrocellulose membranes (Amersham, UK) for 1 h at 100 V using the manufacturer's instructions (Trans-blot cell system, BioRad, UK). Blots were blocked overnight at 4°C in blocking solution (3% milk powder, 3% bovine serum albumin, 1% Tween 20 in PBS) followed by probing with a mouse monoclonal antibody raised against human HO-1 (OSA-110, Stressgen, Victoria, Canada) at 1:200 dilution in blocking solution containing 0.05% Tween 20. The HO-1 antibody was detected with a rabbit anti-mouse IgG-HRP conjugate at a dilution of 1:1000 and visualised on Hyperfilm ECL using the ECL chemiluminescence kit (Amersham). The blots were then stripped by incubating at 65°C for 20 min in a solution containing 0.1 M β-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8) and 2% SDS. This was followed by 3 washes in 0.5% Tween in PBS. Subsequently, the blots were probed for β-actin with a mouse monoclonal antibody (Amersham) under the same conditions as HO-1. The signals on the films were quantified by densitometry (Gene Genius, Syngene Gene Tools software, UK, version 2.11.03). HO-1 was normalised to β-actin and expressed relative to baseline samples in rest and exercise trials, respectively.

Statistical analysis

Unless otherwise stated, a two-way analysis of variance with repeated measures was used to compare results between treatments and over time. Where a significant interaction was observed, a paired Student's *t*-test was used to compare conditions (exercise and rest). When there were only single comparisons, a paired Student's *t*-test was used to determine whether any differences between conditions existed. Values are presented as means ± standard error of the mean (SEM). Values for HO-1 mRNA accumulation were not normally distributed and therefore these results are presented as medians and the individual values for the exercise trial. Values for HO-1 mRNA were analysed using the Wilcoxon signed-rank test. Significance was accepted at the 5% level.

Results

The mean treadmill running speed was 12.0 ± 0.6 km h^{-1}, which elicited an oxygen uptake of 70 ± 1% $\dot{V}O_2$ max (44.9 ± 1.7 ml kg^{-1} min^{-1}). Mean heart rate during exercise was 168 ± 3 b.min^{-1} with the mean rating of perceived exertion being 14 ± 1 a.u. Oxygen uptake and heart rate during the resting trial were 4.7 ± 0.3 ml kg^{-1} min^{-1} and 65 ± 1 b.min^{-1}, respectively. Ambient temperature and humidity were similar in exercise and resting trials, being 19.4 ± 0.3°C vs. 18.7 ± 0.5°C and 43 ± 2% vs. 41 ± 2%, respectively. Total fluid loss was 1.7 ± 0.1 l after the exercise trial and 0.1 ± 0.0 l after the resting trial. There were no differences between trials in the amount or composition of food consumed over each 3-day period (Table I).

Leukocyte numbers

Exercise provoked an increase in total leukocyte count up to 2 h after exercise ($P < 0.05$), which was largely accounted for by an increase in neutrophil count

Table I. Estimated daily dietary composition assessed during exercise and rest trials.

	Energy Intake (MJ)	CHO (%)	Fat (%)	Protein (%)	Vitamin C (mg)	Vitamin E (mg)
Exercise	15.6 ± 1.1	59 ± 2	25 ± 2	16 ± 1	188 ± 19	11 ± 1
Rest	14.9 ± 1.0	57 ± 3	27 ± 3	16 ± 1	172 ± 19	13 ± 3

Values are means ± SEM ($n = 8$). CHO: carbohydrate.

(Table II; $P < 0.05$). There was a small increase in lymphocyte and monocyte count immediately post-exercise ($P < 0.05$) and monocyte number remained above corresponding resting values until 2 h after exercise (Table II; $P < 0.05$).

Lymphocyte HO-1 mRNA accumulation

Six of the eight subjects showed an increase in HO-1 mRNA greater than two-fold after exercise (Figure 1). However, there was considerable variation in terms of the magnitude of this response and the time of peak HO-1 mRNA accumulation. Of those subjects demonstrating an increase in HO-1 mRNA above two-fold, two subjects showed a peak 1 h after exercise, two subjects 2 h after exercise and two subjects 24 h after exercise. One subject showed a particularly pronounced response with HO-1 mRNA increasing progressively so that at 24 h post-exercise HO-1 mRNA was 20-fold greater than pre-exercise. HO-1 mRNA in the rest trial did not change over the period of investigation (Figure 1).

The peak exercise-induced fold-change in HO-1 mRNA from pre-exercise values is presented for each subject and expressed graphically against the fold-change from baseline at the corresponding time point in the rest trial (Figure 2). The median peak fold-change in HO-1 mRNA following exercise was 2.7-fold and was significantly greater than corresponding values in the rest trial ($P < 0.05$).

In order to confirm the extreme response shown by subject eight and to investigate the reproducibility of the technique, cDNA was prepared and analysed on three further occasions (Figure 3). Each independent analysis yielded very similar results in terms of both the pattern and magnitude of response.

Lymphocyte HO-1 protein

HO-1 protein was measured by Western blotting at each time point in the exercise trial and at three time points in the resting trial (Figure 4). Densitometric values for the exercise trial were compared with the mean for the resting trial using a paired Student's *t*-test. HO-1 protein was approximately two-fold higher than resting values 2 h after exercise (Figure 4; $P < 0.05$). However, it should be noted that these values do not include results for the subject with a pronounced HO-1 mRNA response because of technical problems with the analysis of these samples.

Serum IL-6

Interleukin-6 concentration increased immediately after exercise and remained above resting values until 1 h post-exercise (Figure 5; $P < 0.05$).

Discussion

The present study is first to document increased HO-1 mRNA accumulation in human lymphocytes following exercise, although other investigators have shown that freshly harvested human lymphocytes respond to oxidative stress (e.g. hyperbaric oxygen treatment) with an up-regulation of HO-1 mRNA.[3] The present investigation is also consistent with previous reports that an acute bout of prolonged exercise increases HO-1 protein expression in lymphocytes. Niess *et al.*[13] demonstrated that a competitive half-marathon increased HO-1 protein expression in leukocytes (using flow cytometry). Indeed, the change in HO-1 protein expression in lymphocytes reported by these authors was very similar to the findings of the present investigation. It is worth noting, however, that while Niess and co-workers reported peak HO-1 protein

Table II. Blood leukocyte counts in exercise and rest trials.

Cell Type ($10^9 l^{-1}$)	Trial	Pre	Post	1 h Post	2 h Post	24 h Post
Total leukocytes	Exercise	5.6 ± 0.5	9.0 ± 1.5 *	9.8 ± 1.3 *	13.2 ± 1.2 *	5.6 ± 0.6
	Rest	5.1 ± 0.2	5.5 ± 0.4	5.6 ± 0.4	5.9 ± 0.4	5.3 ± 0.3
Neutrophils	Exercise	2.8 ± 0.4	5.7 ± 1.3 *	7.7 ± 1.4 *	10.7 ± 1.2 *	3.2 ± 0.5
	Rest	2.5 ± 0.1	3.0 ± 0.3	3.3 ± 0.4	3.5 ± 0.4	2.8 ± 0.2
Lymphocytes	Exercise	2.1 ± 0.1	2.5 ± 0.2 *	1.6 ± 0.1	1.5 ± 0.1	2.0 ± 0.2
	Rest	2.0 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.9 ± 0.2	1.9 ± 0.2
Monocytes	Exercise	0.5 ± 0.1	0.6 ± 0.1 *	0.7 ± 0.1 *	0.9 ± 0.1 *	0.5 ± 0.1
	Rest	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0

Values are means ± SEM ($n = 8$). * different to corresponding rest values ($P < 0.01$).

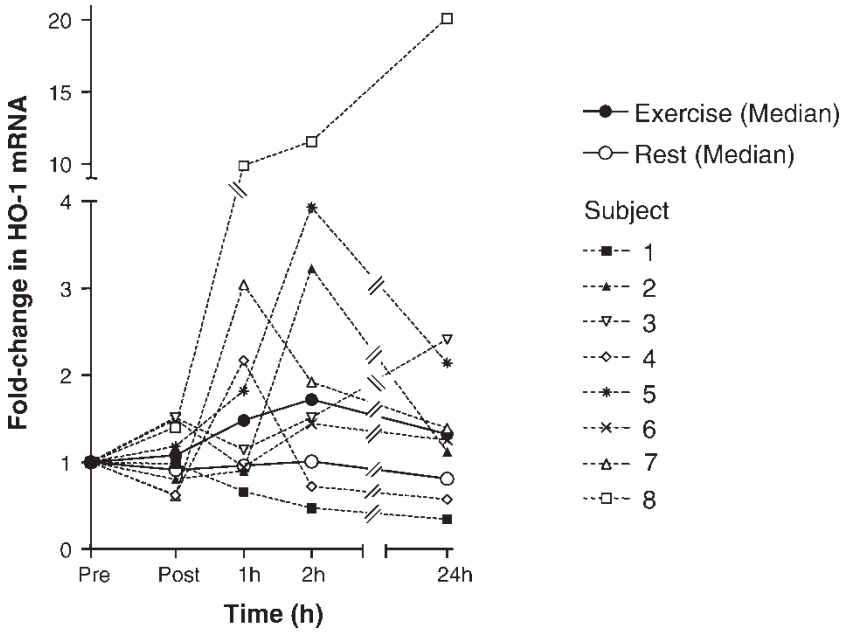

Figure 1. Lymphocyte HO-1 mRNA in exercise and resting trials expressed relative to GAPDH. Median values for exercise and resting trials are shown with the individual values for the exercise trial over time ($n = 8$).

immediately after exercise,[18] we observed the highest mean lymphocyte HO-1 protein at 2 h following exercise. This apparent discrepancy may be explained by the different modes of exercise and/or the different analytical techniques employed in each investigation. Interestingly, this discrepancy is perhaps greater than it might initially seem, since the processing time involved in harvesting and separating cells in the present investigation (~ 2.5 h) should be added to each of the time points as the cells would be metabolically active during this time.

HO-1 is an anti-inflammatory and cytoprotective enzyme.[6–8] The up-regulation of HO-1 has been demonstrated to protect cells against subsequent oxidative stress[1–3] and to help modulate and/or resolve inflammation.[5,6,19,20] Although the precise mechanisms behind these effects remain to be elucidated, an exercise-induced increase in the expression of this enzyme has potentially important implications. It is possible that lymphocyte HO-1 plays a role in responding to the inflammatory response that accompanies an acute bout of exercise.[11,21] HO-1 has been

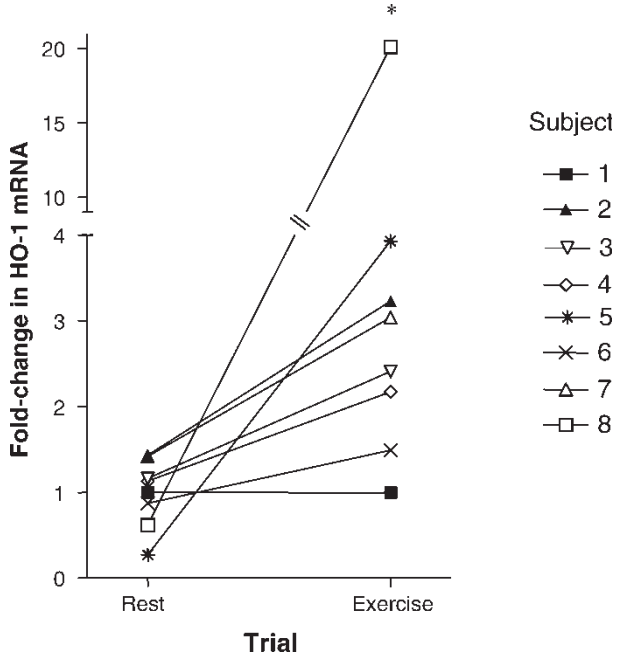

Figure 2. The peak exercise-induced fold-change in HO-1 mRNA from pre-exercise values is expressed graphically against the fold-change from baseline at the corresponding time point in the rest trial. The peak fold-change in HO-1 mRNA following exercise was significantly greater than corresponding values in the rest trial ($P < 0.05$).

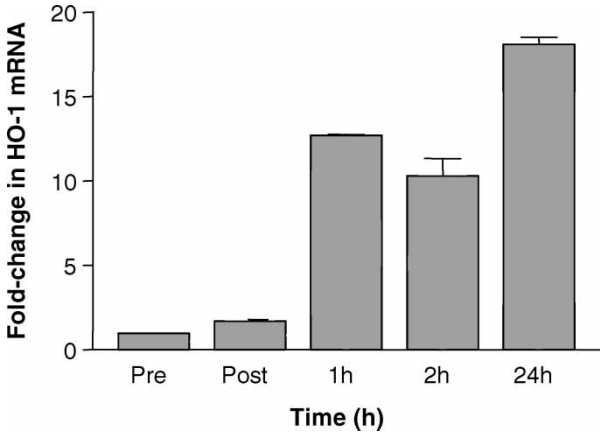

Figure 3. Repeat analysis of lymphocyte HO-1 mRNA in the exercise trial for the subject with the most pronounced response (subject 8). Values are means ± SEM ($n = 3$).

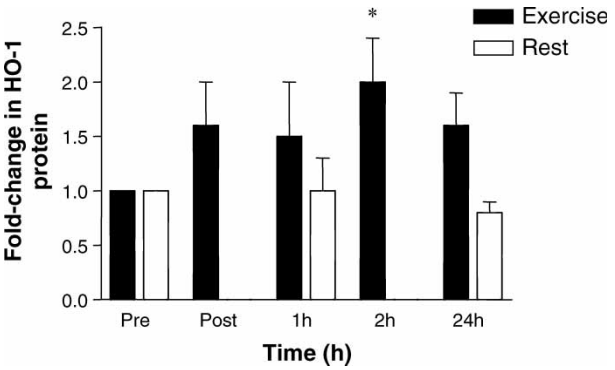

Figure 4. Lymphocyte HO-1 protein in exercise and resting trials. Densitometric values for HO-1 protein are expressed relative to β-actin. Values are means for $n = 7$ (± SEM). * different to mean resting values ($P < 0.05$). Values were only available at three time points during the resting trial.

shown to have anti-inflammatory properties and it has been suggested that HO-1 should be considered a form of acute phase protein.[22] In addition, increased expression of HO-1 is likely to improve lymphocyte protection against subsequent oxidative stress. Abraham *et al.*[22] suggested that the induction of HO-1 might serve to protect against reactive intermediates produced by macrophages at sites of inflammation. As a corollary, recent evidence shows that pre-induction of HO-1 in lymphocytes protects against DNA damage following treatment with hydrogen peroxide.[3] It is also possible that HO-1 might serve to catabolise heme released at sites of microtrauma.[22] Interestingly, it appears that an acute bout of exercise leads to an increase in plasma free hemoglobin,[23] although it is unclear to what extent exercise alters intracellular heme availability in lymphocytes.

The pathways involved in the up-regulation of lymphocyte HO-1 following exercise have not yet been defined. Several authors have demonstrated that active skeletal muscle releases reactive intermediates into the interstitium,[12,24] and it has been proposed that skeletal muscle is the source of reactive intermediates detected in the circulation during exercise.[25] Since the HO-1 promoter is controlled by a regulatory network that is sensitive to oxidative stress,[26] it is possible that increased expression of HO-1 in lymphocytes is a direct product of oxidative stress generated by working skeletal muscle. However, an acute bout of exercise also provokes a transient inflammatory response,[11,21] and this too has the capacity to initiate oxidative stress.[27,28] In the present study, plasma concentrations of the pro-inflammatory cytokine IL-6 increased approximately 4-fold immediately after exercise. Consequently, in addition to a potential direct effect from oxidative stress associated with muscle contraction, it is also possible that increased lymphocyte HO-1 is a response to oxidative stress initiated by exercise-induced inflammation.

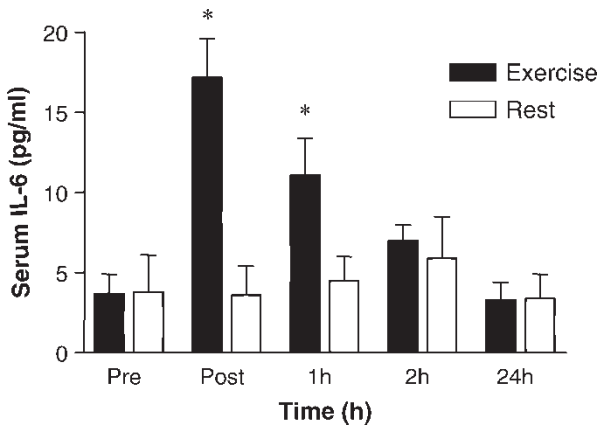

Figure 5. Serum IL-6 concentration in exercise and resting trials. Values are means ± SEM ($n = 8$). * different to corresponding resting values ($P < 0.05$).

The oxidant-responsive property of HO-1 has led to its adoption as a specific and sensitive marker of oxidative stress at the cellular level *in vitro*.[29–32] It has also been suggested that HO-1 gene expression might be a useful marker of oxidative stress *in vivo*.[33,34] Indeed, there is some evidence that antioxidant supplementation modifies expression of HO-1 protein in lymphocytes taken from human volunteers.[35] Furthermore, a recent report found lower HO-1 mRNA in lymphocytes taken from patients with Alzheimer's disease, and the authors speculated that the quantitative assessment of HO-1 mRNA using real time RT-PCR might be a useful measure of oxidative stress *in vivo*.[36] The current consensus is that in addition to being reproducible and sensitive to change, markers of oxidative stress should be part of a well-defined physiological process that plays some role in health or well-being.[34,37] Given the wealth of evidence showing the functional importance of HO-1,[6,8] it is possible that lymphocyte HO-1 may prove to be an excellent and accessible marker of oxidative stress *in vivo*. Nevertheless, given the enormous range of known HO-1 inducers,[8] there is a clear need to improve our understanding of HO-1 induction in lymphocytes in order for this to be exploited as a marker of oxidative stress *in vivo*.

In conclusion, an acute bout of exercise increases expression of HO-1 in human lymphocytes although it is unclear if this is a direct product of exercise-induced oxidative stress or an exercise-induced inflammatory response. One subject showed a particularly marked increase in HO-1 mRNA accumulation, although it is not clear whether this reflects amplified exercise-induced oxidative stress or inter-individual variation in response to similar levels of oxidative stress.

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