Strenuous Exercise-Induced Change in Redox State of Human Serum Albumin during Intensive Kendo Training

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Abstract: A high-performance liquid chromatographic (HPLC) analysis of human serum albumin (HSA) using an ion-exchange (DEAE-form) column shows three components: The principal component corresponds to human mercaptalbumin (HMA); the secondary to nonmercaptalbumin (HNA), having mixed disulfide with cystine (HNA[Cys]), or oxidized glutathione (HNA[Glut]); and the tertiary to HNA, oxidized more highly than mixed disulfide. The purpose of the present study is to clarify the effects of strenuous exercise load on HMA-HNA conversion (i.e., dynamic change in redox state) of HSA from elite kendo athletes (n=30; 20.0±1.1 years old). They participated in an intensive kendo training camp for 5 d. The mean value for the HMA fraction (f(HMA)) of kendo athletes after camp (62.8±2.4%) was significantly lower than before camp (71.9±3.7%) (p<0.0005). In contrast, the mean value for f(HNA-1) (i.e., f(HNA[Cys] and HNA[Glut])) after camp (34.2±2.1%) was significantly higher than before camp (25.7±3.7%) (p<0.0005). These results suggested that strenuous physical exercise markedly increased the oxidized albumin level in extracellular fluids during the intensive training camp. [Japanese Journal of Physiology, 52, 135–140, 2002]

Key words: serum albumin (human), high-performance liquid chromatography, kendo, physical exercise, redox state, training camp.

Serum albumin is the most abundant protein in extracellular fluids such as blood plasma and interstitial fluid. One of the most important features of the molecular structure of albumin is the presence of a highly reactive free sulfhydryl group in position 34 (Cys-34). This residue is a major ligand binding site (site V) for thiol-containing compounds, such as cystine and glutathione, and for various metal ions such as Au⁺, Ag⁺, and Hg⁺ in a reversible manner [1, 2]. Albumin with this sulfhydryl group in its unbound or free state is called mercaptalbumin (in humans, human mercaptalbumin, HMA) or reduced albumin. In contrast, albumin in which the sulfhydryl group is intermolecularly bound with compounds containing thiol or other species in the blood is called nonmercaptalbumin (in humans, human nonmercaptalbumin, HNA) or oxidized albumin. Depending on which ligand is bound with the sulfhydryl group, several kinds of HNA may result. The major HNA compound is mixed disulfide with cystine or oxidized glutathione (HNA[Cys] or HNA[Glut]) [3] (see references therein). Others include oxidation products higher than mixed disulfide,
such as sulfenic (−SOH), sulfinic (−SO₂H), and sulfonic (−SO₃H) states (HNA[Oxi]), which comprise an extremely small proportion of the HNA compound [4, 5]. Thus albumin might have powerful antioxidant properties against reactive oxygen species because it possesses the distinctive sulfhydryl group and is present in relatively high concentrations in extracellular fluids.

Recently, the possible role of oxygen free radicals in exercise-induced tissue damage, such as lipid peroxidation in skeletal muscle and liver, has attracted a great deal of attention [6–8]. Based on several reported results on the relationship between exercise and antioxidants, the protective role of endogenous antioxidant substances, such as enzymes and vitamins, against exercise-induced lipid peroxidation has been reviewed [9]. Inayama et al. [10] reported a relationship between physical exercise and plasma proteins, such as albumin. However, there have been no reports of the direct observation of the relationship between strenuous physical exercise and dynamic changes in the redox state of sulfhydryl groups of plasma proteins, especially those of serum albumin.

We have developed a convenient high-performance liquid chromatographic (HPLC) system for the clear separation of human serum albumin (HSA) to HMA and HNA by using a Shodex-Asahipak GS-520H [3, 4] or an ES-502N [4, 5] column. We have also extensively studied the mercapt̄nonmercapt conversion (i.e., dynamic change in the redox state) of HSA in various physical states [3–5] (see references therein). The purpose of the present study is to investigate the effects of strenuous physical exercise at an intensive training camp on the redox state of HSA from elite kendo athletes (kendo is a traditional Japanese sport), because a large portion of the sulfhydryl groups in human extracellular fluids are primarily derived from the sulfhydryl group (Cys-34) in the HSA molecule.

SUBJECTS, PROCEDURES, AND METHODS

Subjects, sample preparation, and exercise protocol. Thirty male students at the University of Tsukuba, all elite Japanese kendo players (mean age 20.0±1.1 years old), participated in this intense kendo training camp as the training group. The control group was comprised of 20 male students who did not regularly engage in exercise (22.1±1.8 years old). All were healthy male adults with no history of renal or hepatic diseases. Every subject gave his informed consent, and all procedures were performed in accordance with the Helsinki Declaration. The redox states of HSA for the control group and for the training group before and after training camp were comparatively investigated. No kendo player or control subject regularly consumed nutritional supplements during this study.

Blood samples from both groups were obtained by the use of vacuum blood-collecting tubes (EDTA-2K, Terumo Co., Tokyo, Japan). Samples from the training group were obtained twice: on the first day of training just before practice and on the fifth day within 40 min of the end of practice. Samples were then centrifuged for 20 min at 3,000 rpm in a Kubota 2100 centrifuge (Kubota Manufacturing Co., Tokyo, Japan). The resulting plasma was then subjected to pressure filtration by the use of a Cosmonice filter (0.45 μm, Nacalai Tesque, Inc., Kyoto, Japan), and specimens were stored at −80°C until HPLC analysis [3–5].

Although 6 weeks of summer vacation preceded the start of training camp, students trained by themselves according to the instructions of the kendo coach. Practice sessions during camp consisted of about 2-and-a-half hours in the morning and 3 in the afternoon every day. Detailed daily practice consisted of 50 min of kihon-keiko (practicing to acquire the basic movements); 120 min of gokaku-keiko (keiko practiced by persons of almost equal skill, also keiko in which the participants treat one another with equal respect even if their skills differ); 20 min of kakari-keiko (the keiko method in which for a short period the trainee practices striking the motodachi (person acting as instructor) with all his/her might, using all learned waza techniques (motor skills) without thinking of being struck or dodged); and 90 min of shiai-keiko (a method of keiko performed with referees, as in a match). The V̇O₂ max percentages for kihon-keiko, gokaku-keiko, and kakari-keiko were approximately 40, 55, and 70%, respectively, and that for shiai-keiko at its maximum value was nearly the same as the rate for kakari-keiko [11, 12]. All practices were conducted at a facility with a controlled environment; i.e., the average temperature throughout training camp was 25.0±1.3°C, and the humidity averaged 64.2±4.3%.

Serum analysis. The HPLC system consisted of a Model AS-8010 autosampler, a Model CCPM double-plunger pump, and a Model FS-8000 fluorescence detector (excitation wavelength 280 nm; emission wavelength 340 nm) in conjunction with a Model SC-8020 system supercontroller, all from Tosoh Co., Tokyo, Japan. A Shodex-Asahipak ES-502N column (10×0.76 cm I.D., DEAE-form for ion-exchange HPLC, Showa Denko Co., Tokyo, Japan; column temperature 35 ± 0.5°C) was used. Elution was carried out with a linear gradient of increasing ethanol concentration from 0 to 5% in 0.05 M sodium acetate–0.40 M
sodium sulfate (pH 4.85) (acetate-sulfate buffer) at a flow rate of 1.0 ml/min. The following ethanol concentration gradient was applied: 0–5 min, 0%; 5–30 min, linear increase from 0 to 5%; 30–35 min, linear decrease from 5 to 0%; 35–40 min, 0%. Deaeration of the buffer solution was carried out by bubbling it with helium. Samples were injected by means of an autosampler with fixed volumes of 2 μl. All chemicals and reagents were of analytical grade. All solvents and solutions were filtered through a Sterivex-GS filter unit (0.22 μm, Millipore Co., USA) before use.

To determine the value for each fraction of albumin, i.e., \[ f(HMA) = \frac{[HMA]}{[HMA + HNA]} \] and \[ f(HNA) = \frac{[HNA]}{[HMA + HNA]} \], the obtained HPLC profiles were subjected to numerical curve fitting; each peak shape was approximated by a Gaussian function.

**Statistical analysis.** Comparisons were made between the control group and the training group with a Mann-Whitney U-test. Data for the training group before and after camp were evaluated with a Wilcoxon signed-ranks test to determine significance. Values were expressed as means ± SD.

**RESULTS**

HSA is known to be a mixture of HMA and HNA [3–5]. Moreover, there are several kinds of HNA, i.e., HNA(Cys), HNA(Glut), and HNA(Oxi). A clear separation of three HSA fractions, i.e., HMA, (HNA[Cys] and HNA[Glut]) (tentatively called HNA-1 in this study), and HNA(Oxi) (called HNA-2) fractions was performed by the use of our HPLC system in this study. Representative HPLC profiles of the training group before and after camp are shown in Fig. 1A and B, respectively. The mean values (±SD) for each fraction of HSA from healthy young male subjects in the control group \((n=20)\) were 73.2±2.3, 24.9±2.0, and 1.9±0.8%, respectively, as shown in Fig. 2 (shaded columns). These values were not significantly different from those reported previously [5]. The mean value for HMA fraction \((f[HMA])\) in the training group after camp was significantly lower than before camp \((p<0.0005, \text{Fig. 2})\). On the other hand, the mean values for the HNA-1 and HNA-2 fractions in the training group after camp were significantly higher.
than before camp ($p<0.0005$ and $p<0.05$, respectively; Fig. 2), indicating that strenuous physical exercise markedly increased the oxidized albumin level in plasma during the intensive training camp. However, these differences in their HSA redox state were not observed in general training (data not shown).

As shown in Fig. 2, there were no significant differences in the values for $\bar{f}(\text{HMA})$, $\bar{f}(\text{HNA-1})$, or $\bar{f}(\text{HNA-2})$ between the control group and training group before camp. However, the value for $\bar{f}(\text{HMA})$ of the training group after camp showed a significantly lower value than that of the control group ($p<0.0005$), and both $\bar{f}(\text{HNA-1})$ and $\bar{f}(\text{HNA-2})$ values showed significantly higher values than those of the control group ($p<0.0005$).

To compare among $\bar{f}(\text{HMA})$, $\bar{f}(\text{HNA-1})$, and $\bar{f}(\text{HNA-2})$ values before and after camp (after camp/before camp ratio), the correlation coefficients ($r$) between HMA and HNA-1 and between HMA and HNA-2 were calculated to be $-0.927$ ($p<0.0001$) and $-0.216$ (not significant), respectively. These results indicated that the mercapt (HNA) nonmercapt conversion, i.e., dynamic change in the redox state of HSA, during the intensive training camp was mainly due to conversion from HMA to HNA-1, i.e., (HNA[Cys] and HMA[Glut]).

**DISCUSSION**

Evidence is growing that exhaustive physical exercise is associated with increased oxygen consumption in the cells, especially in mitochondria, and results in a marked increase in the endogenous production of free radicals [7]. Consequently, lipid peroxidation and the modification of protein structure initiated by a large amount of free radicals may generate tissue damage, especially in skeletal muscle and liver, during high-intensity exercise [7]. Several studies have demonstrated that the activities of antioxidant enzymes such as catalase and superoxide dismutase (SOD) in skeletal muscle [17], SOD in erythrocytes [18], glutathione peroxidase and catalase in erythrocytes [19], and SOD in serum [20] are increased in response to physical exercise or training, thereby protecting tissues from oxidant-induced damage. Gohil et al. [21] reported that endurance training could result in the enhancement of the extracellular antioxidant system, and Sen et al. [22] also reported that long-term submaximal exercise training elevated the antioxidant and detoxicant status of muscle and liver, respectively.

We emphasize that the elite kendo players belonging to the kendo club at the University of Tsukuba chosen in this study have been training intensively for more than 10 years, and they all have the ability to compete and win at the national championship level in Japan. Based on the above noted results [21, 22], we speculated that the activity of their antioxidant system would be relatively high compared with that for normal subjects who do not regularly engage in strenuous exercise. However, as shown in Fig. 2, there were no significant differences in $\bar{f}(\text{HMA})$, $\bar{f}(\text{HNA-1})$, or $\bar{f}(\text{HNA-2})$ values between both groups, indicating that prolonged physical exercise in humans does not affect the antioxidant ability in the extracellular fluids. This result was consistent with the recent observation by Ørtenblad et al. [23] that the intracellular antioxidant enzyme system adapted to high-intensity training, whereas no difference was observed in blood between trained jumpers and untrained controls.

It is now generally accepted that strenuous physical exercise can induce oxidative stress in humans. As described in the SUBJECTS, PROCEDURES, AND METHODS section, daily physical practice for 5 d in this training camp was highly intense exercise, suggesting that a large amount of exercise produced a large increase in oxidative stress. As shown in Fig. 2, HMA $\rightarrow$ HNA conversion of HSA was a conversion of HMA not to HNA-2 (HNA[Oxi]), but to HNA-1 (HNA[Cys] and HNA[Glut]).

Glutathione is present in large quantities in most mammalian tissues, especially within the cells, and it
also plays a key role in the defense of tissues against many oxidants [24]. Intracellular glutathione is normally in a form over 99% reduced (GSH), but the levels of the oxidized form of glutathione (glutathione disulfide, GSSG) are extremely low [25]. Under conditions of marked oxidative stress, however, such as strenuous physical exercise, the intracellular GSSG level increases substantially and GSSG is released from the cells into the blood [26]. Previous studies [21, 22, 27, 28] have also shown that the redox state of glutathione in humans is influenced by exercise-induced oxidative stress, and our observations of the redox state of HSA from kendo athletes were indirectly concerned with these results.

In summary, the results of HPLC analysis of the fractions of HMA and HNA on HSA during the intensive kendo training camp indicated that the production of reactive oxygen species and free radicals is stimulated by oxidative stress during physical exercise, resulting in an increase of GSSG concentration in the blood, which then converts HMA to HNA, especially HNA(5). It is generally accepted that plasma levels or the ratio of reduced and oxidized compounds, such as those of GSH and GSSG, can be used as markers of oxidative stress in the body [8, 21, 22, 26–28]. Although various analytical methods for glutathione status in biological samples have been published, most current methods have the disadvantages of being either time-consuming or labor-intensive (for review, see Redegeld et al. [29]). In this sense, our HPLC method for HSA analysis offers some advantages, including specificity, speed, sensitivity, and ease of use. The HSA redox state can be used as an index or biomarker of oxidative stress in the body, because albumin is the most abundant protein and is responsible for the largest fraction of reactive sulphydryl in extracellular fluids.

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REFERENCES