Physical Exercise Induces Oxidation of Plasma Protein Thiols to Cysteine Mixed Disulfides in Humans

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We have reported that strenuous physical exercise causes a decrease in protein-bound sulfhydryl groups (p-SHs), such as albumin cysteine residues, in human plasma (Inayama *et al*., 1996, *Life Sci*., **59**, 573–578). We further investigated the fate of plasma protein thiols after moderate exercise. Six untrained healthy female volunteers ran for 30-min at the individual ventilatory threshold. We observed an increase in protein cysteine mixed disulfides (p-S-Cys) after running, as evidenced by reducing plasma proteins with dithiothreitol to detect the increase of cysteine, along with the concomitant decrease in p-SHs in plasma. However, plasma protein-bound glutathione (GSH) and S-nitroso-protein were undetectable before and after exercise. Test tube experiments suggest that p-S-Cys are probably formed by the hydrolysis of protein GSH mixed disulfides by γ-glutamyltranspeptidase and peptidase, and/or by the oxidative addition of p-SHs to cystine.

Key words —— plasma protein thiol, cysteine mixed disulfide, physical exercise, human

INTRODUCTION

The role of thiols as antioxidants has been recognized in protecting cellular functions against oxidative stress.[1–3] Thiols consist of low-molecular-weight (LMW) thiols such as reduced glutathione (GSH) and cysteine, and protein-bound sulfhydryl groups (p-SHs). In human plasma, changes in the redox state of LMW thiols are being investigated as potential indicators of oxidative stress,[4] whereas the physiological importance of p-SHs is poorly understood. The concentration of p-SHs in human plasma, mostly derived from free cysteine residues of albumin, is very high (0.5–0.6 mM)[5] while that of GSH is low (5–25 μM).[5] We found that physical exercise induced a decrease in plasma p-SHs in humans,[6] although the mechanism for this decrease has not been studied. In animal studies, physical exercise enhanced the hepatic excretion of GSH into the circulation.[7] It was reported that the formation of GSH (and cysteine)-bound albumin increased when animals were challenged to oxidative stress,[5] but this phenomenon is unknown in humans.

We hypothesized that the enhanced generation of reactive oxygen species might increase the LMW thiol mixed disulfides with protein in human plasma. In this study, to identify the fate of p-SHs, we used plasma samples from healthy female volunteers who ran for 30-min as described previously,[8] and discovered the formation of protein-S-cysteine, not protein GSH mixed disulfides (p-S-SG), in the circulating plasma. We therefore discussed the possible mechanisms for the formation of protein cysteine mixed disulfides (p-S-Cys) in test tube experiments.

MATERIALS AND METHODS

Experimental Design —— Human plasma samples were obtained from healthy female subjects as described previously.[8] Briefly, six untrained, physically healthy female subjects were non-smokers with normal dietary habits who did not take any form of medication or vitamin supplements. Each volunteer

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ran for 30-min on a treadmill at the individual ventilatory threshold (VT) level. An oil-free breakfast (mostly rice meal) was consumed 4 hr before running. Blood samples were collected in heparinized tubes from the median cubital vein before and after running. The subjects did not consume any special food or drink before or after running except for water. The protocol of the study was approved by the Ethical Committee of National Institute of Health and Nutrition, and conformed to the guidelines of the Helsinki Conference for Research on Human Subjects.

Materials —— Aminopeptidase M and γ-glutamyltranspeptidase (γ-GTP) were purchased from Sigma (MO, U.S.A.). Cysteine was obtained from Takara Kohsan (Tokyo, Japan). Other chemicals were purchased from Wako (Osaka, Japan).

To obtain plasma p-S-SG, plasma samples (500 μM p-SHs) were incubated in the presence of 4 mM GSH and 10 μM $FeCl_2$ for 5 days at 37°C under air, and then dialyzed against 10 mM sodium phosphate (pH 7.5) for 1 day to yield 203 μM p-SHs.

Analytical Procedures —— The plasma contained 4.9–6.3 μM cysteine, 58–61 μM cystine and 2.2–2.6 μM GSH as LMW thiols. An aliquot of the plasma was dialyzed against 50 mM potassium phosphate (pH 6.5) containing 0.01 mM ethylenediaminetetraacetic acid (EDTA) for 1 day to remove LMW thiols and stored at –80°C until analyzed.

For the measurement of plasma p-S-Cys, the dialyzed plasma was incubated with 5 mM dithiothreitol (DTT) for 60 min at 37°C. Sulfosalicylic acid (SSA) was then added to give a final concentration of 5% and the acidified plasma was centrifuged at 10000 × *g* for 5 min. The thiols in the supernatant were determined using high performance liquid chromatography (HPLC) analysis as described previously.[8)]

For the measurement of plasma p-S-SG, 0.2 ml of the dialyzed plasma was added to 0.2 ml of 50 mM glycine-NaOH (pH 10) containing 6 mM sodium tetrahydroborate, 5.7 M urea, and 3 mM EDTA. The mixture was incubated for 15 min at 38°C, and 0.5 ml of 0.14 M potassium dihydrogenphosphate and 0.1 ml of 50% SSA were added. The acidified samples were centrifuged at 10000 × *g* for 5 min. The supernatant fractions were subjected to HPLC analysis.

The plasma concentrations of p-SHs and protein were determined as described previously.[6,9)] All analyses were performed within 3 days of blood sample collection.

Statistics —— All values are expressed as means ± S.E. Statistical analysis was performed using repeated-measures analysis of variance (ANOVA) with Fisher's Protected Least Significant Difference post hoc test to compare the pre-running values. Significance of differences was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Changes in Plasma p-S-Cys after Exercise

In the previous study,[8)] 30-min treadmill running at the individual VT, performed by untrained healthy females, caused a significant decrease in plasma levels of p-SHs after running, but no significant change in plasma LMW thiols such as GSH and cysteine. To quantify the possible protein mixed disulfides, the plasma was treated with DTT or sodium tetrahydroborate after dialysis, and the released amounts of cysteine or GSH were measured by HPLC.

Before running, the plasma level of p-SHs was 570 ± 20 μM and that of p-S-Cys was 107 ± 7 μM, whereas p-S-SG was not detected. This result was consistent with a previous report that human plasma p-SHs formed mixed disulfide with cysteine and cysteine-glycine.[10)]

We observed a decrease in plasma levels of p-SHs at 1 hr (553 ± 19 μM, –17 μM) and 2 hr (556 ± 17 μM, –14 μM), and a significant increase of p-S-Cys at 1 hr (118 ± 8 μM, +11 μM, $p < 0.05$) and 2 hr (127 ± 6 μM, +20 μM, $p < 0.01$) after 30-min treadmill running. The inconsistency of p-SHs at 5 min after running was probably due to dehydration during exercise (Fig. 1). On the other hand, p-S-SG was undetectable at all points (data not shown). It should be noted that no significant change in plasma cyst(e)ine and GSH was observed possibly due to their very rapid turnover.[11)] We therefore concluded that a single bout of exercise of moderate intensity induced the oxidation of plasma protein thiols to p-S-Cys.

Mechanisms for the Formation of Cysteine Mixed Disulfides

It is known that protein cysteine residue forms the mixed disulfide with GSH and cysteine by oxidative stress in *in vivo* animal experiments.[5,12)] Moreover, a rapid binding of ^{35}S-labeled GSH to circulating albumin has been reported in mouse plasma.[13)] It is noteworthy that physical exercise enhanced the hepatic excretion of GSH into the circulation in rats

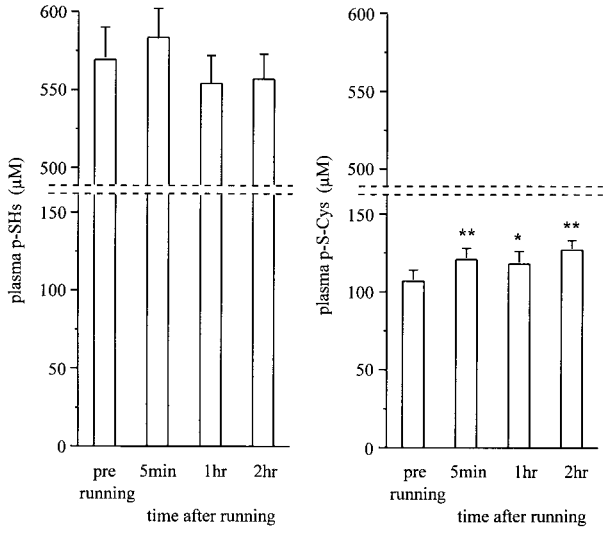

Fig. 1. Changes in Human Plasma Levels of p-SHs and p-S-Cys after 30-min Treadmill Running

Blood samples were collected 30 min before running and 5 min, 1 hr, and 2 hr after running. The values are expressed as means ± S.E. ($n = 6$). * and ** indicate significant difference ($p < 0.05$, $p < 0.01$) from the pre-running value as analyzed by repeated-measures ANOVA with post hoc test.

and, hence, hepatic GSH level decreased with the concomitant increase in plasma GSH level.[7] Investigating the mechanism for the formation of p-S-Cys, not p-S-SG, as shown above, we hypothesized the following mechanisms.

First, we confirmed whether human plasma p-S-SG could be hydrolyzed by γ-GTP and dipeptidase since these enzymes were highly enriched on the apical membrane surface of the kidney, small intestine and bile-tree cells in humans.[14] Animals treated with inhibitors of γ-GTP which hydrolyzes GSH have increased concentrations of GSH in their plasma and excreted substantial amounts of GSH, γ-glutamylcysteine and cysteine in their urine,[11] indicating that γ-GTP fully functions at the inner lumen of blood vessels. In fact, it was reported that nonmercaptalbumin [mainly albumin-S-SG (Alb-S-SG)] injected into normal animals rapidly decreased with a concomitant increase in mercaptalbumin [albumin-SH (Alb-SH)].[15] Figure 2 clearly shows that p-S-SG was converted to p-S-Cys by incubation in the presence of γ-GTP and aminopeptidase M for 30 min, but no significant increase in p-S-Cys level was observed in the absence of the enzymes or p-S-SG.

The mechanism for the formation of p-S-SG is not clear at present. However, S-nitroso-albumin may be a precursor since physical exercise induces

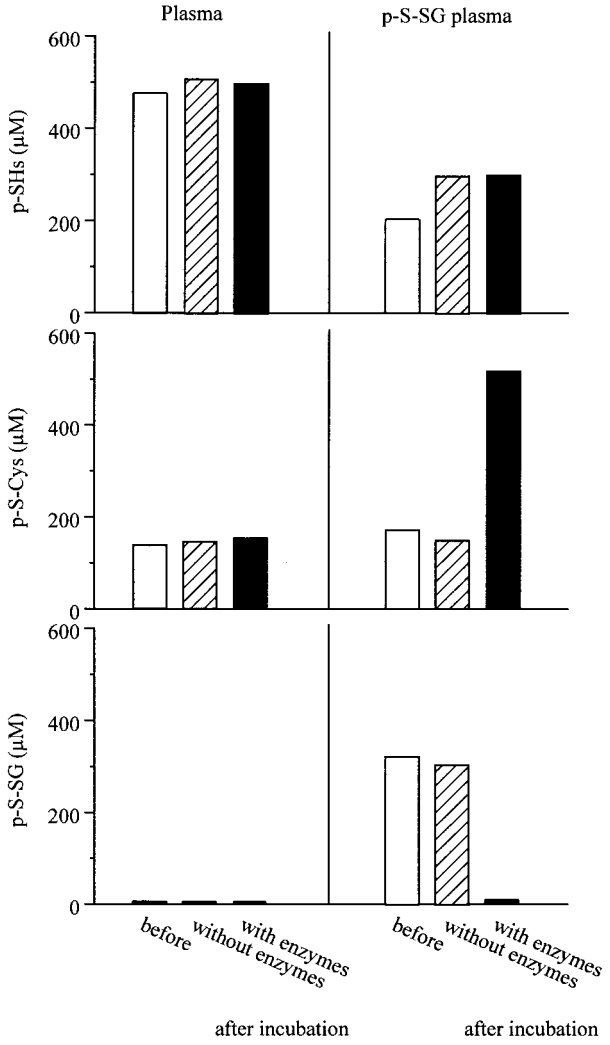

Fig. 2. The Formation of p-S-Cys from Human Plasma (Left Panel) and Plasma Containing 324 μM p-S-SG (Right Panel) Treated with γ-GTP and Aminopeptidase M (0.125 unit Each) at 37°C for 30 min

the production of nitric oxide in human plasma,[16] and nitric oxide in circulation results in S-nitroso-albumin.[17] S-nitroso-albumin is probably converted into the more stable mixed disulfides, p-S-SG and p-S-Cys, after the exchange with GSH and cysteine, respectively.[18] The rates of these reactions must be rapid since we could not detect the increased formation of S-nitroso-albumin as measured by the Saville's method.[19]

We also hypothesized another possible mechanism; the oxidative reaction of p-SHs with cyst(e)ine.[20] The physiological concentration of cystine in human plasma is reported to be around 60 μM. The rate of p-SH decay in plasma was trivial, from 649 μM to 616 μM by 1 hr and to 594 μM by 3 hr (the upper curve in Fig. 3), but this rate was found

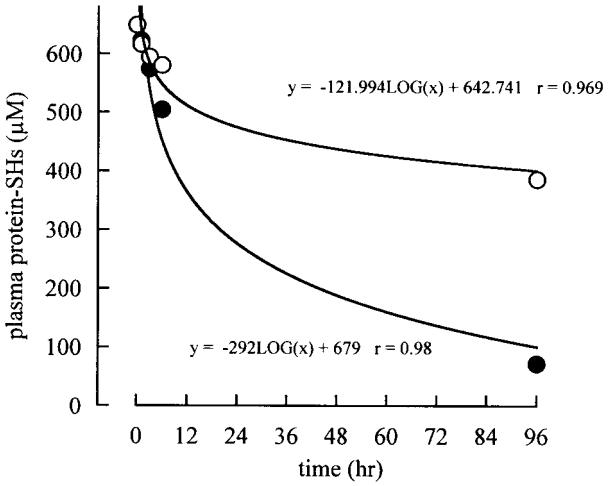

Fig. 3. The Decay of p-SHs during the Incubation of Human Plasma without (Open Circle) or with (Closed Circle) the Addition of 350 μM Cystine at 37°C for 96 hr under Aerobic Conditions

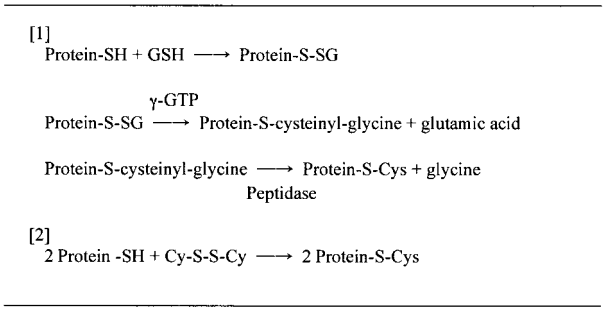

Fig. 4. Plasma Protein Thiols to Cysteine Mixed Disulfides

to be consistent with that observed in the above experiment (Fig. 1). To exaggerate the oxidative reaction of p-SHs with cyst(e)ine, we chose the experimental conditions that we incubated human plasma with the addition of 350 μM cystine at 37°C under aerobic conditions (the lower curve in Fig. 3). The rate of p-SH decay was accelerated by the addition of cystine, indicating that the reaction of p-SHs with cystine is plausible.

Furthermore, we measured plasma p-S-Cys using HPLC analysis at the extreme point of 96 hr later, and confirmed that one molecule of cystine was stoichiometrically converted to two molecules of p-S-Cys. The decay of 60 μM cystine was accompanied by the formation of 115 μM p-S-Cys (the upper curve in Fig. 3), and when 350 μM cystine, though not physiological, was added to plasma, the formation of 414 μM p-S-Cys was observed with a concomitant decay of 210 μM cystine (the lower curve in Fig. 3).

In summary, we demonstrated that moderate exercise induced the oxidation of plasma protein to cysteine mixed disulfides. Based on the results of test tube experiments, we also proposed that p-S-Cys are probably formed by the hydrolysis of protein GSH mixed disulfides, and/or by the oxidative addition of p-SHs to cystine (Fig. 4).

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