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Research article

# Blood glutathione homeostasis as a determinant of resting and exercise-induced oxidative stress in young men

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Although the importance of glutathione in protection against oxidative stress is well recognised, the role of physiological levels of glutathione and other endogenous antioxidants in protecting against exercise-induced oxidative stress is less clear. We evaluated the role of glutathione and selected antioxidant enzymes as determinants of lipid peroxidation at rest and in response to exercise in men ($n$ = 13–14) aged 20–30 years, who cycled for 40 min at 60% of their maximal oxygen consumption ($VO_{2max}$). Levels of plasma thiobarbituric acid reactive substances (plasma TBARS) and blood oxidised glutathione (GSSG) increased by about 50% in response to exercise. Mean blood reduced glutathione (GSH) decreased by 13% with exercise. Of the measured red blood cell (RBC) antioxidant enzyme activities, only selenium-dependent glutathione peroxidase (Se-GPX) activity rose following exercise. In univariate regression analysis, plasma TBARS levels at rest predicted postexercise plasma TBARS and the exercise-induced change in total glutathione (TGSH). Blood GSSG levels at rest were strongly determinant of postexercise levels. Multiple regression analysis showed blood GSH to be a determinant of plasma TBARS at rest. The relative changes in TGSH were determinant of postexercise plasma TBARS. In summary, higher blood GSH and lower plasma TBARS at rest were associated with lower resting, and exercise-induced, lipid peroxidation. Subjects with a favourable blood glutathione redox status at rest maintained a more favourable redox status in response to exercise-induced oxidative stress. Changes in blood GSH and TGSH in response to exercise were closely associated with both resting and exercise-induced plasma lipid peroxidation. These results underscore the critical role of glutathione homeostasis in modulating exercise-induced oxidative stress and, conversely, the effect of oxidative stress at rest on exercise-induced changes in glutathione redox status.

## Introduction

Increasing attention has been placed on the role of glutathione in protecting against resting and exercise-induced oxidative stress.[1–3] Oxygen consumption increases 8–10-fold during even moderate physical exercise, and 100-fold in exercising skeletal muscle. Even moderate exercise may generate reactive oxygen species beyond the capacity of antioxidant defences, inducing lipid, protein and DNA oxidative damage[4]. Uncontrolled oxidative stress may potentially decrease the therapeutic benefits of physical exercise.[4]

Studies have demonstrated increased blood oxidised glutathione (GSSG) and GSSG/total glutathione (TGSH), indices of oxidative stress, in response to exercise of sufficient duration and intensity.[1,2,5,6] Physical training, on the other hand, may strengthen glutathione and other antioxidant defences,[7–9] and may decrease resting and exercise-induced oxidative stress.[10–12] Animal and human studies with supplementation of glutathione or the proglutathione

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drug N-acetylcysteine, and with glutathione deficiency induced by L-buthionine-[S,R]-sulfoximine (BSO), have further emphasised the role of glutathione in protecting against resting and exercise-induced oxidative stress.[1–3]

Glutathione and its dependent enzymes glutathione peroxidase (Se-GPX), glutathione-S-transferase (GST) and glutathione reductase (GRD) work in concert with other antioxidants and antioxidant enzymes such as superoxide dismutase (SOD) and catalase to protect against reactive oxygen species. Reduced glutathione detoxifies reactive oxygen species such as the hydroxyl radical, hydrogen peroxide and lipid peroxides directly or in reactions catalysed by Se-GPX or GST.[13] GST also catalyses the reaction between the –SH group and potential alkylating agents, rendering them suitable for transport out of the cell. GRD catalyses the NADPH-dependent reduction of oxidised glutathione.

Despite growing knowledge about the importance of glutathione homeostasis in protecting against resting and exercise-induced oxidative stress, the relationships of blood glutathione redox status and erythrocyte glutathione dependent enzymes with indices of oxidative stress at rest and after physical exercise have remained unclear. The purpose of this study was to assess the role of antioxidant defences, and glutathione in particular, in protecting against exercise-induced oxidative stress in young men who served for comparison in a larger study assessing oxidative stress in men with insulin-dependent diabetes mellitus.[14]

## Subjects and Methods

Men aged 20–30 years ($n$ = 14) were recruited into the study after giving informed consent.[14] Subjects were volunteers from the local university student population. Clinical and biochemical data are displayed in Table 1. All underwent clinical examination, routine laboratory tests and ECG to rule out significant diseases. Reasons for exclusion included any cardiovascular or pulmonary disease, vitamin supplementation, chronic medication and regular participation in organised athletic events or highly intense physical activity. The study was approved by the Ethics Committee of the Kuopio University Hospital.

**Table 1** Anthropometric and biochemical characteristics of subjects ($n$ = 14)

| | |
|---|---|
| Age (years) | 23 ± 2.9 |
| Body mass index (kg/m$^2$) | 23.3 ± 1.7 |
| $VO_{2max}$ (ml/kg/min) | 45 ± 6.0 |
| Exercise frequency (times/week) | 1.2 ± 1.6 |
| Blood haemoglobin (g/l) | 152 ± 8.8 |
| Serum total cholesterol (mmol/l) | 4.9 ± 1.0 |
| HDL cholesterol (mmol/l) | 1.5 ± 0.5 |
| Serum triglycerides (mmol/l) | 1.1 ± 0.4 |

Data are means ± SD; HDL, high density lipoprotein.

### *Exercise testing*

All subjects underwent a maximal exercise test to determine $VO_{2max}$, using an electrically braked bicycle ergometer, breath by breath gas monitoring and continuous ECG monitoring. Testing began at 60 W and was increased by 30 W every 2 min. Maximal effort was defined subjectively by the subjects' maximal voluntary effort or objectively (oxygen consumption increase of less than 150 ml/min despite increasing workload).

One to two weeks later, subjects exercised for 40 min at 60% of their $VO_2$ max after 5 min warm up at 60 W. Before exercising, all subjects were instructed to refrain from intense exercise and alcohol for at least 3 days before exercise testing, and from smoking for at least 24 h for the one smoker. On the day of exercise, the subjects ate a light, carbohydrate-rich breakfast. Exercise tests were carried out 2–4 h after breakfast.

### *Blood sample collection and preparation*

Routine screening laboratory tests and lipoprotein profiles were determined on blood samples drawn in a fasting state in the morning on a separate day from the exercise tests. Samples for blood glutathione and plasma TBARS assays were taken from an antecubital vein 5 min before, and within 2 min after, exercising at 60% $VO_{2\,max}$ for 40 min.

Blood TGSH and GSSG analyses were done as described before.[1,7] Briefly, for TGSH determinations, EDTA-blood was precipitated with perchloric acid and the deproteinised supernatant was used. For blood GSSG, the clear supernatant obtained from EDTA-blood treated with 5-sulfosalicylic acid was neutralised and reacted with 2-vinylpyridine. Treated samples were frozen at –20°C until spectrophotometric determination.

Plasma TBARS were assayed as described previously[1]. Briefly, non-haemolysed EDTA-blood was added immediately after being drawn to ice-cold Chelex (Bio-Rad Laboratories, CA, USA) treated potassium phosphate buffer containing $Na_2EDTA$ and centrifuged to obtain plasma. The plasma was immediately treated with ethanolic butylated hydroxytoluene, added to 25% (w/v) trichloroacetic acid to precipitate plasma proteins and to bring the pH to about 2 and then placed in a water bath at 90°C for 30 min to release protein-bound malondialdehyde (MDA). After centrifugation, supernatants were frozen at –75°C until TBARS could be determined as described previously.[1] Some of the criticisms arising from the use of the TBARS assay to assess lipid peroxidation

**Table 2** Plasma thiobarbituric acid reactive substances (TBARS), blood glutathione redox status and red blood cell antioxidant enzyme activities before and after physical exercise at 60% $VO_{2max}$

| | Before exercise | After exercise | *P* |
|---|---|---|---|
| Plasma TBARS (μmol/l) | 0.86 ± 0.37 | 1.44 ± 0.54 | 0.012 |
| Blood TGSH[a] (μmol/l) | 936 ± 151 | 915 ± 102 | NS[b] |
| Blood GSH[c] (μmol/l) | 775 ± 118 | 672 ± 154 | 0.032 |
| Blood GSSG[d] (μmol/l) | 80 ± 48 | 122 ± 58 | 0.002 |
| Blood GSSG/TGSH (× 1000) | 84 ± 42 | 135 ± 66 | 0.002 |
| RBC[e] Se-GPX[f] (μmol/min·g Hb[g]) | 25 ± 3.0 | 28 ± 2.1 | 0.003 |
| RBC GRD[h] (μmol/min·g Hb) | 5.0 ± 1.2 | 5.0 ± 1.1 | NS |
| RBC GST[i] (μmol/min·g Hb) | 9.2 ± 2.1 | 9.7 ± 1.3 | NS |
| RBC SOD[j] (Units/mg Hb) | 51 ± 3.8 | 50 ± 4.5 | NS |
| RBC catalase (Units/mg Hb) | 67 ± 6.4 | 66 ± 9.9 | NS |

[a]TGSH, total blood glutathione; [b]NS, nonsignificant; [c]GSH, reduced glutathione; [d]GSSG, oxidised glutathione; [e]RBC, red blood cell; [f]Se-GPX, glutathione peroxidase; [g]Hb, haemoglobin; [h]GRD, glutathione reductase; [i]GST, glutathione-S-transferase; and [j]SOD, superoxide dismutase. Data are means (± SD).

stem from the many modifications of the assay and the differing assay conditions employed. When proper precautions are taken, however, the TBARS assay remains a valid and sensitive if relatively non-specific index of lipid peroxidation.[15]

Determination of erythrocyte Se-GPX, GRD and GST activity was carried out using washed red blood cell (RBC) haemolysates as described before,[7] with the following modifications. For Se-GPX measurement, the haemolysate was treated with a 1.2-fold excess of hexacyanoferrate (III) and a 12-fold excess of cyanide over haem concentration to avoid interference of methaemoglobin.[16] Se-GPX activity was determined using 50 μmol/l hydrogen peroxide.

Determination of erythrocyte Cu,Zn-SOD activity was based on the reduction of nitroblue tetrazolium in a xanthine/xanthine oxidase-dependent superoxide generating system.[17] The activity of SOD that could cause a 50% inhibition of superoxide produced as assessed by the reduction of nitroblue tetrazolium was defined as 1 unit (U).

Red cell catalase activity was determined by monitoring the decrease in absorbance at 240 nm in the presence of 10 mM hydrogen peroxide.[18] Blood haemoglobin was measured photometrically using the cyanmethaemoglobin method.[19] Serum cholesterol and triglycerides were measured enzymatically using a Hitachi 717 analyser (Tokyo, Japan). The same method was also used for high-density lipoproteins (HDL) after removal of LDL and very low-density lipoproteins (VLDL) by dextran sulphate/$MgCl_2$.[20]

### *Data analysis*

The SPSS/PC+ software (SPSS, Chicago, IL, USA) was used for statistical analyses. Results for the groups are expressed as means ± (SD). Data from 14 subjects were available for blood GSSG and TGSH analyses; in other analyses, data from 13 subjects were available. Differences before and after exercise were tested with repeated measures ANOVA. Pearson's correlation analysis was used to assess the associations between selected variables. When data were sequential, univariate regression analysis was used to assess the associations between selected variables. Data for pre-exercise plasma TBARS and the relative change in GSSG and GSSG/TGSH with exercise were log transformed for correlation and regression analyses to correct for skewedness. For multivariate analyses, stepwise multiple regression was done using various four variable combinations of the following: plasma TBARS; blood TGSH, GSH and GSSG; RBC Se-GPX, GST, GRD, catalase and SOD; and $VO_{2\ max}$. Statistical significance was defined as $P < 0.05$.

## RESULTS

### *Maximal oxygen consumption*

The mean $VO_{2max}$ was normal (Table 1).

### *Lipid peroxidation at rest and after exercise*

Plasma TBARS levels immediately after 5 min warm up and 40 min of physical exercise at 60% of $VO_{2\ max}$ are depicted in Table 2. Mean plasma TBARS increased by approximately 50% ($P = 0.012$).

### *Blood glutathione redox status at rest and after exercise*

Mean blood TGSH levels did not change with exercise (Table 2). Mean GSSG, however, increased by about

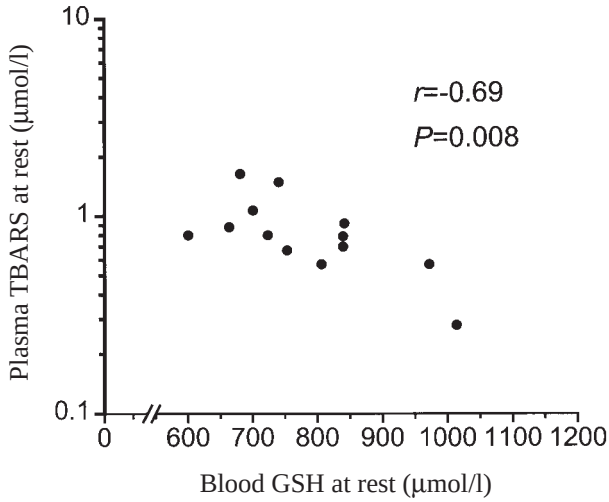


**Fig. 1** Plasma thiobarbituric acid reactive substances (TBARS) at rest *vs.* blood reduced glutathione (GSH) levels at rest in the young men.

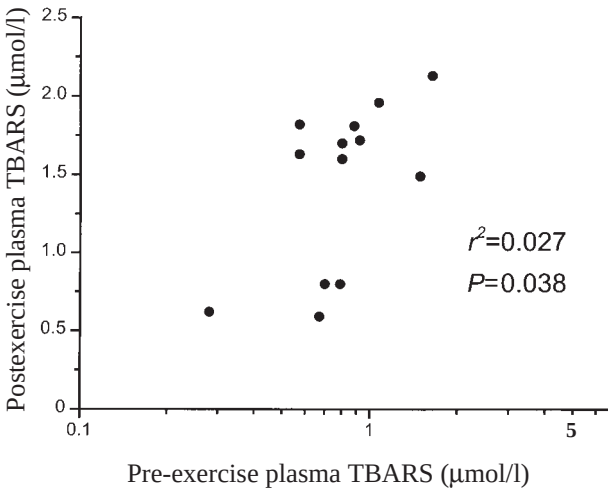


**Fig. 2** Plasma thiobarbituric acid reactive substances (TBARS) after 40 min of exercise at 60% of maximal oxygen consumption as a function of resting plasma TBARS levels.

50% ($P$ = 0.002). The GSSG/TGSH ratio showed a similar response to exercise ($P$ = 0.002). Calculated reduced GSH (TGSH – 2 × GSSG), conversely, decreased by 13% with exercise ($P$ = 0.032).

*Glutathione dependent enzymes at rest and after exercise*

Postexercise RBC Se-GPX activity rose modestly following exercise (Table 2, $P$ = 0.003). Exercise did not significantly affect RBC GRD or GST activity.

*Erythrocyte Cu,Zn-SOD and catalase activity at rest and after exercise*

There were no significant exercise-induced changes in either Cu,Zn-SOD or catalase activity (Table 2).

*Correlation and univariate regression analysis*

At rest, blood GSSG levels did not correlate with blood GSH levels ($r$ = 0.01, $P$ = 0.98). After exercise, blood GSSG correlated inversely with blood GSH ($r$ = –0.75, $P$ = 0.002). Blood GSH levels at rest correlated inversely with plasma TBARS levels at rest ($r$ = –0.69, $P$ = 0.008, Fig. 1).

Univariate regression analysis showed pre-exercise plasma TBARS levels to be weakly predictive of postexercise plasma TBARS levels (adjusted $r^2$ = 0.27, β = 0.58, $P$ = 0.038, Fig. 2). Blood GSSG (adjusted $r^2$ = 0.51, β = 0.74, $P$ = 0.002, Fig. 3) and GSSG/TGSH (adjusted $r^2$ = 0.43, β = 0.69, $P$ = 0.006) values at rest were predictive of their respective post-exercise values. Because blood GSSG and GSSG/TGSH values correlated highly with each other both before and after exercise ($r$ = 0.93–0.97, $P$ < 0.001), and results were virtually identical when substituting GSSG/TGSH for GSSG in the correlation and regression analyses, further results are presented using only GSSG. Plasma TBARS at rest was determinant of the relative change in TGSH (adjusted $r^2$ = 0.73, β = 0.85, $P$ < 0.001, Fig. 4) and GSH (adjusted $r^2$ = 0.45, β = 0.67, $P$ = 0.01) with exercise.

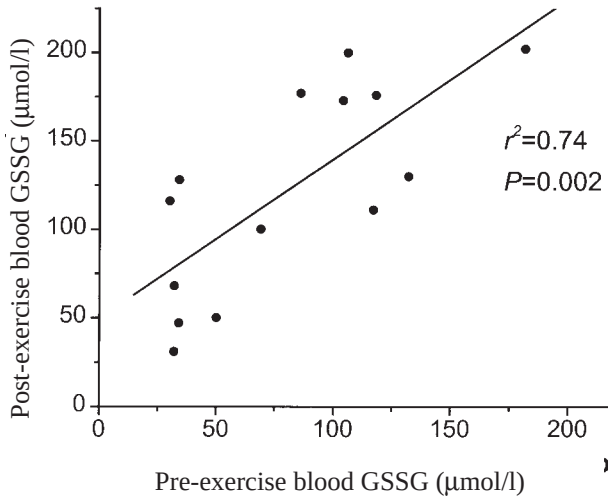


**Fig. 3** Blood glutathione disulphide (GSSG) after 40 min of exercise at 60% of maximal oxygen consumption as a function of pre-exercise GSSG in the young men.

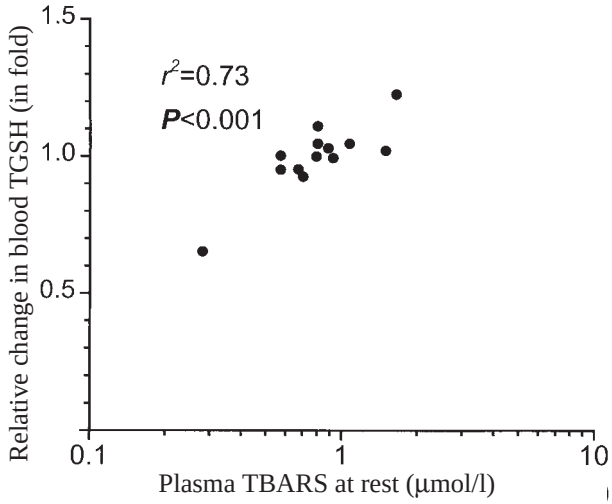


**Fig. 4** Relative change in blood total glutathione (TGSH) after 40 min of exercise at 60% of maximal oxygen consumption *vs.* plasma TBARS at rest in the young men.

When the outlying point closest to the origin (Fig. 4) was excluded from statistical analyses, plasma TBARS levels at rest remained predictive of TGSH (adjusted $r^2$ = 0.41, β = 0.68, $P$ = 0.014).

The exercise-induced relative change in TGSH ($r$ = 0.71, $P$ = 0.006; Fig. 5) and postexercise blood TGSH levels ($r$ = 0.58, $P$ = 0.039) correlated positively with postexercise plasma TBARS levels. Exclusion of the outlier closest to the origin (Fig. 5) only slightly decreased the correlation ($r$ = 0.65, $P$ = 0.022). The exercise-induced relative change in blood GSH ($r$ = 0.76, $P$ = 0.003), but not postexercise GSH ($r$ = 0.49, $P$ = 0.091), also correlated with postexercise plasma TBARS levels.

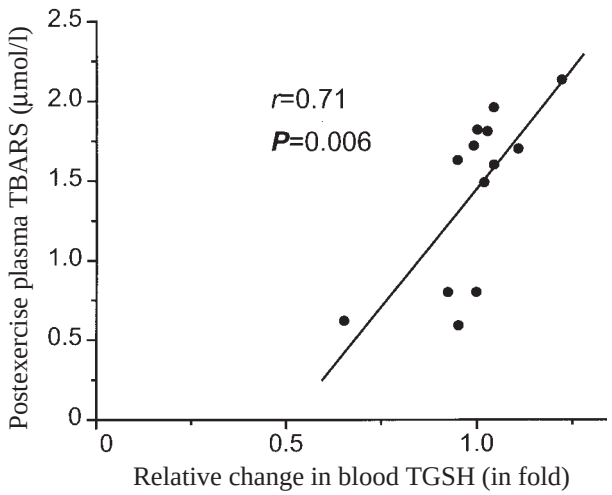


**Fig. 5** Relative change in blood total glutathione (TGSH) after 40 min of exercise at 60% of maximal oxygen consumption *vs.* plasma thiobarbituric acid reactive substances (TBARS) after exercise.

*Multivariate regression analysis*

Blood GSH levels at rest were the only determinant of plasma TBARS at rest (adjusted $r^2$ = 0.44, β = –0.70, $P$ = 0.008) using stepwise multivariate analysis with different combinations of indices of physical fitness, glutathione redox status and antioxidant enzyme activities as explanatory variables.

The relative change in GSSG with exercise was the only variable determinant of the relative change in plasma TBARS with exercise (adjusted $r^2$ = 0.39, β = –0.66, $P$ = 0.014) when included with other indices of glutathione redox status and antioxidant enzyme activities at rest or with their changes with exercise as explanatory variables.

Of the postexercise explanatory variables, only postexercise blood TGSH was determinant of postexercise plasma TBARS levels (adjusted $r^2$ = 0.27, β = 0.58, $P$ = 0.039). The relative changes in TGSH and GSSG with exercise were also determinant of postexercise plasma TBARS (adjusted $r^2$ = 0.69, $P$ = 0.001; change in TGSH, β = -0.70, $P$ = 0.001; change in GSSG, β = –0.49, $P$ = 0.012) when included with changes with exercise of other indices of glutathione redox status and antioxidant enzyme activities as explanatory variables. Only changes in blood GSH with exercise were determinant of postexercise plasma TBARS (adjusted $r^2$ = 0.54, β = –0.76, $P$ = 0.003) when the change in GSH with exercise was substituted for the change in TGSH with exercise. GSSG levels and antioxidant enzyme activities at rest were not predictive of postexercise plasma TBARS levels.

## Discussion

Sustained moderate intensity physical exercise induced oxidative stress, as measured by increased plasma TBARS levels and increased oxidised blood glutathione levels[14]. Higher blood GSH levels at rest were associated with less plasma lipid peroxidation, and changes in blood glutathione with exercise were associated with resting and exercise-induced lipid peroxidation, observations never before reported to our knowledge. This study has some important limitations, however. TBARS was measured only in plasma, and glutathione was measured only in whole blood, representing essentially red cell glutathione since virtually all blood glutathione is within the red cells. Furthermore, lipid peroxidation was estimated only by the TBARS assay, widely used but often criticised for its nonspecificity. Still, such links between blood glutathione homeostasis and plasma lipid peroxidation are biologically plausible, unlikely to be due entirely to chance, and potentially important.

The protective effect of blood GSH levels at rest against resting plasma TBARS suggested by both simple correlation and multivariate regression analysis may be indirect,

since blood GSH and GSSG is contained primarily in erythrocytes. Of the reactive oxygen species most likely generated by exercise,[21] superoxide and hydrogen peroxide are relatively long lived, and can readily cross cell membranes.[22,23] RBCs have been proposed to act as 'sinks' for superoxide and hydrogen peroxide in the plasma,[24] with depletion of GSH then occurring intracellularly. Extracellular oxidative stress, as measured by plasma TBARS, may deplete erythrocyte GSH through thiol disulphide transduction. Red cell membranes have been shown to contain transmembrane protein thiols that can transduce disulphide formation and GSH depletion from the extracellular space to the intracellular space in response to extracellular oxidative stress.[25] Lipid peroxidation could also spread from the outer RBC membrane into the RBC interior, resulting in depletion of GSH. Blood GSH may also reflect glutathione stores in other tissues.[26]

Moderate intensity exercise increased plasma TBARS by roughly 50%, consistent with exercise-induced oxidative stress and in agreement with many other studies.[1,4,27] Plasma TBARS at rest was weakly predictive of postexercise plasma TBARS, suggesting that lower levels of lipid peroxidation at rest decreased susceptibility to exercise-induced lipid peroxidation.

Sustained exercise also increased blood GSSG and GSSG/TGSH values by about 50%. In response to exercise, changes in blood GSSG and GSSG/TGSH values are among the most consistent markers of oxidative stress in humans.[27] Blood GSSG and GSSG/TGSH values at rest were strongly predictive of postexercise values. Those with a favourable glutathione redox status at rest were thus able to maintain a more favourable glutathione redox status despite exercise-induced oxidative stress. This may be important because glutathione and thiol redox status have been implicated in the regulation of genes involved in the pathogenesis of diseases such as atherosclerosis, diabetic complications, cancer and AIDS.[27] Furthermore, glutathione homeostasis can be positively affected by regular training[7] and certain antioxidant supplements, such as α-lipoate.[28]

Mean blood GSH decreased with exercise. Because mean blood TGSH did not change significantly and postexercise GSH was strongly inversely correlated with postexercise GSSG, most of the GSSG formation probably originated from blood GSH oxidation. The response of TGSH and GSH to exercise, however, ranged from decreases of 35% and 51% to increases of 23% and 13%, suggesting that, in at least some cases, GSSG and GSH originate from elsewhere. We[1] and others[5] have previously shown marked exhaustive exercise-induced TGSH depletion in rat skeletal muscle, and smaller decreases in the liver. Blood TGSH and GSSG increased in response to 30 min[2] and 134 min[29] of cycling exercise of slightly higher intensity (anaerobic threshold and 70% $VO_{2max}$, respectively) than the current study in humans, and in response to exhaustive treadmill running[1] in rats. On the other hand, in response to 30 min of exercise at aerobic threshold or during a $VO_{2max}$ test lasting on average 14 min, blood TGSH did not change.[1]

It has been hypothesised that during exercise-induced oxidative stress of sufficient intensity and duration, GSSG from exercising skeletal muscle and GSH from the liver (which also serves as a reservoir of glutathione for other tissues[30]) are exported into the general circulation.[1,2,5,29] These mechanisms increase blood TGSH levels. GSH released into the circulation has been shown to transduce its reducing power across the red cell membrane via transmembrane thiol proteins into the cell interior.[31] In some subjects in the current study, both blood TGSH and GSH decreased following exercise. Although blood protein thiols were not determined, decreases in both blood TGSH and GSH could represent mixed disulphide formation in response to milder exercise-induced oxidative stress, which outweigh possible gains in TGSH from skeletal muscle and liver.

In contrast, GSH decreased by well over 50% already after 15 min exercise in 8 moderately trained men during 90 min exercise at 65% $VO_{2max}$, with little change in blood TGSH and large increases in GSSG.[32] This discrepancy is difficult to explain. Also difficult to explain are the many fold higher levels of blood GSSG both at rest and with exercise in that study.[32] Duthie and colleagues[33] found marked decreases in both erythrocyte TGSH and GSH in seven trained athletes after a half marathon. RBC TGSH and GSH may have decreased in that study because exercise was of relatively long duration (76–84 min), and apparently exhaustive, in clear contrast to the present study and the human studies cited above.[1,29,32]

A novel finding was that plasma TBARS at rest were determinant of changes in blood TGSH and GSH. In turn, both changes in TGSH and GSH and postexercise blood TGSH were determinants of postexercise plasma TBARS. Therefore, in subjects with lower levels of oxidative stress at rest as measured by plasma TBARS, blood TGSH and GSH decreased in response to exercise, and decreases in blood TGSH and GSH also were associated with lower levels of postexercise plasma TBARS. Although in this study no changes in mean TGSH were noted, our results are consistent with the hypothesis that, in response to sufficient exercise-induced oxidative stress, blood TGSH increases via GSH and GSSG export from skeletal muscle and liver, particularly in those with higher levels of oxidative stress as measured by plasma TBARS. Conversely, in subjects with lower levels of resting and exercise-induced lipid peroxidation, blood TGSH and GSH decreased in response to exercise.

Red cell Se-GPX activity increased significantly with exercise. Se-GPX activity increases with increasing concentrations of peroxides,[6,29,34] and has been interpreted as a marker of oxidative stress also in response to exercise.[6,29,34]

Although in animal studies skeletal muscle Se-GPX activity has generally increased in response to sustained exercise,[6] exercise-induced changes in erythrocyte Se-GPX activity in human studies have been less consistent, either increasing,[35] or remaining unchanged.[33]

At rest, higher blood reduced glutathione levels were associated with lower levels of lipid peroxidation, as measured by plasma TBARS. Changes in reduced, oxidised and total blood glutathione with exercise were closely coupled with both resting and exercise-induced plasma lipid peroxidation. These results underscore the critical role of glutathione homeostasis in modulating exercise-induced oxidative stress and, conversely, the effect of oxidative stress at rest on exercise-induced changes in glutathione redox status.

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