Oxidative stress after human exercise: effect of *N*-acetylcysteine supplementation

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Sen, Chandan K., Tuomo Rankinen, Sari Väisänen, and Rainer Rauramaa. Oxidative stress after human exercise: effect of *N*-acetylcysteine supplementation. *J. Appl. Physiol.* 76(6): 2570–2577, 1994.—The association between exercise intensity and related oxidative stress was investigated in nine men who exercised for 30 min at their aerobic (AeT) and anaerobic (AnaeT) thresholds. We also tested the effect of oral *N*-acetylcysteine (NAC) on exercise-associated rapid blood glutathione (GSH) oxidation in subjects performing two identical maximal bicycle ergometer exercise (Max) tests. Before the second test {Max with NAC supplementation [Max(NAC)]}, the men took 200 × 4 mg/day of NAC tablets for 2 days and an additional 800 mg on the test morning. Blood samples were drawn before, immediately after, and 24 h after the tests. Total and oxidized GSH levels in blood were determined. Plasma thiobarbituric acid-reactive substances and net peroxyl radical scavenging capacity (PSC) were assayed. Exercise-associated damage in leukocyte DNA was estimated by fluorometric analysis of DNA unwinding. A single bout of exercise at Max, AeT, and AnaeT resulted in a significant increase in blood GSH oxidation but did not influence net PSC of plasma. Although an association between a single bout of exercise and leukocyte DNA damage was apparent, this study suggests that the parameter may not serve as a sensitive index to assess the role of exercise intensity in the extent of exercise-associated oxidative stress. Plasma thiobarbituric acid-reactive substances did not change after either Max or Max(NAC) tests. NAC supplementation resulted in an increase in preexercise PSC, indicating a higher net antioxidant capacity of the plasma, but did not affect blood GSH. Max-associated rapid decline in blood thiol redox status was markedly attenuated by NAC supplementation, indicating that the supplementation may have spared exercise-associated blood GSH oxidation and the thiol redox status perturbation.

antioxidant; glutathione; thiol; lipid peroxidation; aerobic; anaerobic; deoxyribonucleic acid; leukocyte; free radical; redox; blood; plasma

THE HYPOTHESIS that increased O_2 influx, which is known to occur during moderate and exhaustive exercise, may be potentially toxic to the body is authenticated by a large number of studies, some of which have been recently reviewed (1). The association between exercise intensity and related O_2 toxicity in humans is a fundamental problem that is yet to be pertinently addressed. Data of exercise-induced oxidative injury in humans are not only scanty but, in certain instances, especially those related to the exercise-associated response of blood glutathione (GSH) status, findings are inconsistent (14, 18). In a recent study (19), young men exercised at 60% of maximal O_2 consumption ($\dot{V}O_{2\,max}$) for 30 min, had a brief rest period for sample collection, further exercised for 5 min as the exercise intensity was progressively increased to 90% of $\dot{V}O_{2\,max}$, and then exercised at 90% of their $\dot{V}O_{2\,max}$ during the subsequent >2.5 min. Compared with levels detected after exercising at 60% of $\dot{V}O_{2\,max}$, expired pentane and serum malonaldehyde levels were higher after the 90% of $\dot{V}O_{2\,max}$ exercise challenge. Although the study design was not aimed at a direct comparison of exercise intensity-dependent effect on lipid peroxidation, the results present evidence that exercise-associated oxidative stress is related to the intensity of exercise.

GSH (L-γ-glutamyl-L-cysteinylglycine) is a ubiquitous endogenous thiol with well-established antioxidant properties (27). The antioxidative function of the thiol is implicated through two general mechanisms of reaction with reactive O_2 species (ROS): *1*) direct or spontaneous and *2*) GSH peroxidase (EC 1.11.1.9) catalyzed. As a major byproduct of such antioxidative reactions, glutathione disulfide (GSSG) is produced. Intracellular GSSG thus formed is cytotoxic and may be either reduced back to GSH by GSSG reductase (EC 1.6.4.2) or released to the extracellular compartment. GSH peroxidase-dependent reactions can decompose hydrogen peroxide and lipid peroxides. Also, GSH participates in the enzymatic decomposition of phospholipid hydroperoxide. Apart from the GSH-dependent antioxidant protection described above, GSH is also suggested to play a crucial role in replenishing antioxidants that get oxidized during their course of action. The water-soluble antioxidants ascorbic acid and GSH are suggested to be involved in regenerating α-tocopherol from their radical byproduct (13, 26, 37) by an antioxidant chain reaction. Mammalian erythrocytes are rich in GSH (~2 mM) and account for most of the blood GSH. At rest, ~20% of the total GSH (TGSH; GSH + GSSG) in human blood is present in the oxidized form (14, 18). The ratio of the concentrations of GSSG and TGSH is often used to represent the GSH redox ratio of tissues (24). A sharp increase in blood GSSG was observed after human exercise (14). Consistent data related to exercise-associated increases in either blood or plasma GSSG were also obtained from a number of animal experiments (22–24). An exercise-associated increase in blood GSSG reflects GSH oxidation in the erythrocytes and other tissues. Lew et al. (23) and Sen et al. (29) observed a consistent fall in the level of skeletal muscle TGSH after exercise. Sen et al. (30) also observed a sharp decline in intracellular TGSH content of myogenic cells subjected to oxidant challenge. The exercise-associated decline in the GSH redox ratio may therefore diminish GSH-dependent antioxidant protection and impair the antioxidant chain reaction as well. Therefore, efforts to attenuate such perturbations in the redox ratio should be of crucial importance.

During light or moderate exercise, energy is provided by aerobic metabolism. However, when the work inten-

sity is such that the rate of energy demand exceeds the capacity of aerobic metabolism, the anaerobic component of metabolism causes blood lactate and bicarbonate concentrations to sharply increase and decrease, respectively. The threshold at which such changes are observed is commonly referred to as the anaerobic threshold (AnaeT). In the present study, nine healthy men performed a graded exercise to maximum capacity (Max), allowing determination of their aerobic threshold (AeT) and AnaeT, two parameters largely dependent on individual physiological response to exercise (32). When expressed as a percentage of $\dot{V}O_{2\,max}$, AeT and AnaeT may vary between individuals (see Table 1). However, compared with levels after AeT exercise, the induced increase in blood lactate concentration is markedly higher after AnaeT exercise. Previously it was discussed that lactate may act as a buffer to radical formation in vivo (14). We were therefore interested in selecting those exercise intensities at which a uniform relative change in blood lactate concentration would occur in all subjects. Changes in oxidative stress indexes were compared after subjects exercised at AeT, AnaeT, and Max. In an attempt to control the exercise-associated rapid blood GSH oxidation, we examined the effect of oral *N*-acetylcysteine (NAC), a pro-GSH drug, supplementation (28).

METHODS AND PROCEDURE

Subjects and study design. Nine men aged 23–37 yr, who were nonsmokers, volunteered for the study. None of the subjects had chronic disease or was receiving permanent medication. All subjects showed normal left ventricular function in resting echocardiography. None had any abnormal findings in 24-h holter electrocardiogram (ECG) or a depressed ST segment in exercise ECG.

The study consisted of four separate exercise tests. First, the subjects completed a maximal ergospirometry test, allowing determination of their $\dot{V}O_{2\,max}$ as well as AeT and AnaeT. During the 2nd and 3rd wk, the subjects performed two submaximal tests (AeT and AnaeT), each lasting 30 min. The order of the two tests was randomly assigned. The AeT test was preceded by a 3-min warm-up period, and the AnaeT test was preceded by a 4-min warm-up period. Heart rate and respiratory gas exchange were measured continuously. During the last week, the maximal test was identically repeated after NAC supplementation. The exercise tests were separated by 7 days to ensure complete recovery and were performed at the same time of day to control possible diurnal differences. The subjects were also asked to refrain from alcoholic beverages and strenuous physical exercise for ≥3 days preceding each exercise test.

Exercise tests. $\dot{V}O_{2\,max}$, AeT, and AnaeT were assessed in maximal ergospirometry test using an electrically braked bicycle ergometer. Work load was increased by 20 W/min, and respiratory gas exchange was analyzed breath by breath. Subjective (Borg scale rating of perceived exertion of 19–20) or objective [increase in O_2 consumption ($\dot{V}O_2$) of <150 ml/min despite increasing work load] maximum was used as stopping criteria for the test. ECG was continuously monitored and recorded every minute during the exercise. Maximal aerobic capacity was determined as a plateauing off despite the increased work load, and AeT and AnaeT were estimated based on the characteristic changes in minute ventilation, end-tidal P_{O_2}, and respiratory quotient (32).

NAC supplementation protocol. Before the fourth exercise test [Max(NAC)], men took four NAC tablets (200 mg of NAC; Mucoporetta, Leiras, Turku, Finland) per day (2 tablets twice a day) for 2 days. On the day of exercise, two tablets were taken 2 h before the exercise test and an additional two tablets were taken 30 min before the commencement of the test.

Standard breakfast. The subjects ate a standard breakfast 3–4 h before each preexercise blood sampling to ensure sufficient energy supply for the exercise test, to avoid excess lipemia of the blood samples, and to standardize the effect of meal on respiratory gas exchange during the exercise test. The breakfast consisted of four slices of bread, four teaspoons of margarine, one glass (2 dl) of fat-free milk, and one apple. The energy content of the breakfast was ~500 kcal, with 55% of energy derived from carbohydrates, 30% from fat, and 15% from protein. Subjects were asked not to consume any caffeine-containing beverages during the 12 h before each exercise test.

Preparation of blood samples. Blood samples were drawn from an antecubital vein at 5 min before exercise, 2 min after exercise, and 24 h after exercise. Immediately after sample collection, EDTA-blood mixture was added to 2 vol of ice-cold 0.5 N perchloric acid and 0.5 vol of ice-cold 10% 5-sulfosalicyclic acid contained in separate Eppendorf tubes. The resultant mixtures were immediately vortexed. Eppendorf tubes containing the mixture were then briefly centrifuged at 10,000 *g* at 4°C. Immediately after the centrifugation, the clear supernatant obtained from 5-sulfosalicyclic acid-treated blood was neutralized, reacted with 2-vinylpyridine to derivatize GSH as recommended by Griffith (15), and frozen at −20°C for the determination of GSSG. The protein-free supernatant obtained from the perchloric acid-treated blood was stored at −20°C for the determination of TGSH. For the fluorometric analysis of DNA unwinding (FADU) in leukocytes, 5 ml of EDTA-blood mixture (pre- and 2-min postexercise samples) were added to 15 ml of ice-cold solution of ammonium chloride containing 0.87% NH_4Cl and 10 mM $NaHCO_3$ in 10 mM tris(hydroxymethyl)aminomethane · HCl buffer, pH 7.2. The mixture was gently mixed and allowed to stand at 4°C (to avoid DNA repair) until it turned dark, indicating lysis of erythrocytes. The lysate was centrifuged (600 *g*) in 50-ml polypropylene tubes at 2°C for 15 min. The leukocyte pellet was washed with ice-cold balanced salt solution containing 137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, and 5 mM D-glucose in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4. The residual erythrocytes in the suspension were removed by hypotonic lysis (2 vol of water at 0°C for 60 s). The suspension was centrifuged at 2°C for 15 min at 600 *g* to obtain the pellet of leukocytes. The pellet was resuspended in 3 ml of ice-cold balanced salt solution for FADU.

Plasma was separated from freshly collected heparinized whole blood and frozen at −80°C for the assay of peroxyl radical scavenging capacity (PSC). For the determination of thiobarbituric acid (TBA)-reactive substances (TBARS) in the plasma, the sample was processed as follows: EDTA-blood mixture was added to 1 vol of Chelex (Bio-Rad Laboratories)-treated 100 mM potassium phosphate buffer containing 2 mM Na_2EDTA, pH 7.4. Before use, the phosphate buffer was deaerated with He. The mixture was gently mixed and briefly centrifuged at 2°C to obtain plasma. Immediately after the isolation of blood plasma, ethanolic butylated hydroxytoluene (BHT) was added (final concn of 0.04% wt/vol) to the plasma. Four volumes of the treated plasma were added to 1 vol of 25% trichloroacetic acid contained in an acid-washed glass tube with cap. The mixture was immediately vortexed to precipitate the plasma proteins. The capped tube was then placed on a boiling water bath for 30 min to release protein-bound malonaldehyde. After the 30 min, the tubes were centrifuged at 1,000 *g* for 10 min to obtain a clear supernatant that was frozen at −80°C for the determination of TBARS.

Determination of TGSH and GSSG. TGSH in the acidified blood extract was determined by a GSSG reductase recycling

method as described previously (29). GSSG, from the 2-vinylpyridine-treated blood extract, was determined according to Griffith (15). GSSG reductase (type 3), GSSG (grade 3), reduced GSH (free acid), β-NADPH (tetrasodium salt, type 3), and 5,5′-dithio-bis(2-nitrobenzoic acid) were purchased from Sigma Chemical (St. Louis, MO). 2-Vinylpyridine was obtained from Aldrich-Chemie (Steinhein, Germany). All other chemicals were of the highest analytic grade. A Shimadzu UV-240 double-beam spectrophotometer or a Perkin-Elmer (Norwalk, CT) Lambda 2 UV/VIS spectrophotometer was used.

FADU. The fresh suspension of leukocytes obtained as described above was used for FADU (7). The cells were rapidly and uniformly distributed in 200-μl aliquots to a series of 12 disposable fluorometric cuvettes (1 ml) at ice temperature. Four of the 12 cuvettes were designated as T (total), 4 as P (partial), and the remaining 4 as B (blanks). Sets of four were used as quadruplicates to improve precision. Cells in the P cuvettes were subjected to an alkaline environment (pH 12.6), and DNA unwinding was allowed for a period of 60 min at 15°C. Cells in the T cuvettes were brought to pH 11 without exposure to pH 12.6. Cells in the B cuvettes were subjected to an alkaline environment (pH 12.6) and subsequently ultrasonicated for 5 s to introduce large numbers of DNA strand breaks so that there would be complete unwinding of the DNA. All cells were treated with alkaline ethidium bromide, and fluorescence from each cuvette was read directly in a spectrofluorometer (excitation wavelength of 520 nm, emission wavelength of 590 nm). Not more than two samples were handled per day. Pre- and postexercise sets of readings of the quadruplicates were averaged to give a single T, P, and B value for each cell sample. Percent double-stranded DNA (DNA_{pre} or DNA_{post} for pre- or postexercise samples, respectively) was given by $100(P - B)/(T - B)$. The quantity of DNA strand breaks induced by a single bout of exercise was estimated as $100[\log (DNA_{pre}) - \log (DNA_{post})]$ (7).

Assay of plasma PSC. PSC of the rapidly reacting antioxidants in the plasma was determined by a phycoerythrin fluorescence-based assay (10). Samples were deproteinized and diluted as recommended. The working solutions prepared were *1*) 75 mM sodium phosphate buffer (pH 7.0) in distilled deionized water [metal ions were removed from the buffer by passing it through a Chelex membrane (*buffer A*)]; *2*) a 1.7×10^{-3} mM solution of B-phycoerythrin (Sigma Chemical) in ice-cold *buffer A*, considering the molecular weight to be 25×10^4; *3*) a 0.5 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Aldrich Chemical, Milwaukee, WI) solution (reference peroxyl radical scavenger, 1 mol of which is known to trap 2 mol of peroxyl radical) in 50% methanol-50% water (vol/vol); and *4*) a 40 mM solution of the free radical initiator 2,2′-azobis[2-amidinopropane]hydrochloride (AAPH) (Polysciences, Warrington, PA) in ice-cold *buffer A*. The final reaction mixture contained 1.7×10^{-5} mM B-phycoerythrin, 4 mM AAPH, and any other additive (Trolox while standardizing the assay, deproteinized plasma supernatant while measuring samples) in 2 ml of *buffer A* in a 10-mm fluorometer cuvette. The reaction was initiated by adding 0.2 ml of 40 mM AAPH to 1.8 ml of the other components at 37°C. After the addition of AAPH, the fluorometric measurement (excitation wavelength of 540 nm, emission wavelength of 575 nm) was started after a pause of 2 min to compensate for the drop in temperature of the reaction mixture due to the addition of AAPH. A personal computer-assisted Shimadzu RF-5001PC (Shimadzu Europa, Duisburg, Germany) spectrofluorophotometer and software were used for the acquisition and analysis of data.

Detection of TBARS in the plasma. Determination of TBARS in the plasma was carried out spectrophotometrically according to recommendations published previously (6, 12). The EDTA-blood mixture was treated and frozen as described above. On the day of measurement, samples were thawed on ice and 1 vol of the sample was added to 1 vol of TBA solution. TBA solution was prepared by dissolving 1% (wt/vol) TBA in distilled deionized water initially passed through the Chelex membrane to remove traces of free metal contamination. TBA solution was deaerated by passing He through it. The pH of the mixture of the sample and TBA solution was adjusted to 1.5 with HCl. The pH-adjusted mixture was placed in boiling water for 30 min. The reaction mixture was then cooled to room temperature, and absorbance was measured at 532 nm. For the standard curve, a 10 mM stock of 1,1,3,3-tetramethoxypropane (Sigma Chemical) was prepared by adding 1 mmol of the compound to 100 ml of 1% (vol/vol) sulfuric acid. The mixture was left at room temperature for 2 h to allow complete hydrolysis of the reference compound. Standard solutions of different strengths (0.5–10.0 μM) of tetramethoxypropane were prepared to obtain the standard curve.

Statistical analysis. Changes during each exercise bout, as well as the differences in the changes between the tests, were analyzed using nonparametric Friedman two-way analysis of variance. Results of the second maximal test [Max(NAC)] were compared with those of the first exercise test. Results are given as means ± SE. Statistical analyses were done using SPSS/PC+ software (SPSS, Chicago, IL).

RESULTS

Max. During Max, preexercise $\dot{V}O_2$ (mean 0.34 l/min) increased by 760% within a mean time interval of 14.06 min (Table 1). Such a bout of exercise did not cause any change in blood TGSH level (Fig. 1). Although the blood level of reduced GSH (TGSH − 2GSSG) remained unchanged after the bout of exercise (Fig. 2), there was a significant (100%) Max-associated increase in the blood level of GSSG and therefore an increase in the GSSG/TGSH ratio. After 24 h of recovery, blood GSSG level and GSSG/TGSH ratio were not any higher compared with preexercise values (Figs. 3 and 4). Exercise-associated change in the level of lipid peroxide in the plasma, as measured by the TBARS, was not significant (Fig. 5). The net PSC of the plasma was also not affected by the exercise challenge (Fig. 6). Max-associated injury in leukocyte DNA was detected in seven of nine subjects (Table 2).

Exercising at AeT for 30 min. The rate of $\dot{V}O_2$ when exercising at AeT corresponded to 50% of $\dot{V}O_{2\,max}$ (Table 1). Compared with Max, this bout of submaximal exercise was twice as long in time and the total volume of $\dot{V}O_2$ during the exercise test was just double compared with that consumed during Max. Similar to that observed after Max, AeT was not followed by any significant change in the blood level of TGSH (Fig. 1) or GSH (Fig. 2). However, after the exercise test, a significant increase in the level of blood GSSG and GSSG/TGSH ratio was observed (Figs. 3 and 4). AeT also resulted in a significant increase in plasma TBARS (Fig. 5). AeT-associated increases in blood GSSG and plasma TBARS were no longer seen in the 24-h recovery samples (Figs. 3 and 5). Compared with the preexercise data, no change in PSC was observed (Fig. 6). AeT-associated injury in leukocyte DNA was detected in five of nine subjects (Table 2).

Exercising at AnaeT for 30 min. The rate of $\dot{V}O_2$ when exercising at AnaeT corresponded to 77% of $\dot{V}O_{2\,max}$ (Table 1). Although the duration of the exercise test was

TABLE 1. *Description of human subjects and their O_2 consumption during different exercise test protocols*

Subj No.	Age, yr	Weight, kg	$\dot{V}O_{2\,max}$, l/min	Duration of $\dot{V}O_{2\,max}$, min	$\dot{V}O_{2\,AeT}$, l/min	$\dot{V}O_{2\,AnaeT}$, l/min
1	34	93	3.36 (32.4)	16	1.84 (55.2)	2.62 (78.6)
2	25	78.3	2.48 (21.5)	12	1.43 (42.9)	1.97 (59.1)
3	26	62	2.08 (14.7)	11	1.27 (38.1)	1.67 (50.1)
4	37	88.8	3.44 (32.3)	15.5	1.46 (43.8)	2.55 (76.5)
5	29	65.4	2.41 (19.5)	12	1.08 (32.4)	1.99 (59.7)
6	37	70.3	2.65 (21.0)	13	1.21 (36.3)	2.06 (61.8)
7	30	78.9	2.64 (21.0)	13	1.24 (37.2)	2.09 (62.7)
8	29	75.4	3.09 (29.1)	15	1.43 (42.9)	2.34 (70.2)
9	23	73.1	4.24 (45.5)	19	2.07 (62.1)	3.03 (90.9)
Mean ± SE	30.00±1.69	76.13±3.37	2.93±0.22 (26.3±3.1)	14.06±0.84	1.45±0.11 (43.4±3.2)	2.26±0.14 (67.7±4.2)

$\dot{V}O_{2\,max}$, maximum O_2 uptake; $\dot{V}O_{2\,Aet}$, rate of O_2 uptake when exercising at aerobic threshold (AeT); $\dot{V}O_{2\,Anaet}$, rate of O_2 uptake when exercising at anaerobic threshold (AnaeT). Duration of AeT and AnaeT tests were 30 min each. Weight was measured before each of 4 wk of study; no significant difference was observed. Total O_2 consumed (in l) given in parentheses.

similar to that for AeT, the total volume of $\dot{V}O_2$ during the test was 157 and 56% higher than that consumed during Max and AeT, respectively. Unlike that observed after Max and AeT, AnaeT was followed by a significant increase in the blood level of TGSH (Fig. 1) but not of GSH (Fig. 2). After the exercise test, significant increases in the level of blood GSSG and GSSG/TGSH ratio were observed (Figs. 3 and 4). AnaeT also resulted in a significant increase in plasma TBARS (Fig. 5). AnaeT-associated increases in blood TGSH, GSSG, and plasma TBARS were no longer seen in the 24-h recovery samples (Figs. 1, 3, and 5), indicating a complete recovery. Compared with the preexercise data, no change in PSC was observed (Fig. 6). AnaeT-associated injury in leukocyte DNA was detected in seven of nine subjects (Table 2).

Max(NAC). This exercise protocol is identical in intensity and time with Max. Exercise-associated increases in blood TGSH after Max and Max(NAC) were apparent; however, the change was not significant (Fig. 1). No difference in the preexercise values of blood TGSH, GSH, GSSG, GSSG/TGSH ratio, or plasma TBARS was observed in the Max and Max(NAC) tests (Figs. 1–5). A remarkable observation was that exercise-associated blood GSH oxidation (i.e., increase in blood GSSG and GSSG/TGSH ratio) as observed after Max was attenuated by supplementation of NAC (Figs. 3 and 4). Also, compared with the data obtained from Max, NAC supplementation resulted in a significant increase in preexercise PSC of the plasma. During the other three experiments (Max, AeT, and AnaeT), we observed that a single bout of exercise did not affect plasma PSC. Compared

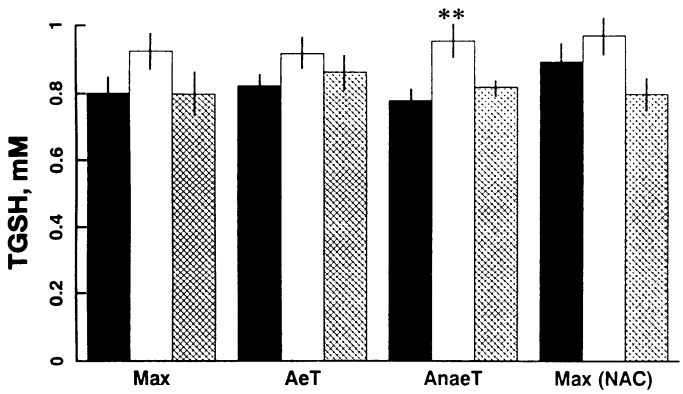

FIG. 1. Blood total glutathione (TGSH) levels 5 min before (solid bars), 2 min after (open bars), and 24 h after (crosshatched bars) continuous progressive cycle ergometer exercise. Values are means ± SE. Max, maximal O_2 uptake capacity determination test; AeT, 30 min of bicycling at aerobic threshold; AnaeT, 30 min of bicycling at anaerobic threshold; Max(NAC), Max after *N*-acetylcysteine (NAC) supplementation. AnaeT was followed by a significant increase in blood TGSH level (** $P < 0.01$).

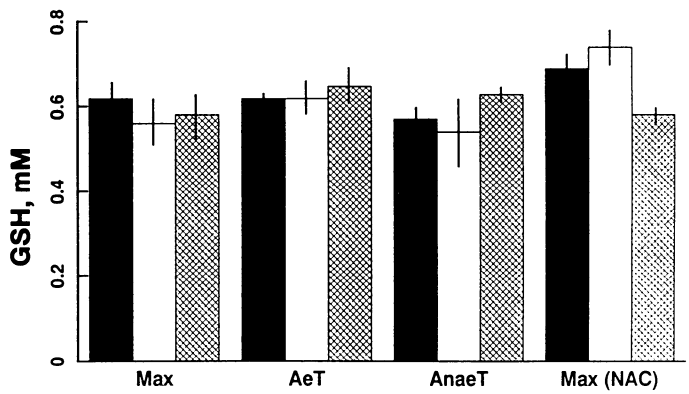

FIG. 2. Blood reduced glutathione (GSH) levels 5 min before (solid bars), 2 min after (open bars), and 24 h after (crosshatched bars) continuous progressive cycle ergometer exercise. Values are means ± SE. GSH was calculated as TGSH − 2GSSG, where GSSG is oxidized GSH. A single bout of exercise did not cause any significant change. After administration of NAC, preexercise blood GSH was slightly higher compared with preexercise GSH values of preceding tests. However, difference was not significant.

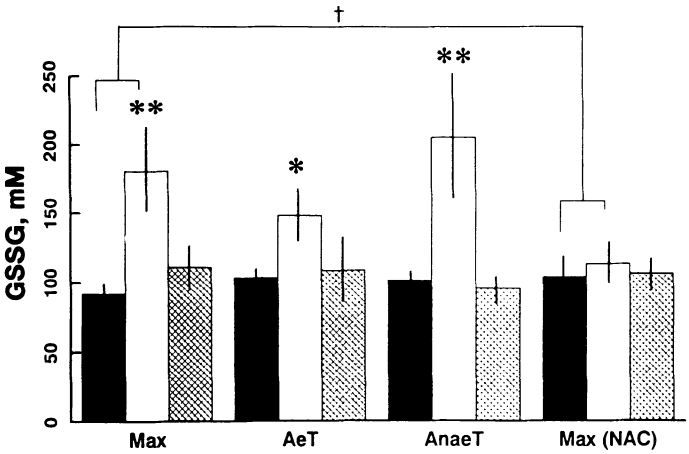

FIG. 3. Blood GSSG levels 5 min before (solid bars), 2 min after (open bars), and 24 h after (crosshatched bars) continuous progressive cycle ergometer exercise. Values are means ± SE. All exercise tests except Max(NAC) resulted in a significant increase in blood GSSG level (* $P < 0.05$ and ** $P < 0.01$). Exercise-associated increase in blood GSSG level was significantly higher after Max compared with that after Max(NAC) († $P < 0.05$).

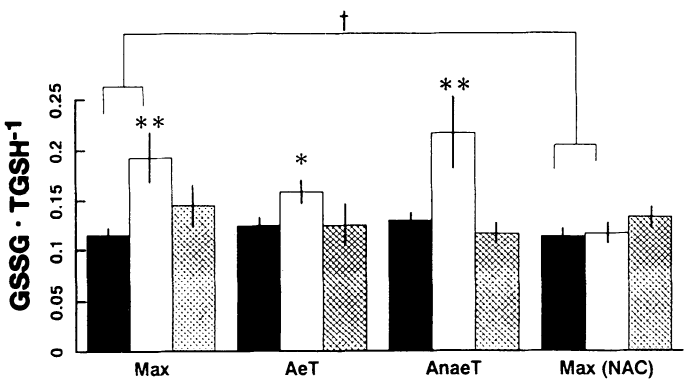

FIG. 4. Blood GSSG/TGSH index 5 min before (solid bars), 2 min after (open bars), and 24 h after (crosshatched bars) continuous progressive cycle ergometer exercise. Values are means ± SE. Increase in GSSG/TGSH ratio indicates a decline in GSH redox status. All exercise tests except Max(NAC) resulted in a significant decrease in blood GSH redox status (* $P < 0.05$ and ** $P < 0.01$). Exercise-associated decline in blood GSH redox status was significantly higher after Max than after Max(NAC) († $P < 0.05$).

with the preexercise data, PSC was significantly lower in the 24-h recovery samples (Fig. 6). Injury in leukocyte DNA was detected in six of nine subjects (Table 2).

DISCUSSION

Blood GSH status after Max, AeT, and AnaeT. The primary article on human blood GSH oxidation during exercise, by Gohil et al. (14), reported that submaximal exercise influences blood GSH status. A sharp increase (100%) in blood GSSG was observed within the first 15 min of exercising at 65% of $\dot{V}O_{2\,max}$ (14, 36). In a recent study by Ji et al. (18), eight healthy cyclists exercised at 70% of $\dot{V}O_{2\,max}$. In contrast to the finding of Gohil et al., the bout of exercise, which lasted for a mean duration of 134 min, did not affect blood GSSG level. Studies related to human blood GSH oxidation during exercise are limited; however, previous studies did reveal that exhaustive exercising of rats remarkably increases GSSG level in the plasma (23, 24). Compared with the subjects of the

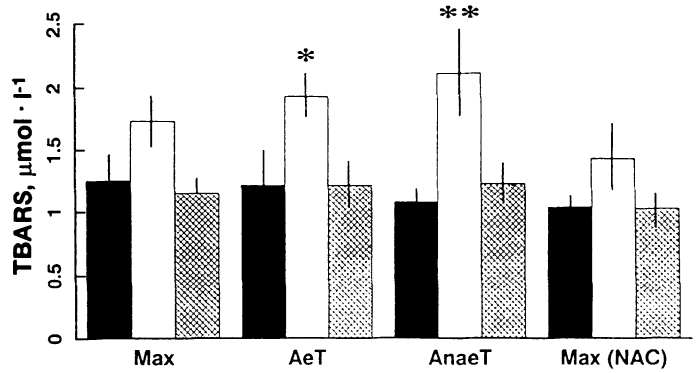

FIG. 5. Thiobarbituric acid-reactive substances (TBARS; byproducts of lipid peroxidation) in blood plasma 5 min before (solid bars), 2 min after (open bars), and 24 h after (crosshatched bars) continuous progressive cycle ergometer exercise. Values are means ± SE. AeT and AnaeT were followed by a significant increase in plasma TBARS level (* $P < 0.05$ and ** $P < 0.01$).

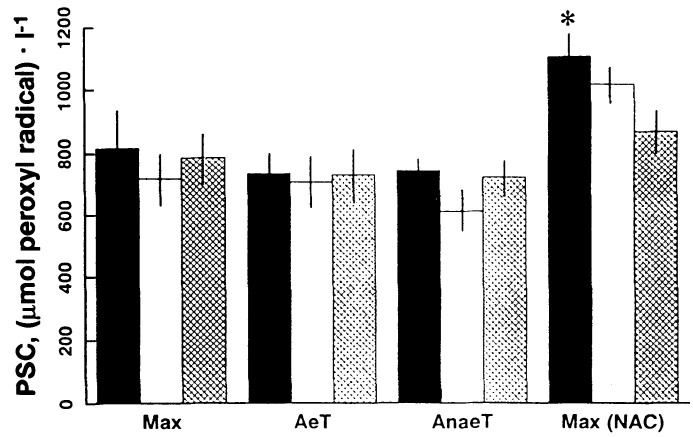

FIG. 6. Net peroxyl radical scavenging capacity (PSC) of blood plasma 5 min before (solid bars), 2 min after (open bars), and 24 h after (crosshatched bars) continuous progressive cycle ergometer exercise. Values are means ± SE. Compared with preexercise data from Max, AeT, and AnaeT, preexercise net PSC of blood plasma was significantly higher before Max(NAC), indicating that NAC supplementation resulted in improvement in fast-reacting antioxidant status of blood plasma (* $P < 0.05$).

study of Gohil et al., the subjects of the present study had a lower aerobic capacity (mean $\dot{V}O_{2\,max}$ of 39 vs. 48 $ml \cdot kg^{-1} \cdot min^{-1}$). We observed a significant (100%) increase in blood GSSG after Max (Fig. 3) that lasted for a mean duration of 14 min (Table 1). The increase in blood GSSG was significant after each of the three test (Max, AeT, and AnaeT) protocols. Although the increase in blood GSSG after AnaeT was apparently higher than that observed after AeT, the difference was not significant (Fig. 3). After 24 h of recovery, blood GSSG levels were restored to preexercise values, indicating that such an interval of time is sufficient for the restoration of exercise-associated perturbation of the blood thiol redox status (Fig. 3). Such a finding is in accordance with that reported by Gohil et al. Previous observations related to the exercise-associated change in blood GSH are also inconsistent. Ji et al. reported that, although there was a trend to increase, no significant change in blood GSH was observed until the 120th min of bicycling at 70% of $\dot{V}O_{2\,max}$; however, a significant increase in the parameter was seen after the completion of the exercise that lasted

TABLE 2. *Leukocyte DNA strand break after single bout of exercise at different intensities*

Subj No.	Max	AeT	AnaeT	Max(NAC)
1	ND	16.92	65.35	5.96
2	7.61	ND	0.48	ND
3	10	37.35	57.68	43.21
4	ND	0.56	ND	ND
5	9.53	ND	10.92	ND
6	37.34	48.98	62.29	44.13
7	28.5	12.16	ND	1
8	5.19	ND	12.01	24.91
9	38.59	ND	10.43	18

Nos. of strand break have been estimated as recommended by Birnboim (7). Max, $\dot{V}O_{2\,max}$ test; AeT, 30 min of bicycling at AeT; AnaeT, 30 min of bicycling at AnaeT; Max(NAC), $\dot{V}O_{2\,max}$ test after *N*-acetylcysteine supplementation; ND, not detected.

for a mean duration of 134 min. In contrast, Gohil et al. and Viguie et al. (36) observed a sharp fall in blood GSH within the first 15 min of bicycling at 65% of $\dot{V}O_{2\,max}$. No change in blood GSH was observed during Max (14). Consistent with the findings of Gohil et al., we observed no change in blood GSH after Max. Blood GSH level remained unchanged after AeT and AnaeT tests, which lasted for 30 min each. After subjects exercised at 70% of $\dot{V}O_{2\,max}$, Ji et al. observed a trend to increase but no significant change in blood TGSH up to the first 120 min. The difference was distinct after the completion of the exercise, which lasted for a mean duration of 134 min. Although there was a trend to increase, we did not observe any significant increase in TGSH after either Max or AeT, indicating that there was no net flow of either GSH or GSSG from other tissues into the blood. The remarkable increase in blood GSSG after Max and AeT was therefore dependent on oxidation of GSH in the blood itself. Because the plasma level of GSH is almost undetectably low in humans (<0.01 μM; Ref. 14) and quite low in rats (22–27 μM; Ref. 4), the oxidation of GSH may have taken place mostly in the erythrocytes. Human erythrocytes possess a specific energy-dependent system for the export of excess intracellular GSSG. Therefore, increase in plasma GSSG, as has been observed in previous studies with rats (23, 24), should be largely dependent on oxidation of GSH in erythrocytes and its subsequent export to the plasma. Similar to that observed by Ji et al. after subjects exercised at 70% of $\dot{V}O_{2\,max}$ for a mean duration of 134 min, we observed a significant increase in blood TGSH after subjects underwent strenuous exercise at AnaeT. Because AnaeT was not associated with an increase in blood GSH (Fig. 2), the increase in TGSH may be expected to be due to export of GSSG from tissues other than blood. Plasma GSH level is about three orders of magnitude lower than the blood level (14). Therefore, it is quite likely that changes in plasma GSH due to accelerated hepatic GSH efflux that may occur during exercise (23, 29) will not have a revealing effect on the blood level of GSH or TGSH. In early studies, Lew et al. (23) and Sen et al. (29) observed a consistent fall in the level of skeletal muscle TGSH after exercise. We have also previously reported that skeletal muscle-derived cells experience a rapid loss in intracellular TGSH after exposure to oxidant stress. It was explained that exposure of the muscle cells to oxidant challenge resulted in a rapid oxidation of intracellular GSH, which in turn was followed by a rapid efflux of GSSG. Oxidant stress-associated decrease in intracellular TGSH was partly subdued in the presence of KCN, suggesting that the GSSG efflux mechanism may be energy dependent (30). In another recent study performed using hepatectomized rats, a significant contribution of the skeletal musculature to the blood GSH status was evident (21). Therefore, it may be assumed that during prolonged strenuous exercise, when the total $\dot{V}O_2$ of the body is sufficiently large, substantial amounts of GSH in active peripheral tissues such as the skeletal muscle may be oxidized to GSSG and exported to the general circulation. However, such a hypothesis demands further evidence for authentication. Irrespective of the intensity or duration of exercise used in this study, exercise-associated perturbation of the blood thiol redox status, as reflected by the GSSG/TGSH ratio, was restored within 24 h of recovery.

Plasma lipid peroxides after Max, AeT, and AnaeT. Exercise-associated increases in secondary products of lipid peroxidation in tissues as well as in the plasma, urine, and expired air have often been used as a marker of oxidative stress (3, 11, 17–19, 25). Absolute values of the lipid peroxidation byproducts may vary considerably depending on the assay method employed. However, it is critically important to stabilize lipid peroxidation byproducts in biological samples by immediate treatment with excess EDTA and/or BHT during sample collection. In an early study carried out in our laboratory, it was observed that Max of untrained men did not result in any change of plasma TBARS (20). However, Lovlin et al. (25) observed a significant increase in plasma levels of lipid peroxidation byproduct after Max. We observed that, although Max was associated with a marginal increase in plasma TBARS, there was no significant change (Fig. 5). In a very recent study by Kanter et al. (19), young nonsmoking men ran at 60% of $\dot{V}O_{2\,max}$ for 30 min on a treadmill followed by a 5-min bout of running at 90% of $\dot{V}O_{2\,max}$. The bout of 30 min of exercise closely resembles the AeT test used in this study. Kanter et al. observed elevated levels of serum TBARS after the 30-min bout of treadmill running at 60% of $\dot{V}O_{2\,max}$. Our finding of elevated plasma TBARS in post-AeT samples are consistent with the above-described report. Comparing the increase in plasma TBARS after AeT with that after AnaeT, we observed that the exercise-associated increase in plasma TBARS was apparently more after the higher-intensity AnaeT. However, two-way ANOVA did not reveal any statistical significance of such an intensity-dependent response. In earlier studies, based on results obtained from the determination of TBARS from rat skeletal muscle and analysis of expired ethane, it was suggested that exercise intensity influences the extent of lipid peroxidation (3, 11). Endogenous malonaldehyde, a principal component of TBARS, undergoes rapid oxidative metabolism either in its primary form or after formation of an adduct with various macromolecules, e.g., lysine, guanine, and phospholipid bases (12). Therefore, our observation that an exercise-associated increase in plasma TBARS level was not detectable in the 24-h re-

covery sample was not unexpected. Lipid peroxides are not only markers of oxidative stress but are themselves strong prooxidants. Lipid peroxyl radicals possess sufficient energy to initiate further lipid peroxidation chain reactions. Our observation suggests that 24 h of recovery should be sufficient to neutralize the deleterious effect of exercise manifested by increased levels of plasma TBARS (Fig. 5).

PSC of plasma after Max, AeT, and AnaeT. Plasma PSC quantifies the total amount of rapidly reacting peroxyl scavengers in the plasma and may therefore be employed to assess the collective contribution of all rapidly reacting antioxidants in the plasma at a given time (10). PSC values from pre- and postexercise plasma samples were therefore expected to be useful in the assessment of exercise-associated oxidative stress. The fast-reacting antioxidants in the plasma include vitamin E, ascorbate, urate, bilirubin, and reduced thiols. The slow-reacting scavengers are mainly the plasma proteins. The phycoerythrin-based method for the determination of PSC has been recently introduced and has not yet been employed to assess exercise-associated oxidative stress. A large number of early studies have shown that a single bout of exercise remarkably elevates the prooxidant status of the body. Blood and plasma antioxidants, e.g., GSH and vitamin E, are significantly consumed and increased amounts of lipid peroxidation byproducts are produced; therefore we expected to observe a significant exercise-associated decrease in plasma PSC. In the present study, although we did observe a tendency of plasma PSC to decrease after a bout of exercise, no significant changes were seen (Fig. 6). In recent experiments, it was observed that a single bout of exercise is associated with a significant increase in plasma uric acid (31) and an increase in the level of plasma ascorbate (36). Both urate and ascorbate are components of the fast-reacting peroxyl scavengers in the plasma, and their increased level in the plasma may have masked the exercise-associated decrease in plasma PSC due to oxidation of plasma GSH and vitamin E and an increased level of lipid peroxides. Urate and ascorbate are known to be capable of scavenging 2.0 and 0.7 peroxyl radicals per molecule, respectively.

Leukocyte DNA damage after Max, AeT, and AnaeT. The FADU technique was initially developed to allow the study of DNA strand breaks in cells, such as leukocytes, in which DNA cannot be easily radiolabeled. Early studies have revealed that exercise-induced oxidative stress may cause DNA damage. Ten hours after marathon running by subjects, the ratio of oxidized nucleosides per creatinine increased 1.3-fold above the resting level (2). Myeloperoxidase-dependent superoxide production in neutrophils is a physiological process having microbicidal activity. Neutrophils represent 50–60% of the total circulating leukocytes, and Smith et al. (34) showed that a single bout of exercise may remarkably increase ROS production by the neutrophils. We were therefore interested to see how different intensities of exercise may affect leukocyte DNA. Results from the present study indicate the possibility that exercise-associated oxidative stress may initiate DNA damage in the leukocytes (Table 2). However, of the 36 measurements carried out with 9 subjects during the 4 exercise tests, DNA damage was not detected in 11 cases. Interestingly, with one exception (*subject 2*), leukocyte DNA strand break caused by AnaeT was noticeably lower in the most fit (higher aerobic capacity, Table 1) subjects (Table 2). The extent of DNA damage did not appear to be related to exercise intensity. These first results in the area of exercise-associated oxidative stress and related leukocyte DNA damage as detected by FADU indicate that the parameter may not serve as a sensitive index to assess the effect of exercise intensity on the extent of exercise-associated oxidative stress.

Effect of NAC supplementation. Oral NAC has been found to elevate GSH levels in the plasma and bronchoalveolar lavage fluid (8). Exogenous NAC may act to rescue GSH by *1*) promoting GSH synthesis in vivo by providing cysteine, a precursor of GSH that limits GSH synthesis (9, 27), and/or *2*) directly scavenging ROS (5). NAC is very effective in controlling perturbation of the thiol redox status after acetaminophen toxicity and has been successfully used for clinical purposes (33). The GSH replenishing and sparing aspects of NAC have been recently reviewed (28). Bioavailability of oral NAC has been shown to be the highest when fast-dissolving effervescent tablets, as used in this study, are taken. Studies on clinical pharmacokinetics of NAC reveal that after an oral dose of 200–400 mg the peak plasma concentration is achieved in ≤1–2 h, after which NAC from the plasma is rapidly cleared (16). The potent radical scavenging property of NAC (5) explains the difference in plasma PSC that was observed in this study after NAC supplementation (Fig. 6). The decrease in plasma PSC after 24 h of the last administration is consistent with data obtained from the pharmacokinetic studies on the clearance of exogenous NAC from the plasma (16). Oxidation of GSH in the erythrocytes is a major contributor to the exercise-associated increase in blood GSSG. Recently, Udupi and Rice-Evans (35) have shown that NAC is particularly effective in protecting erythrocytes exposed to oxidative stress. Because NAC supplementation did not have any revealing effect in elevating blood TGSH level, it may be expected that the protective action of NAC during exercise was not mediated by promotion of de novo synthesis of GSH but by the direct antioxidant properties of the drug. Therefore, our findings suggest that NAC supplementation may spare exercise-associated blood GSH oxidation.

In summary, this study presents the results from a controlled experiment where the effects of different intensities (classified with respect to individual physiological response) of exercise on changes in blood GSH status, plasma lipid peroxides, and leukocyte DNA strand injury have been investigated in humans. Compared with AeT, exercising at AnaeT tended to be associated with a higher extent of oxidative stress. Although AnaeT induced perturbation of blood GSH status and the increase in plasma TBARS was slightly higher compared with that after exercising at AeT, the differences were not statistically significant. A single bout of moderate or strenuous exercise did not influence the net PSC of the plasma. Exercise-associated changes in leukocyte DNA damage have not been studied earlier. Although a weak associa-

tion between a single bout of exercise and leukocyte DNA damage was apparent, this study suggests that the parameter may not serve as a sensitive index to assess the role of exercise intensity in the extent of exercise-associated oxidative stress. NAC supplementation increased the net PSC of the plasma, indicating that the antioxidant properties of the drug may augment the net antioxidative capacity of the plasma. It is possible that the effect of supplementation persisted for a short duration and tended to dissipate after a 24-h postsupplementation interval. Findings of this study also suggest the possibility that exercise-associated perturbation of blood GSH redox status may be circumvented by the supplementation of NAC.

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REFERENCES

1. **Alessio, H. M.** Exercise-induced oxidative stress. *Med. Sci. Sports Exercise* 25: 218–224, 1993.
2. **Alessio, H. M., and R. G. Cutler.** Evidence that DNA damage and repair cycle activity increases following a marathon race (Abstract). *Med. Sci. Sports Exercise* 22: 751, 1990.
3. **Alessio, H. M., and A. H. Goldfarb.** Lipid peroxidation and scavenger enzymes during exercise. Adaptive response to training. *J. Appl. Physiol.* 64: 1333–1336, 1988.
4. **Anderson, M. E., and A. Meister.** Dynamic state of glutathione in blood plasma. *J. Biol. Chem.* 255: 9530–9533, 1980.
5. **Aruoma, O. I., B. Halliwell, B. M. Hoey, and J. Butler.** The antioxidant action of *N*-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radical Biol. Med.* 6: 593–597, 1989.
6. **Bird, R. P., and H. H. Draper.** Comparative studies on different methods of malonaldehyde determination. In: *Methods in Enzymology*, edited by L. Packer. New York: Academic, 1984, p. 299–305.
7. **Birnboim, H. C.** Fluorometric analysis of DNA unwinding to study strand breaks and repair in mammalian cells. In: *Methods in Enzymology*, edited by L. Packer and A. N. Glazer. New York: Academic, 1990, p. 550–555.
8. **Bridgeman, M. E., M. Marsden, W. MacNee, D. C. Flenley, and P. A. Ryle.** Cysteine and glutathione concentrations in plasma and bronchoalveolar lavage fluid after treatment with *N*-acetylcysteine. *Thorax* 46: 39–42, 1991.
9. **Cotgreave, I. A., R. C. Grafström, and P. Moldéus.** Modulation of pneumotoxicity by cellular glutathione and precursors. *Bull. Eur. Physiopathol. Respir.* 22, *Suppl.*: 263S–266S, 1986.
10. **Delange, R. J., and A. N. Glazer.** Phycoerythrin fluorescence-based assay for peroxy radicals: a screen for biologically relevant protective agents. *Anal. Biochem.* 177: 300–306, 1989.
11. **Dillard, C. J., R. E. Litov, W. M. Savin, E. E. Dumelin, and A. L. Tappel.** Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. *J. Appl. Physiol.* 45: 927–932, 1978.
12. **Draper, H. H., and M. Hadley.** Malondialdehyde determination as index of lipid peroxidation. In: *Methods in Enzymology*, edited by L. Packer and A. N. Glazer. New York: Academic, 1990, p. 421–431.
13. **Esterbauer, H., G. Striegl, H. Puhl, and M. Rotheneder.** Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radical Res. Commun.* 6: 67–75, 1989.
14. **Gohil, K., C. Viguie, W. C. Stanley, G. A. Brooks, and L. Packer.** Blood glutathione oxidation during human exercise. *J. Appl. Physiol.* 64: 115–119, 1988.
15. **Griffith, O. W.** Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106: 207–212, 1980.
16. **Holdiness, M. R.** Clinical pharmacokinetics of *N*-acetylcysteine. *Clin. Pharmacokinet.* 20: 123–134, 1991.
17. **Jenkins, R. R., K. Krause, and L. S. Schofield.** Influence of exercise on clearance of oxidant stress products and loosely bound iron. *Med. Sci. Sports Exercise* 25: 213–217, 1993.
18. **Ji, L. L., A. Katz, M. Griffiths, and M. Spencer.** Blood glutathione status during exercise: effect of carbohydrate supplementation. *J. Appl. Physiol.* 74: 788–792, 1993.
19. **Kanter, M. M., L. A. Nolte, and J. O. Holloszy.** Effects of an antioxidant vitamin mixture on lipid peroxidation at rest and postexercise. *J. Appl. Physiol.* 74: 965–969, 1993.
20. **Kretzschmar, M., D. Müller, J. Hübscher, E. Marin, and W. Klinger.** Influence of aging, training and acute physical exercise on plasma glutathione and lipid peroxides in man. *Int. J. Sports Med.* 12: 218–222, 1991.
21. **Kretzschmar, M., U. Pfeifer, G. Machnik, and W. Klinger.** Glutathione homeostasis and turnover in the totally hepatectomized rat: evidence for a high glutathione export capacity by extrahepatic tissues. *Exp. Toxicol. Pathol.* 44: 273–281, 1992.
22. **Lang, J. K., K. Gohil, L. Packer, and R. F. Burk.** Selenium deficiency, endurance exercise capacity, and antioxidant status in rats. *J. Appl. Physiol.* 63: 2532–2535, 1987.
23. **Lew, H., S. Pyke, and A. Quintanilha.** Changes in glutathione status of plasma, liver and muscle following exhaustive exercise in rats. *FEBS Lett.* 185: 262–266, 1985.
24. **Lew, H., and A. Quintanilha.** Effects of endurance training and exercise on tissue antioxidative capacity and acetaminophen detoxification. *Eur. J. Drug Metab. Pharmacokinet.* 16: 59–68, 1991.
25. **Lovlin, R., W. Cottle, M. Kavanagh, and A. L. Belcastro.** Are indices of free radical damage related to exercise intensity? *Eur. J. Appl. Physiol. Occup. Physiol.* 56: 313–316, 1987.
26. **McCay, P. B.** Vitamin E: interactions with free radicals and ascorbate. *Annu. Rev. Nutr.* 5: 323–340, 1985.
27. **Reed, D. J.** Glutathione toxicological implications. *Annu. Rev. Pharmacol. Toxicol.* 30: 603–631, 1990.
28. **Ruffmann, R., and A. Wendel.** GSH rescue by *N*-acetylcysteine. *Klin. Wochenschr.* 69: 857–862, 1991.
29. **Sen, C. K., E. Marin, M. Kretzschmar, and O. Hänninen.** Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization. *J. Appl. Physiol.* 73: 1265–1272, 1992.
30. **Sen, C. K., P. Rahkila, and O. Hänninen.** Glutathione metabolism in skeletal muscle derived cells of the L6 line. *Acta Physiol. Scand.* 148: 21–26, 1993.
31. **Sjödin, B., and Y. Hellsten Westing.** Changes in plasma concentration of hypoxanthine and uric acid in man with short-distance running at various intensities. *Int. J. Sports Med.* 11: 493–495, 1990.
32. **Skinner, J. S., and T. M. McLellan.** The transition from aerobic to anaerobic metabolism. *Res. Q. Exercise Sport* 51: 234–248, 1980.
33. **Smilkstein, M. J., G. L. Knapp, K. W. Kulig, and B. H. Rumack.** Efficacy of oral *N*-acetylcysteine in the treatment of acetaminophen overdose: analysis of the national multicenter study (1976 to 1985). *N. Engl. J. Med.* 319: 1557–1562, 1988.
34. **Smith, J. A., R. D. Telford, I. B. Mason, and M. J. Weidemann.** Exercise, training and neutrophil microbicidal activity. *Int. J. Sports Med.* 11: 179–187, 1990.
35. **Udupi, V., and C. Rice-Evans.** Thiol compounds as protective agents in erythrocytes under oxidative stress. *Free Radical Res. Commun.* 16: 315–323, 1992.
36. **Viguie, C. A., B. Frei, M. K. Shigenaga, B. N. Ames, L. Packer, and G. A. Brooks.** Antioxidant status and indexes of oxidative stress during consecutive days of exercise. *J. Appl. Physiol.* 75: 566–572, 1993.
37. **Wefers, H., and H. Sies.** The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. *Eur. J. Biochem.* 174: 353–357, 1988.