Intense Exercise Increases Protein Oxidation in Spleen and Liver of Mice

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ABSTRACT: Studies have indicated that sports anemia is mainly associated with intravascular hemolysis induced by exercise. We hypothesized that such exercise-induced hemolysis leads to oxidative damage due to an increase in free iron caused by hematocyte destruction. Thirty-one male ICR mice were randomly divided into 3 groups: a rested control group, an intense-exercise group, and a group rested for 24 hours after intense exercise. The serum haptoglobin level of the intense-exercise group decreased compared with that of the rested control group, suggesting hemolysis. Tissue iron and protein carbonyl levels in the liver were increased after exercise, and the protein carbonyl level in the spleen on the day after exercise was significantly increased compared with that of the resting state. These results suggest that the spleen and liver, where extravascular hemolysis occurs, were subjected to oxidative modification by the free iron, which was released from large numbers of hemocytes that were destroyed due to the intense exercise.

KEY WORDS: sports anemia, hemolysis, oxidative stress, spleen, intense exercise

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Introduction

Anemia is a state in which the amount of hemoglobin in the total blood volume is reduced.[1] There are three causes of anemia: impairment of red blood cell production, an increase in red blood cell destruction, and bleeding. Anemia causes a lack of oxygen in the body, and, accordingly, it not only impairs health, but also deceases the ability to exercise or perform competitively.[2–4] As a major social and health issue, a solution for anemia is highly desirable. Studies have indicated that sports anemia (anemia induced by vigorous physical training) is mainly associated with intravascular hemolysis, which develops due to an increase in red blood cell destruction induced by exercise.[5–7] Various studies have been performed on the factors involved in hemolysis and exceeding the normal exercise tolerance, and it was reported that intravascular hemolysis may develop due to physical factors, such as the bursting of red blood cells in the circulation due to the impact of footfalls,[8,9] or an increase in friction between red blood cells and vessel walls due to increased blood flow.[10,11] Furthermore, several studies have indicated that erythrocyte membrane compromise due to factors such as lactic acid, lysolecithin, or oxidative stress, which increase in blood when the normal exercise tolerance is exceeded, could also be involved in hemolysis.[12–17] Therefore, the development of exercise-induced hemolysis has been attributed to not only physical, but also chemical factors.

On the other hand, it is known that in extravascular hemolysis, the reticuloendothelial systems of the spleen and liver play a role in the destruction of senescent or pathological erythrocytes through phagocytosis by macrophages.[18,19] We hypothesized that the hemolysis resulting from exceeding the exercise tolerance may contribute to the impairment of organs by oxidative damage due to the rise in free iron caused by a rapid increase in hematocyte destruction in the spleen and liver. However, the relationship between intense exercise and extravascular hemolysis has not been examined to date. Therefore, the objective of the present study was to investigate whether or not intense exercise is associated with intravascular and/or extravascular hemolysis resulting in severe muscle damage, through the observation of physiological changes

and oxidative damage of organs after intense exercise and at 24 hours after exercise.

Materials and Methods

Animals and experimental protocol. This experimental study was performed following the Guidelines for Animal Experimentation of Kyoto Prefectural University (No. 2306), compiled from the Guidelines for Animal Experimentation published by the Japanese Association for Laboratory Animal Science. Thirty-one male ICR mice aged 7 weeks (body weight: 32–34 g) were employed in the study (Japan SLC, Hamamatsu, Japan). The mice were housed in plastic cages at a controlled temperature of 22–24°C, a relative humidity of 40–60%, and a light cycle of 12 hours with free access to food (MF; Oriental Yeast, Tokyo, Japan) and distilled water. The mice were randomly divided into three groups: a rested control group (R, n = 10), an intense exercise group (E, n = 11), and a group rested for 24 hours after intense exercise (ER, n = 10). The exercise training and tolerance protocols employed were prepared as previously described.[20,21] All of the mice were acclimated to running on a motor-driven treadmill for 3 weeks: 15–18 m/min for 4 min for 3 days in the first week, followed by 15–25 m/min for 4 min for 5 days in the second and third weeks. After being acclimated to running at 25 m/min, the E and ER groups underwent single acute treadmill-running sessions for 30 min at 30–32 m/min. It has been shown that this speed promotes slightly less than 80% of the maximal oxygen consumption (VO_2 max).[22,23] The mice in the E group were euthanized under ether anesthesia immediately after the exercise, and the mice in the ER group were euthanized 24 hours after the exercise. The R group was euthanized without any exercise. Blood samples were drawn from the tails and hearts of the mice in all three groups and placed in tubes with heparin. Samples of the right gastrocnemius muscle, liver, and spleen were also collected.

Blood analysis. The hemoglobin concentration, hematocrit level, red blood cell count, mean cell volume, mean corpuscular hemoglobin, and mean cell hemoglobin concentration in blood samples drawn from the heart were measured using an automatic hematology analyzer (KX-21NV; Sysmex, Kobe, Japan). The partial pressures of oxygen (pO_2) and carbon dioxide (pCO_2) were measured using an analyzer (i-STAT; Abbott Point of Care, IL, USA). Lactic acid in the blood samples drawn from the tail was measured using a simplified analyzer (Lactate Pro; Arkray, Kyoto, Japan).

Blood samples were centrifuged at 1,500 g for 10 min at 4°C to obtain serum samples. Serum haptoglobin was measured using an ELISA Kit (Life Diagnostics, PA, USA). The serum hemoglobin level was measured using an assay kit (Hemoglobin Colorimetric Assay Kit; Cayman Chemical, MI, USA). The serum level of creatine kinase was quantified using a kit (Max Discovery; Bioo Scientific, USA). Serum iron, the unsaturated iron-binding capacity (UIBC), and lactate dehydrogenase were measured using Detaminer Fe and UIBC (Kyowa Medix,

Tokyo, Japan) and LDH–L (Serotec, Sapporo, Japan) with an automatic biochemical analyzer (CL-8000; Shimadzu, Kyoto, Japan). The total iron-binding capacity (TIBC) and serum transferrin saturation were calculated as follows:

$$TIBC = serum\ iron + UIBC$$

$$Serum\ transferrin\ saturation = serum\ iron/TIBC \times 100$$

Protein carbonyl assay of tissues. Protein was isolated from the homogenized tissue of the right gastrocnemius, liver, and spleen using a tissue-dissolving reagent (Cellytic; Sigma-Aldrich, MO, USA). The protein carbonyl level in the tissue samples was measured using an assay kit (JaICA, Shizuoka, Japan).

Iron content of the liver. The samples were perfused with saline, and treated employing the wet ash method using a microwave extraction system (Ethos; Milestone, Sorisole, Italy). The ash was suspended in dilute hydrochloric acid solution after evaporation, and then left to dry. Iron concentrations were measured using the same method as employed for the serum iron, as described above, after appropriate dilution. The iron concentrations in the samples are expressed on a wet-weight basis.

Statistical analysis. Before assessing the different variables, we carried out the Kolmogorov-Smirnov test to verify the normal distribution of the variables. Data fitting the normal distribution were compared by one-way analysis of variance. Levene's test for homogeneity was used to test for equal variance between samples. When equal variance could be assumed, the Bonferroni post-hoc test was used to identify significant differences between multiple test groups. Data are presented as the mean ± standard error (SEM). The level of significance was set at $P < 0.05$. Analyses were performed using PASW statistics 18.0 (IBM, IL, USA).

Results

The effects of the single session of intense exercise on the blood parameters and liver iron content are shown in Table 1. The blood lactic acid and serum lactate dehydrogenase levels of the E group (5.7 ± 0.8 mmol/L and 933 ± 84 U/L, respectively) showed a significant increase after the intense exercise compared with the R group (2.7 ± 0.1 mmol/L and 566 ± 49 U/L, respectively), and the levels in the ER group (3.2 ± 0.4 mmol/L and 624 ± 70 U/L, respectively) showed a significant decrease compared with the E group. No significant differences in serum creatine kinase were observed among the three groups; however, the levels in the ER group (470 ± 97 U/L) were higher than those in the R and E groups (226 ± 30 and 256 ± 42 U/L, respectively). The blood pO_2 of the E and ER groups (15.5 ± 2.5 and 13.8 ± 3.4 mmHg, respectively) was higher than that of the R group (11.6 ± 2.5 mmHg), but no significant difference was observed among the three groups.

Table 1. Effects of single-time intense exercise tolerance on blood parameters and liver iron content in mice.

	R (n = 10)	E (n = 11)	ER (n = 10)
Lactic acid (mmol/L)	2.7 ± 0.1[a]	5.7 ± 0.8[b]	3.2 ± 0.4[a]
Lactate dehydrogenase (U/L)	566 ± 49[a]	933 ± 84[b]	624 ± 70[a]
Creatine kinase (U/L)	226 ± 30	256 ± 42	470 ± 97
pO_2 (mmHg)	11.6 ± 2.5	15.5 ± 2.5	13.8 ± 3.4
pCO_2 (mmHg)	114.2 ± 4.1[a]	85.2 ± 6.3[b]	117.6 ± 7.3[a]
Serum haptoglobin (μg/mL)	9.0 ± 4.7[a,b]	0.8 ± 0.2[a]	4.0 ± 1.5[b]
Serum hemoglobin concentration (mg/dL)	17.4 ± 1.8	14.5 ± 1.7	22.4 ± 3.8
Red blood cell count (×10⁴/μL)	995 ± 15	802 ± 28	929 ± 26
Hematocrit level (%)	49.0 ± 1.7	50.1 ± 1.5	48.6 ± 2.6
Blood hemoglobin concentration (g/dL)	16.7 ± 0.6	17.0 ± 0.5	16.5 ± 0.9
Mean cell volume (fL)	54.8 ± 0.3	55.4 ± 0.5	56.0 ± 0.5
Mean corpuscular hemoglobin (pg)	16.3 ± 0.1[a]	16.8 ± 0.2[b]	16.6 ± 0.2[a,b]
Mean cell hemoglobin concentration (g/dL)	29.7 ± 0.1[a]	30.3 ± 0.2[b]	29.7 ± 0.2[a]
Total iron-binding capacity (μg/dL)	353 ± 33	392 ± 16	394 ± 15
Serum iron (μg/dL)	287 ± 31	311 ± 14	251 ± 15
Serum transferrin saturation (%)	81.4 ± 4.3[a]	79.3 ± 2.4[a]	63.9 ± 2.1[b]
Liver iron content (μg/g liver, wet weight)	81.5 ± 7.3	91.0 ± 8.9	75.6 ± 5.5

Rested control group; R, intense exercise group; E or 24-hour rested after intense exercise group; ER. Data that fit the normal distribution were compared by 1-way analysis of variance. Levene's test for homogeneity was used to test for equal variance between samples. When equal variance could be assumed, the Bonferroni post-hoc test was used to identify significant differences between multiple test groups. Data are presented as the mean ± standard error (SEM). Values with a different letter were significant: $P < 0.05$.

The blood pCO_2 of the E group (85.2 ± 6.3 mmHg) significantly decreased after the intense exercise compared with that of the R group (114.2 ± 4.1 mmHg), and the ER group (117.6 ± 7.3 mmHg) showed a highly significant increase compared with the E group.

The serum haptoglobin level of the E group (0.8 ± 0.2 μg/mL) decreased compared with the R group (9.0 ± 4.7 μg/mL), and the level in the ER group (4.0 ± 1.5 μg/mL) increased significantly compared with the E group. The serum hemoglobin concentration of the E group (14.5 ± 1.7 mg/dL) decreased compared with the R group (17.4 ± 1.8 mg/dL), and that of the ER group (22.4 ± 3.8 mg/dL) increased compared with the E group, but no significant differences were shown among the three groups. No significant differences were noted in the red blood cell count, hematocrit level, blood hemoglobin concentration, or mean cell volume between the three groups. However, the red blood cell count of the E group (802 ± 28 × 10⁴/μL) decreased compared with the R group (995 ± 15 × 10⁴/μL), and the ER group (929 ± 26 × 10⁴/μL) showed recovery of the levels, compared with the E group. The mean corpuscular hemoglobin of the E group (16.8 ± 0.2 pg) increased significantly, compared with the R group (16.3 ± 0.1 pg), but no significant difference was noted between the E and ER groups. The mean cell hemoglobin concentration of the E group (30.3 ± 0.2 g/dL) increased significantly, compared with the R group (29.7 ± 0.1 g/dL),

and the ER group (29.7 ± 0.2 g/dL) showed favorable recovery of the levels compared with the E group.

No significant differences were observed in the TIBC or serum iron level between the three groups, but the TIBC of the E and ER groups was higher than that of the R group, while the serum iron level of the E group was higher and that of the ER group was lower than in the R group. The serum transferrin saturation level of the ER group (63.9 ± 2.1%) decreased markedly compared with the R and E groups (81.4 ± 3.4 and 79.3 ± 2.4%, respectively). The liver iron content of the E group (91.0 ± 8.9 μg/g liver) showed an increase compared with the R group (81.5 ± 7.3 μg/g liver), and the level in the ER group (75.6 ± 5.5 μg/g liver) was lower than that in the E group, but no significant differences were observed between the three groups.

Figure 1 shows the changes in the tissue protein carbonyl levels. The protein carbonyl level in the right gastrocnemius muscle samples of the E group increased compared with the R group, and that of the ER group increased compared with the E and R groups. In the liver, the protein carbonyl level of the E group increased significantly compared with the R group, and the level in the ER group showed a significant decrease compared with the E group. In the spleen, the protein carbonyl level of the ER group showed a marked increase compared with the levels in the R and E groups, but no significant difference was noted between the R and E groups.
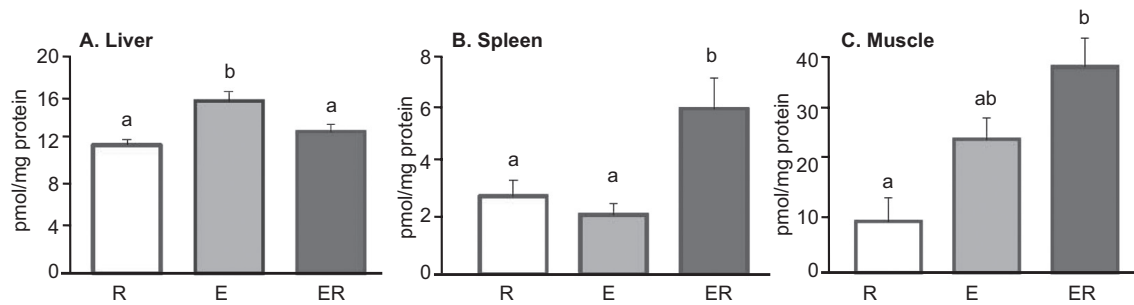
Figure 1. Effects of single-time intense exercise tolerance on the protein carbonyl level of the liver (**A**), spleen (**B**), and right gastrocnemius muscle (**C**) in mice. Rested control group (R: n = 10), intense exercise group (E: n = 11), or 24-hour rested after intense exercise group (ER: n = 10). Data that fit the normal distribution were compared by 1-way analysis of variance. Levene's test for homogeneity was used to test for equal variance between samples. When equal variance could be assumed, the Bonferroni post hoc test was used to identify significant differences between multiple test groups. Data are presented as the mean ± standard error (SEM). Values with a different letter were significant: $P < 0.05$.

Discussion

In the present study, we examined the relationship between intense exercise and extravascular and/or intravascular hemolysis by observing physiological changes. Immediately after intense exercise, blood lactic acid increased significantly compared with the resting state, confirming exercise tolerance had been exceeded. The serum lactate dehydrogenase immediately after intense exercise increased remarkably compared with the resting state, and the serum creatine kinase value at 24 hours after exercise was higher than that of the resting state. These results suggest that muscle damage was induced by the exercise. Additionally, the protein carbonyl level in the gastrocnemius muscle after exercise showed an increase compared with the level in the resting state. Therefore, muscle protein oxidation may have been induced by the exercise. Our data demonstrated that pO_2 in the blood samples increased after the exercise and pCO_2 decreased significantly, compared with the resting state. Moreover, pO_2 and pCO_2 recovered to almost the same levels shown in the rested control group at 24 hours after the exercise. During exercise, the energy demand increases rapidly, and blood flow to the skeletal muscle is increased by response adjustments in the circulation.[24,25] Thus, it appears that the mice exceeding the exercise tolerance in this experiment were in a state of oxygen debt, which supplied a large volume of oxygen to the body. These results demonstrated that the mice in this study underwent physiological changes promoted by the intense exercise.

Haptoglobin is an abundant hemoglobin-binding protein present in the plasma. The function of haptoglobin is primarily to determine the fate of hemoglobin released from the red blood cells after either intra- or extravascular hemolysis.[26] Serum haptoglobin is markedly decreased by exercise-induced hemolysis. It appears that this phenomenon functions to prevent leakage of the hemoglobin out of the blood vessels, because haptoglobin binds with free plasma hemoglobin.[27–29] The findings shown in the present study suggest that hemolysis developed immediately after the intense exercise, as shown by the decrease in haptoglobin and plasma hemoglobin in the E and ER groups, compared with the resting state R group. Some recovery of these levels was evident in the ER group. On the other hand, anemia decreases the red blood cell count, hematocrit level, and hemoglobin concentration. In the present study, while these levels were lower immediately after the intense exercise than they were in the resting state, no significant differences were shown in these results among the three groups, suggesting that hemolysis developed without anemia due to the intense exercise, which induced an exercise tolerance state in the mice.

On the other hand, the serum iron level after exercise was less than that of the resting state, and the same result was also shown in the amount of liver iron, which reflects the storage iron content in the body. The serum transferrin saturation after exercise was higher than that in the resting state, suggesting mobilization of the serum or storage iron in order to maintain the red blood cell characteristics. Additionally, the TIBC of the exercise tolerance groups increased, compared with the resting state, suggesting the increased generation of transferrin carrying iron throughout the body. It has been reported that multiple exercise tolerance states can alter expression of the iron metabolism gene.[30] Therefore, even a single-session, intense running exercise-induced exercise tolerance state is likely to influence iron metabolism.

Hemolysis, which develops *in vivo*, has been divided into intra- and extravascular hemolysis. As one factor of intravascular hemolysis, it has been reported that the level of serum lactic acid is increased by exercise, the blood is acidified, and the erythrocyte membrane resistance is consequently decreased.[12] One of the causes of hemolysis shown in this study may be that the level of blood lactic acid after the intense exercise was increased. However, no decrease was shown in the hydrogen ion exponent, perhaps because the blood lactic acid and hydrogen ion exponent were measured using blood samples obtained at different points in the study. Next, the level of lysolecithin and oxidative stress disorders

in the blood.[13–17] which reportedly weaken the erythrocyte membrane, were not measured in this study. The possibility that the red blood cell membranes developed oxidative stress disorder cannot be denied because a state of oxygen debt was induced by exercise tolerance and oxidative stress was caused by muscle damage. Moreover, because it is expected that blood flow will increase due to exercise, one cause of the hemolysis might be the physical destruction of blood cells due to the impact of the plantaris site and friction between capillary walls and red blood cells. The relevance of these factors must be clarified in future studies.

On the other hand, destroyed blood cells have their iron and hemoglobin degraded after phagocytosis by macrophages of the reticuloendothelial system in the spleen and Kupffer cells in the liver. In the present study, the amount of tissue iron and protein carbonyl level in the liver increased after exercise, but the protein carbonyl level in the spleen on the day after exercise was significantly increased compared with that of the resting state. Protein carbonyl modification is one of the forms of oxidative damage, and the protein carbonyl level is a sensitive biomarker of protein oxidative modification.[31,32] Therefore, the results we obtained may have indicated that the spleen and liver were subjected to oxidative modification at the protein level by free iron, which was accumulated because the large amounts of hemocyte destroyed by the intense exercise were processed in the spleen and liver. Additionally, we consider that the destroyed red blood cells were processed in the liver immediately after the intense exercise, while in the spleen, they were processed the day after exercise. It is known that the spleen and liver have a different erythrocyte capture mechanism due to differences in vessel construction.[33,34] In fact, while red blood cells are trapped in the spleen when the level of impairment is low, red blood cells are only trapped in the liver when impairment is marked. Therefore, the degree of red blood cell impairment due to exercise may be relatively low. Furthermore, an increase in transferrin production in the liver was observed immediately after exercise in this study. The liver might be able to avoid oxidative stress the day after exercise, because the accumulated iron bound to transferrin has already been transferred throughout the body. On the other hand, reports on any increase in oxidative stress in the spleen after intense exercise are limited, and our results thus provided new findings that the spleen demonstrated an increase in the oxidative modification of the protein level after exercise. Therefore, there is no denying the possibility that the decrease in the processivity of extravascular hemolysis can occur due to the hypoactivity of the spleen, if the spleen continues receiving an accumulation of iron, oxidative stress, and protein oxidative modifications due to repeated intense exercise. Eventually, this might be one of the causes of sports anemia.

There were several limitations to the present study. We used protein carbonyl level as an oxidative damage biomarker. However, it is insufficient to measure oxidative damage by this marker alone. Furthermore, it is necessary to synthetically assess oxidative stress and/or oxidative damage response to exercise in the liver and spleen in order to add other specific markers for oxidative stress (Malondialdehyde, GSH/GSSG ratio, Isoprostanes, and others). Next, despite the preparation of the intense exercise tolerance state according to previously published reports, the blood lactic acid level immediately after the intense exercise was approximately twice that of the level of the resting state. The VO_2 max of mice was not measured in the present study, and the exercise intensity may have been somewhat lower than our assumption.

Conclusions
We examined the relationship between intense exercise and extra- and/or intravascular hemolysis by observing the physiological changes after intense exercise. The results demonstrated that protein oxidation in the liver and spleen, where extravascular hemolysis occurs, were increased by the intense exercise. Moreover, the results suggest that intense exercise causes hemolysis, the organs and/or red blood cells are subjected to oxidative stress, and iron metabolism regulated to maintain the shapes of red blood cells, even following a single session of intense exercise. Therefore, repeated intense exercise is likely to exacerbate hemolysis and iron metabolism, and lead to the development of sports anemia. We should examine the changes in iron metabolism and oxidative stress caused by chronic intense exercise tolerance in future studies.

Author Contributions
Designed the study: YKo, WA. Conducted the experiments: YKo, AN, WA, SW, MK, YKa. Conducted analysis: YKo, AN, WA. Interpretation of data: YKo, AN, WA, SW, MK, YKa. Prepared the manuscript: YKo. All authors reviewed and approved of the final manuscript.

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