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ORIGINAL ARTICLE

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Glutathione-dependent modulation of exhausting exercise-induced changes in neutrophil function of rats

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Abstract Reduced glutathione (GSH) plays a central role in maintaining an effective synergism between various physiological and exogenous antioxidants. We tested the effects of GSH and *N*-acetylcysteine (NAC, a pro-GSH clinical drug), intraperitoneal (i.p.) supplementation and GSH deficiency on exercise-induced leucocyte margination and neutrophil oxidative burst activity. GSH, NAC ($1 g \cdot kg^{-1}$) or placebo saline was i.p. injected (one or eight times) to male rats ($n \geqslant$ seven per group). The GSH-deficient rats were prepared by i.p. injections of L-buthionine-[SR]-sulphoximine (BSO, $6 mmol \cdot 1^{-1} \cdot kg^{-1}$) twice daily for 4 days. Exercised animals were subjected to treadmill run to exhaustion. Exhausting treadmill exercise significantly decreased peripheral blood leucocyte count in the controls ($P < 0.001$). Such exercise-associated leucocyte margination was prevented by GSH supplementation. Peripheral blood neutrophil counts were significantly higher ($P < 0.02$) in the GSH-supplemented groups compared to the placebo control groups. Exercise-induced increase in peripheral blood neutrophil oxidative burst activity as measured by luminol-enhanced chemiluminescence per volume of blood tended to be higher in the GSH-supplemented group ($P < 0.10$), and lower in the GSH-deficient rats ($P < 0.02$). In these experiments, for the first time we have shown that GSH supplementation can induce neutrophil mobilization and decrease exercise-induced leucocyte margination, and that exogenous and endogenous GSH can regulate exercise-induced stimulation of the neutrophil oxidative burst.

Key words Antioxidant · Free radical · Thiols
Immune system · *N*-Acetylcysteine

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Introduction

It has been shown that strenuous physical exercise, especially of the eccentric type, initiates a stereotyped series of host defence reactions involving reactive oxygen species (ROS) similar to those induced by bacterial infection (Smith et al. 1990; Cannon and Blumberg 1994). This response, collectively referred to as the acute phase immune response, is characterized by a substantial increase in circulating leucocytes, body temperature and serum factor concentrations that activate phagocyte function in vitro (Cannon and Blumberg 1994).

The acute phase immune response triggered by strenuous exercise has also been found to include the activation and mobilization of neutrophils as in ischaemia and reperfusion (McCord and Roy 1982; Komatsu et al. 1992). Neutrophils accumulate at the site of injury, clear cellular fragments through phagocytosis and release proteolytic enzymes and ROS into the phagosome and into the extracellular space. In a process called the respiratory burst, activated neutrophils and other phagocytes have been shown to produce an excess of superoxide anions, which are subsequently converted to strong physiological oxidants, e.g. hydrogen peroxide and hypochlorite (Weiss 1989). This neutrophil oxidative burst has been shown to serve as a physiological defence mechanism for pathogen killing and wound healing (Besner et al. 1992). Under certain conditions, however, it has been reported that it may contribute to oxidative damage of host tissues (Weiss 1989; Ward and Michigan 1991).

Although a short bout of physical exercise has been found to cause leucocyte demargination leading to elevated leucocyte counts in the peripheral blood (Ferry et al. 1990; Hack et al. 1992), long-lasting endurance exercise has been shown to cause peripheral blood leucopoenia due to leucocyte margination into sites of tissue damage (Galun et al. 1987; Michna 1989). In vitro and in vivo studies have suggested that ROS are implicated in such leucocyte margination (Shingu and Nobunaga 1984; Leff et al. 1993).

Reduced glutathione (GSH L-γ-glutamyl-L-cysteinylglycine) is implicated in the circumvention of cellular oxidative stress and maintenance of a favourable redox milieu of other lipid- and aqueous-phase antioxidants of the antioxidant chain reaction (Sen 1995). Apart from its direct antioxidant properties, GSH has been shown to be capable of markedly influencing neutrophil functions. A combined deficiency of GSH and glutathione peroxidase has been shown to increase chemo-attractant activity of rat liver cytosol during ethanol metabolism towards neutrophils by about 500-fold (Nueschwander and Roll 1990). *N*-acetylcysteine (NAC) is an effective free radical scavenger and a precursor and upregulator of GSH synthesis, with effects on neutrophil kinetics that are similar to GSH (Leff et al. 1993). We have previously shown that NAC can markedly attenuate exercise-induced blood glutathione oxidation in humans (Sen et al. 1994b).

Control of exercise-induced oxidative stress and tissue damage may be important in enhancing the therapeutic benefits of regular exercise and in increasing physical performance of athletes (Sen and Hänninen 1994). Despite a growing knowledge of the role of antioxidants in regulating muscle damage and leucocyte function, information concerning the effect of antioxidant supplementation and deficiency on leucocyte function and muscle damage during strenuous exercise is scanty. We have previously reported the effects of i.p. GSH and NAC supplementation and GSH deficiency on tissue glutathione homeostasis and lipid peroxidation (Sen et al. 1994a). We now report the effects of i.p. GSH and NAC supplementation and GSH deficiency on exercise-induced leucocyte margination and neutrophil oxidative burst activity in rats with the objective of evaluating the role of thiol antioxidants in regulating these processes. An animal model was used because GSH depletion cannot be carried out safely in humans.

Methods

Animals, GSH, L-buthionine-[S,R]-sulphoximine administration and exercise protocol

Male Han Wistar rats aged 8 weeks (body mass 170–180 g; $n = 52$) were used in these experiments. GSH and NAC were supplemented ($n = 7$ per group) with one single i.p. injection ($1\ g \cdot kg^{-1}$ in 0.8–1.0 ml solution; pH was adjusted to 6.5–6.8 before use). A group of rats ($n = 7$) receiving one placebo saline injection was considered as the corresponding control. The GSH-deficient rats ($n = 8$–9 per group) received i.p. injections of L-buthionine-[S,R]-sulphoximine (BSO, $6\ mmol \cdot kg^{-1}$ body mass, in 0.8–1.0 ml solution, twice daily for 4 days; Sigma, St. Louis, Mo., USA; see Sen et al. 1994a). The corresponding control group ($n = 7$) received eight i.p. saline injections. BSO, a selective inhibitor of GSH synthesis, has been shown not to be directly toxic at high ($33\ mmol \cdot l^{-1} \cdot kg^{-1}$) doses and not to react with GSH (Meister 1991).

Exercising animals were subjected to treadmill running 0.5 h after the last injection, and killed immediately after exhausting treadmill exercise. Exercise was performed on a ten-lane rodent treadmill (10% uphill gradient). The running speed was $1.2\ km \cdot h^{-1}$ for the first 10 min. After that period, speed was maintained at $1.8\ km \cdot h^{-1}$ until the rats were exhausted. The loss of the righting reflex when the rats were turned on their backs was the criterion of exhaustion. All animals were acquainted with treadmill running (10% uphill gradient, 1.0–$1.2\ km \cdot h^{-1}$, $0.5\ h \cdot day^{-1}$) for 4 days. Test and control groups of rats were matched for body mass as this may influence endurance capacity. The study protocol was approved by the Ethics Committee of the University of Kuopio. Guide for the care and use of laboratory animals (NIH 1985) was followed for animal care and experiment procedure.

Sample collection

Before decapitation of the animals peripheral blood was drawn from the femoral vein using capillary pipettes (Capilette, Boehringer Mannheim, Germany). In exercised animals blood samples were collected immediately (< 5 min) after the bout of exhausting exercise. Collection of samples from test and control animals was matched for time of day to control for possible ultradian variations.

Chemiluminescence assay

For chemiluminescence (CL) measurements, collected blood was treated with $6\ mmol \cdot l^{-1}$ Na_2-ethylenediaminetetra-acetic acid as anticoagulant. Oxidative burst activity in opsonized zymosan-stimulated peripheral blood was measured as luminol (5-amino-2,3-dihydro-1,-phthalazinedione, Sigma) enhanced CL emission. Whole blood was used for CL measurements instead of isolated leucocytes as the amount of blood taken from rats was limited and it has been found that the isolation procedure may affect activation processes and receptor expression (Fearon and Collins 1983; Glasser and Fiederlein 1990). The reaction mixture consisted of whole blood diluted (1:500) in Hank's balanced salt solution containing $1\ mmol \cdot l^{-1}$ luminol. The reaction was stimulated with 250 µg zymosan (prepared from the membrane of Saccharomyces cerevisiae) opsonized in rat serum. The CL determinations were done at 38°C within 1 h of blood collection using a Luminoskan luminometer (Labsystems, Helsinki, Finland).

The samples were incubated for 7–8 min in the measuring chamber of the luminometer to raise the sample temperature before starting the reaction by adding the zymosan. The CL emissions were measured at 1.5–min intervals for 50 min to obtain the response kinetics. The observed peak CL value (expressed as relative light units, rlu) was taken as the CL value. The CL reaction kinetics is expressed as the time (minutes) from the start of the reaction by addition of zymosan to the time at which the peak CL value was observed. The coefficient of variance was 7.4% when three parallel measurements from one blood sample were made as four replicates.

Leucocyte counts

Peripheral blood leucocytes were counted using methyl-violet-stained fresh blood samples in a Bürker chamber under a microscope. Differential counts were made from peripheral blood smears after conventional May-Grünwald and Giemsa staining.

Biochemical analyses

Details of GSH, oxidized glutathione (GSSG) and thiobarbituric acid reactive substances (TBARS) tissue measurements and results have been previously published (Sen et al. 1994a). Briefly, for GSH measurement, collected peripheral blood was deproteinized with two volumes of 0.5 N perchloric acid. For GSSG measurement, blood was treated with 10% 5-sulphosalicylic acid. The resultant acidic supernatant was neutralized and reacted with 2-vinylpyridine. Blood plasma was separated and processed for the determination of lipid peroxidation byproducts as measured by TBARS.

Statistics

The BMPD software was used for data analysis (BMPD Statistical Software Inc., version 1990 for VAX/VMS). The results were analysed by two-way analysis of variance using variables exercise and drug or number of i.p. injections and exercise. Student's unpaired *t*-test with the Bonferroni correction was used for further comparisons of the rest and exercise subgroups within the drug, control or i.p. injection groups, respectively, and for comparison of the NAC exercise group and its corresponding control group.

Results

Leucocyte and neutrophil counts

Exhausting exercise markedly decreased the peripheral blood total leucocyte count in both control groups (saline injection one vs eight times groups, $P < 0.001$, Fig. 1). Such exercise-induced leucocyte margination was not seen in the GSH-supplemented animals. Furthermore, postexercise leucocyte counts in peripheral blood were substantially higher in the NAC-supplemented rats compared to the corresponding postexercise control group ($P < 0.01$). GSH deficiency resulted in a significantly smaller decrease in postexercise blood total leucocyte counts compared to controls ($P < 0.01$).

Exhausting exercise did not significantly influence peripheral blood neutrophil count in the single i.p. control group or in the GSH-supplemented group (Fig. 2). However, a substantial increase in exercise-induced neutrophil margination was observed in both eight i.p. groups ($P < 0.005$). Also when two control groups were compared, neutrophil counts tended to be higher at rest in the eight i.p. injection control groups ($P < 0.10$). Neutrophil counts were significantly higher in the GSH group ($P < 0.02$), and tended to be higher in the NAC-supplemented group ($P < 0.10$) compared to the control groups. GSH deficiency had no effect on neutrophil counts.

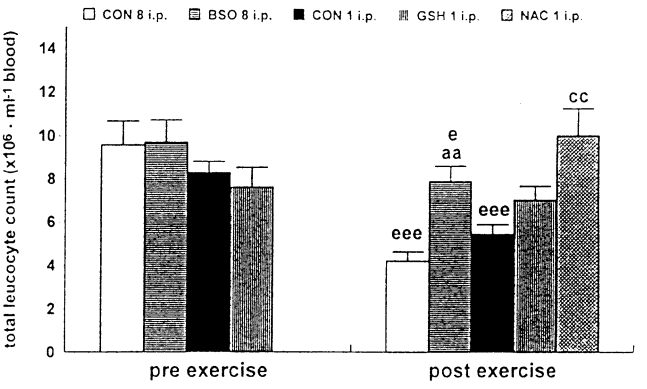

Fig. 1 Effects of glutathione (GSH) and *N*-acetylcysteine supplementation and GSH deficiency on exhausting exercise-induced changes in total leucocyte count in peripheral blood. *Con 1 i.p.*, control group that received 1 saline intraperitoneal injection;*Con 8 i.p.*, control group that received 8 saline i.p. injections; *BSO 8 i.p.*, rats with GSH deficiency induced by 8 i.p. injections of buthionine sulphoxamine (6 $mmol \cdot l^{-1} \cdot kg^{-1}$ twice daily for 4 days); *GSH 1 i.p.*, group supplemented with GSH given as one single i.p. injection (1 $g \cdot kg^{-1}$); *NAC 1 i.p.*, group supplemented with *N*-acetylcysteine given as one single i.p. injection (1 $g \cdot kg^{-1}$). Values are means and SEM. Letters represent significant changes due to *a* GSH deficiency, *c* NAC supplementation, *e* exercise, *e* $P < 0.05$; *aa*, *cc* $P < 0.01$; *eee* $P < 0.001$

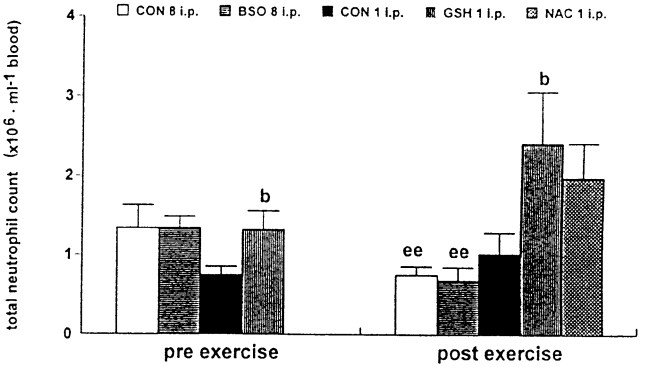

Fig. 2 Effects of glutathione and *N*-acetylcysteine supplementation and glutathione deficiency on exhausting exercise-induced changes in neutrophil count in peripheral blood. Values are means and SEM. Letters represent significant changes due to *b* GSH supplementation, *e* exercise. *b* $P < 0.05$; *ee* $P < 0.01$. For definitions see Fig. 1

Oxidative burst activity

Neutrophil oxidative burst activity measured as CL response per volume of blood and per number of phagocytes was decreased significantly in eight i.p. controls by exhausting exercise ($P < 0.02$, $P < 0.03$ respectively, Fig. 3). Exhausting exercise tended to increase CL activity per volume of blood in the GSH supplemented rats ($P < 0.10$). This effect was not seen when results were expressed as CL activity per phagocyte. In the GSH-deficient rats, exercise significantly decreased blood CL activity expressed as per volume of blood as

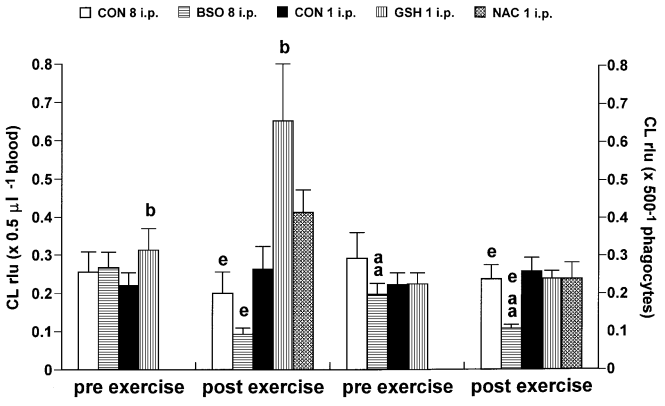

Fig 3 Effects of glutathione and *N*-acetylcysteine supplementation and glutathione deficiency on exhausting exercise-induced changes in chemiluminescence (*CL*) activity in peripheral blood following opsonised zymosan stimulation. Data are expressed as per blood volume and per phagocyte. Values are means and SEM. Letters represent significant changes due to *a* GSH deficiency, *b* GSH supplementation, *e* exercise. *b*, *e* $P < 0.05$; *aa* $P < 0.01$. For definitions see Fig. 1

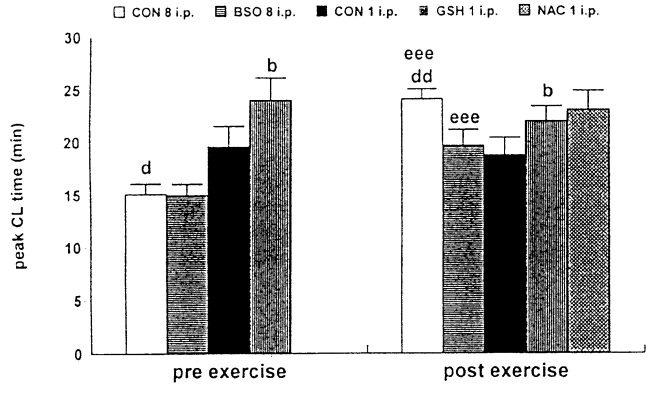

Fig. 4 Effects of glutathione and *N*-acetylcysteine supplementation and glutathione deficiency on exhausting exercise-induced changes in neutrophil oxidative burst peak time. Values are means and SEM. Letters represent significant changes due to *b* GSH supplementation, *d* multiple injections, *e* exercise, *b*, *d* $P < 0.05$; *dd* $P < 0.01$; *eee* $P < 0.001$. For definitions see Fig. 1

well as per phagocyte ($P < 0.02$ and $P < 0.03$, respectively). Blood CL activity was observed to be significantly higher in the GSH groups ($P < 0.02$), and tended to be higher in the postexercise NAC-supplemented group ($P < 0.10$). The CL activity per phagocyte was lower in GSH-deficient animals ($P < 0.002$).

In animals that had received multiple injections, exhausting exercise substantially increased the CL peak time, indicating slower CL reaction kinetics ($P < 0.0001$, Fig. 4). We did not observe any significant changes in rest and postexercise CL kinetics in the rats that received a single i.p. injection irrespective of the substance administered. Exercise resulted in a marked increase in CL peak times in the eight i.p. control group, in contrast to little change in the single i.p. control group ($P = 0.003$). In contrast, multiple injections decreased the CL peak time at rest compared to the single injection control group ($P = 0.05$). GSH deficiency had virtually no effect on CL reaction kinetics. GSH supplementation, however, slowed down CL reaction kinetics ($P < 0.05$).

Discussion

GSH and NAC supplementations prevented exhausting exercise induced leucocyte margination to peripheral tissues. GSH deficiency depressed neutrophil oxidative burst activity. Exhausting exercise markedly decreased total leucocyte counts in the control groups of animals. It has been reported that long-lasting, exhausting exercise may alter the number of leucocytes in the circulation in different ways due to margination (Galun et al. 1987; Hack et al. 1992) into the sites of tissue damage such as skeletal muscle, tendon and lung (Michna 1989; Peters et al. 1992).

Prevention of exercise-induced leucocyte margination and increased neutrophil counts in the GSH-supplemented animals indicated a tight regulatory effect of thiol antioxidants on exercise induced changes in leucocyte function. ROS-dependent chemotaxis has been reported to be strongly implicated in leucocyte-mediated inflammatory events (Petrone et al. 1980; McCord and Roy 1982; Shingu and Nobunaga, 1984). We have previously reported that in all of these animals exhausting exercise significantly decreased total GSH concentrations in liver, skeletal muscles and heart; it increased the GSH redox ratio (oxidized/total GSH) in skeletal muscles, lung, blood and plasma; and it increased muscle and plasma lipid peroxide concentrations, all consistent with increased oxidative stress after exercise (Sen et al. 1994a). Such increased ROS concentrations may be implicated in eliciting chemotactic signals for leucocyte accumulation to the sites of free radical injury (Neuschwander and Roll 1990). Tissue antioxidant defence status and GSH concentrations are known to be inversely related to neutrophil CL activity and mobilization to tissues in response to a challenge. Smith et al. (1989) have shown that after 1 h of reperfusion injury, tissue neutrophil content increased significantly. This was associated with a 26-fold increase of myeloperoxidase activity and a 50% decrease in reduced GSH content in the neutrophils.

Increased neutrophil counts and inhibition of exercise-induced leucocyte margination to peripheral tissues in the GSH- and NAC-supplemented animals may not be completely explained by the antioxidant properties of the thiols. This is especially so because we have previously observed that i.p. administered GSH was poorly available to tissues, e.g. skeletal muscle and heart, and only resulted in a temporary increase of plasma GSH concentrations (Sen et al. 1994a). Furthermore, exercise-induced responses of total and oxidized

GSH, and tissue lipid peroxidation have not been found to be significantly different among the control, NAC and GSH supplementation groups (Sen et al. 1994a). However, a recent proton-nuclear magnetic resonance spin-echo study of erythrocytes showed that although added extracellular GSH may not directly available to the intracellular compartment of erythrocytes, GSH can transduce its reducing power to the intracellular compartment through a thiol-disulphide exchange mechanism that sequentially involves sulphur-rich proteins spanning the erythrocyte membrane (Ciriolo et al. 1993).

Apart from their antioxidant properties, both GSH and to a less extent its oxidized form, GSSG, have been found to influence peripheral blood neutrophil kinetics (Elferink and de-Koster 1991). The effect is mainly due to a chemokinetic effect (random locomotion) and partly due to a chemotactic effect of thiols. Sulphydryl groups on the extracellular surface of the membrane have been shown to play a decisive role in the effects of GSH on neutrophil locomotion (Bridges 1985; Elferink and de-Koster 1991). Such effects of GSH, however, have been observed only at much higher concentrations of GSH (Elferink and de-Koster 1991) than seen in our previous study (Sen et al. 1994a).

According to our preliminary observations with NAC supplementation post exercise leucocyte counts were significantly higher in NAC-supplemented animals than in corresponding post-exercise controls. In our previous studies, NAC have decreased exercise-induced GSH oxidation in the lung and blood in rats (Sen et al. 1994a).

The CL activities of total blood were significantly higher in the GSH-supplemented group and tended to be higher in the NAC-supplemented group than in the control animals. Neutrophil oxidative burst activity per cell, however, remained unchanged after GSH and NAC supplementation. The different effects of GSH and possibly NAC on neutrophil mobilisation and neutrophil oxidative burst activity can be explained by the different mechanisms involved in these processes as shown by Scott et al. (1990) and Elferink and de-Koster (1991).

In contrast to a single injection, repeated i.p. injections tended to increase the blood neutrophil count at rest and caused a substantial exercise induced neutrophil margination. At rest the eight i.p. injection control group had faster reaction kinetics than the single i.p. injected control group. Postexercise CL activity and CL kinetics were, however, depressed in the eight i.p. group compared to the single i,p. injected group. In addition, postexercise neutrophil margination was similar in the multiple injection control group and the GSH-deficient rats. A possible explanation of this effect of multiple injection could be that peritoneal injury due to multiple injection primes neutrophils such that they more readily adhere to the endothelial lining and leave the peripheral circulation in response to exercise.

GSH deficiency markedly suppressed neutrophil CL activity, although GSH supplementation did not have any effect. Such suppression in the CL response has been shown to occur during immune dysfunction and certain diseases (Allen et al. 1981; Lilius and Marnila 1992). Thiols may modulate certain aspects of the cytoskeleton of leucocytes, and thus their phagocytic property and degranulation (Bridges 1985). We observed an influence of GSH on neutrophil phagocytic activity only in its deficiency, suggesting different relative roles of GSH supplementation and deficiency on neutrophil oxidative burst activity. Consistent with our findings. Scott et al. (1990) have shown that GSH deficiency caused by the electrophile chlorodinitrobenzene impaired the phagocytic ability of neutrophils. The BSO treatment protocol employed in our study resulted in an approximately 50–90% decrease in the tissue and blood total GSH pools (Sen et al. 1994a). Plasma and tissue TBARS in the resting and exercise GSH-depleted groups was markedly elevated compared to the control and supplemented animals, indicating increased oxidative stress as found by Sen et al. (1994a). Increased ROS production has been found to decrease neutrophil mobility and phagocytic activity by auto-oxidative membrane damage (Baehner et al. 1977). A significant decrease of CL activity and slower postexercise CL kinetics was observed in GSH-deficient animals despite a markedly decreased duration of exercise (50% that of the control group, Sen et al. 1994a). Our findings and those of others (Scott et al. 1990) have demonstrated a clear role of physiological levels of GSH in the regulation of neutrophil oxidative burst activity in more oxidative stress-susceptible GSH-deficient animals.

In brief, our study showed for the first time that GSH circumvented exhausting exercise-induced leucocyte margination and enhanced CL activity of the blood. BSO-induced GSH deficiency served as an excellent model to study the role of physiological levels of endogenous GSH. GSH deficiency decreased postexercise neutrophil oxidative burst activity. Thus, as a novel aspect of thiol antioxidant function, thiols appear to regulate exercise-induced leucocyte margination and neutrophil activation.

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