Exercise-induced hemolysis is caused by protein modification and most evident during the early phase of an ultraendurance race

Ashril Yusof, Renate M. Leithauser, Heinz J. Roth, Holger Finkernagel, Michael T. Wilson, and Ralph Beneke

1Centre for Sports and Exercise Science, Department of Biological Sciences, University of Essex, Colchester, United Kingdom; 2Labor Limbach, Heidelberg, Germany; 3Institut für Leistungsdiaagnostik, Bad Berleburg, Germany; and 4Biophysics and Chemical Biology, Department of Biological Sciences, University of Essex, Colchester, United Kingdom

Submitted 24 May 2006; accepted in final form 22 September 2006

Yusof A, Leithauser RM, Roth HJ, Finkernagel H, Wilson MT, Beneke R. Exercise-induced hemolysis is caused by protein modification and most evident during the early phase of an ultraendurance race. J Appl Physiol 102: 582–586, 2007. doi:10.1152/japplphysiol.00580.2006.—Whether structural changes of the erythrocyte membrane increase the susceptibility to hemolysis particularly of the relatively older cell population during the early phase of a 216-km ultrarace was tested in six male runners (age 53.6 ± 10.4 yr, height 175.8 ± 11.1 cm, body mass 75.9 ± 8.4 kg). Erythrocyte membrane spectrins were lowest (P < 0.001) after 42 km (75.9 ± 5.25% of prerace) and increased (P < 0.001) toward 216 km (88.27 ± 3.37%). Susceptibility to osmotic hemolysis was highest (P < 0.01) after 42 km (107.34 ± 3.02 mOsm sodium phosphate buffer) with almost identical (P > 0.05) values prerace (97.98 ± 3.41 mOsm) and postrace (98.61 ± 3.26 mOsm). Haptoglobin indicated intravascular hemolysis of 9.27 ± 3.41 cells/l (P < 0.05) during the initial 84 km. Changes in hematocrit and plasma proteins indicated an estimated total net erythrocyte loss of 3.47 ± 10^11 cells/l (P < 0.05) after 21 km. This was compensated by a gain in erythrocytes (P < 0.05) of 3.31 ± 10^11 cells/l during the final 132 km. A main effect (P < 0.05) on erythropoietin suggests increased erythropoiesis throughout the race. Exercise-induced hemolysis reflects alterations in erythrocyte membrane spectrins and occurs particularly in the early phase of an ultraendurance race because of a relative older cell population.

SYMPTOMS OF ENDURANCE exercise-related hemolysis were first described more than 120 years ago (15). Meanwhile, numerous research groups have confirmed this observation (12, 16, 17, 27, 33, 36). Hemolysis may result from 1) intrinsic abnormalities of erythrocyte contents such as modified hemoglobin or enzymes (5) or 2) membrane alterations with respect to cytoskeletal structure, permeability, or protein and/or lipid content (6); or 3) extrinsically based on serum antibodies, trauma in the circulation, or infectious agents (3, 11). Damage to the erythrocyte membrane may cause impairment of the cell’s capability to deform and increases its rigidity, which leads to hemolysis (19, 21). Cytoskeletal proteins of erythrocytes were modified following endurance exercise, resulting in loss of membrane spectrins (2, 18). Spectrin is a protein exposed to the cytoplasmic surface of the membrane bilayer. It has springlike properties that function in regulating cell shape, membrane deformability, and stability (1). However, very little is known about the changes in erythrocyte membrane during ultradistance running.

We used the Badwater ultramarathon, a 216-km single-stage footrace, as an exercise model that provides a high level of exercise-induced hemolysis to elucidate the chronology of erythrocyte membrane damage during such extreme events. We tested the hypotheses 1) that structural changes of the membrane increase the susceptibility of erythrocytes to hemolysis, and 2) that erythrocyte destruction is maximal during the early phase of the race because of the relatively older cell population at the start of the event.

MATERIALS AND METHODS

Subjects and protocol. Six male nonsmoking experienced ultramarathon runners (mean ± SD: age 53.8 ± 10.4 yr, height 175.8 ± 11.1 cm, body mass: 75.9 ± 8.4 kg) were fully informed about the nature and risks involved with participation in this study approved by the Ethical Committee of the University of Münster, Germany, and provided written informed consent. All subjects ran regularly 40 to 100 km/wk for more than 2 yr and had participated in at least 2 ultramarathon races longer than 100 km previously. The study included running the Badwater Ultramarathon.

The Badwater Ultramarathon is a continuous 216-km, one-stage race consisting of 110.4 flat km, 73.6 uphill km (incline approximately +4,000 m total) and 32 downhill km (slope approximately −1,400 m total), which takes place annually in mid-July. Air temperatures during the race are up to 55°C. Time limit for finishing the race, as set by the race organizer, is 60 h (www.badwaterultra.com).

Blood sampling. Blood samples were collected (20 ml) from the antecubital vein 1 h before the event (prerace), after about 21, 42, 84, and 126 km, and immediately after terminating the race at 216 km, in sterile tubes, some of them containing EDTA. All blood samples were obtained within ~2 min after the subject being seated. Samples were stored and transported at 4–8°C and processed within 6 h.

Hematocrit and hemoglobin concentrations as well as leukocyte count (Coulter Counter) were determined from the samples in an accredited medical laboratory in Las Vegas, NV (Quest Diagnostics). Serum haptoglobin concentration was measured nephelometrically (Dade Behring, Germany). Furthermore, erythropoietin concentration was measured using a chemiluminescence immunoassay (Nichols Advantage, Nichols Institute Diagnostics). Total protein was analyzed to calculate hemoconcentration after exercise using the Biuret method (Automatic Analyser 747, Hitachi, Japan).

For further analyses, EDTA-blood samples were centrifuged at 600 rpm for 15 min to separate the plasma and blood cells. Samples were tested and processed immediately for hemolysis and preparation of

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
erythrocyte ghost membranes. Processed samples were kept at −80°C and transported back to our laboratory in the United Kingdom.

**Urine samples.** Urine samples were collected after each blood sample to detect the presence of hemoglobinuria using the HPLC (Agilent HP with diode array detector).

**Determination of hemolysis curve.** The osmotic fragility assays were performed in phosphate buffer at pH 7.4 solutions at increasing tonicity ranging from 0 to 250 mOsm. The hemolysis experiments were carried out in 1 ml of fresh erythrocyte suspensions with hematocrit of 0.33% and incubated for 30 min. Following incubation, the suspensions were centrifuged at 6,000 rpm for 5 min, and the concentrations of hemoglobin released into the supernatant were calculated from absorbance readings taken at 577 nm (oxygenhemoglobin, $E_{577} = 14.6$ mM$^{-1} \cdot$cm$^{-1}$). Hemolysis concentrations were normalized, and the half-maximal values ($H_{50}$) were calculated using the GraphPad Prism 4.0.

**Preparation of erythrocyte ghosts.** Unless otherwise stated, all operations were carried out on ice. Erythrocytes were washed three times in PBS with 1 mM of EDTA. White cells were removed by thorough aspiration of the buffy coat after the first centrifugation. Following subsequent centrifugation, the remaining white cells were carefully aspirated again. Erythrocyte ghosts were prepared according to method described by Dodge et al. (10). After the final wash with isotonic buffer, the erythrocytes were lysed in Dodge buffer at pH 8.0 with addition of phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, at a final concentration of 0.1 M. The lysate was then spun at 12,000 rpm for 20 min at 4°C. The pellet recovered was resuspended in the lysis buffer. The process was repeated four times to remove the remaining hemoglobin and excess PMSF. Prepared ghosts were stored at −80°C.

**SDS gel separation.** Prepared ghosts were treated with an equal volume of solubilization buffer containing 0.125 M Tris - HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, heat denatured at 95°C for 5 min, and subjected to SDS-PAGE using the discontinuous Laemmli system (20). The slab gels were composed of a 12% separating gel and an 8% polyacylamide stacking gel. Spectrins were identified using a standard molecular mass marker of a molecular mass of 220–240 kDa (14). The percentage of membrane proteins was calculated by integrating the Coomasie blue stain profile on the densitometric scans (ImageJ 1.32j, National Institutes of Health). Three independent repeats were carried out to ensure consistency with average variability coefficient of ± 1.65% between tests.

**Data processing and statistical analysis.** The plasma volume changes were estimated using the total serum protein based on the method described by Ohira et al. (24). The hemolysis index of erythrocytes was calculated from the concentrations of serum haptoglobin and hemoglobin, according to the method described by Waks et al. (35). Total loss of erythrocytes was estimated based on changes in plasma protein concentration and hematocrit as described by Reinhart et al. (26).

All data are described as means and SD. Within-subject effects and effect size eta-squared were analyzed using an ANOVA model for repeated measurements, t-tests, and Bonferroni adjustment. The level of significance of $P < 0.05$ was chosen for all statistical comparisons. All data were analyzed using GraphPad Prism program and graph package (V4.0, GraphPad, San Diego, CA).

**RESULTS**

Runners finished the 216-km race after 48.27 ± 9.03 h. Hematocrit was higher ($P < 0.05$, eta squared $> 0.698$) at 21, 42, and 84 km than after 216 km. Hemoglobin was increased ($P < 0.05$, eta squared $= 0.760$) after 42 km compared with the 216-km level. Mean corpuscular hemoglobin (MCH) was lower ($P < 0.05$, eta squared $> 0.741$) after 84 km than after 126 and 216 km; MCH concentration was lower ($P < 0.05$, eta squared $= 0.541$) than at the end of the race. There was a main effect on mean corpuscular volume (MCV) ($P = 0.013$, eta squared $= 0.489$) and erythrocytes ($P = 0.006$, eta squared $= 0.53$); however, no partial differences could be confirmed. Total protein indicated a decrease ($P < 0.05$, eta squared $> 0.793$) in plasma volume to 94.26 ± 3.28% during the initial 42 km and a slight overhydration of 101.91 ± 6.11% at the end of the run. Haptoglobin decreased ($P < 0.05$, eta squared $> 0.883$) almost continuously during the initial 84 km (Table 1). Consideration of haptoglobin and plasma volume indicated intravascular loss of erythrocytes throughout the initial 84 km of the race ($P < 0.05$, eta squared $> 0.922$) and a gain ($P < 0.05$, eta squared $> 0.762$) in erythrocytes during the final 132 km (Fig. 1). A total loss of erythrocytes after 21 km ($P < 0.05$, eta squared $= 0.801$) was almost compensated by a gain toward the end of the race ($P < 0.05$, eta squared $= 0.651$) as shown in Fig. 2, combined with a main effect on erythropoietin ($P = 0.025$, eta squared $= 0.452$). No partial differences in erythropoietin could be confirmed. However, erythropoietin tended ($P = 0.015$, eta squared $= 0.849$) to be higher after 84 km than after 21 km. Hemoglobinuria did not occur. Leukocytes were significantly elevated following 84, 126, and 216 km from the prerace level (Table 1).

Susceptibility to osmotic hemolysis was highest after 42 km ($P < 0.01$, eta squared $= 0.974$) with almost identical ($P > 0.05$) $H_{50}$ values prerace and at the end of the race (Fig. 3). Spectrins were reduced throughout the race compared with prerace level and showed a minimum after 42 km ($P < 0.001$).

<table>
<thead>
<tr>
<th>Running Distance, km</th>
<th>Prerace</th>
<th>21</th>
<th>42</th>
<th>84</th>
<th>126</th>
<th>216</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit, %</td>
<td>41.54±1.89</td>
<td>42.96±2.02</td>
<td>42.94±2.45</td>
<td>42.4±3.08</td>
<td>41.62±3.73</td>
<td>39.54±3.69&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>14.18±0.66</td>
<td>14.68±0.75</td>
<td>14.7±0.81</td>
<td>14.48±1.34</td>
<td>14.46±1.26</td>
<td>13.70±1.34&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>92.48±4.28</td>
<td>92.32±4.69</td>
<td>92.14±4.54</td>
<td>91.94±4.26</td>
<td>91.8±4.20</td>
<td>91.90±4.47</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>31.60±1.75</td>
<td>31.62±1.85</td>
<td>31.62±1.78</td>
<td>31.44±1.70</td>
<td>31.94±1.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.86±1.71&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCHC, g/dl</td>
<td>34.06±0.51</td>
<td>34.28±0.39</td>
<td>34.3±0.39</td>
<td>34.35±0.25</td>
<td>34.83±0.41</td>
<td>34.64±0.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Erythrocytes, 10&lt;sup&gt;6&lt;/sup&gt;/μl</td>
<td>4.49±0.31</td>
<td>4.66±0.40</td>
<td>4.67±0.40</td>
<td>4.62±0.50</td>
<td>4.54±0.51</td>
<td>4.31±0.46</td>
</tr>
<tr>
<td>Total plasma protein, g/l</td>
<td>69.67±3.93</td>
<td>74.00±5.96</td>
<td>73.80±6.76</td>
<td>74.60±8.01</td>
<td>71.40±5.90</td>
<td>68.20±4.32</td>
</tr>
<tr>
<td>Haptoglobin, g/l&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87±0.24</td>
<td>0.71±0.19</td>
<td>0.59±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.46±0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64±0.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leukocytes, 10&lt;sup&gt;3&lt;/sup&gt;/μl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.86±1.18</td>
<td>7.22±1.35</td>
<td>8.42±2.88</td>
<td>10.6±4.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.6±1.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.04±2.38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Erythropoietin, mU/ml&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.01±5.16</td>
<td>10.40±2.84</td>
<td>15.92±8.12</td>
<td>18.69±7.51</td>
<td>15.25±7.39</td>
<td>31.01±22.57</td>
</tr>
</tbody>
</table>

Table 1. Hematological measures and total protein after given running distances

Values are means ± SD. *Values are corrected for %blood volume changes (Δplasma volume, %) according to Ohira et al. (24). MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, MCH concentration. Significantly different: a:vs. prerace; b:vs. 21 km; c:vs. 42 km; d:vs. 84 km.
eta squared = 0.968) and apparent recovery thereafter (Figs. 4 and 5). Spectrins and susceptibility to osmotic hemolysis were highly correlated (Fig. 6).

DISCUSSION

Use of an ultraendurance model has enabled us to demonstrate for the first time that alterations in membrane proteins increased the susceptibility of erythrocytes to hemolysis occurring particularly in the early phase of such events. Our results are consistent with damage occurring primarily to the older cohort of erythrocytes, which are subsequently removed and replaced by younger cells.

Prerace hemoglobin and hematocrit values were within the normal ranges, suggesting the runners were well hydrated and not suffering from acute anemia. Hematocrit and total protein values indicated hemoconcentration during the initial 84 km and subsequent hemodilution toward the end of the race similar to observations during a continuous 6-day track race (13). Decrease in plasma haptoglobin concentrations has also been observed following a 100-km race (8) and during the initial part of a 6-day track race (13), suggesting intravascular hemolysis during ultraendurance events. Intravascular destruction of erythrocytes releases free hemoglobin forming stable complexes with haptoglobin by a 1:1 molar ratio (35). If the valency of haptoglobin is exceeded, hemoglobinuria occurs. The hemoglobin-binding capacity of haptoglobin had not been exceeded in the present study. This enabled us to estimate the intravascular loss of erythrocytes. Poortmans and Haralambie (25) reported a loss of about 2 x 10^9 cells/l due to intravascular hemolysis after a 100-km run, which is comparable with our findings after 84 km under different physiological and environmental conditions. The total erythrocyte loss calculated also indicates high losses during the initial period of the race and a gain in the later part of the race. Reinhart et al. (26) reported a maximum loss of 6% of erythrocytes following a 100-km race, which is comparable to the estimated loss in this study after 84 km (8.8% loss). This substantial amount of erythrocyte loss is attributed to preferential filtration of old and damaged cells.
from the circulation rather than hemolysed intravascularly, explaining the conspicuous absence of hemoglobinuria (26). The estimated erythrocyte losses shown in Figs. 1 and 2 strongly support the hypothesis that newer red blood cells have entered the circulation particularly during the second half of the race. However, an acute phase reaction of haptoglobin cannot be excluded as indicated by an increase in white blood cell count in the later part of the race. Nevertheless, increase ($P < 0.05$) in MCH seen after 126 and 216 km indicated presence of hyperchromic, and thus younger, cells. No significant changes in MCV were observed during the race even though the calculated increase in cell production was similar to erythropoiesis found in iron deficiency and microcytic anemia (4), which suggests loss of iron from the hemolysed erythrocytes. The tendency of a higher ($P = 0.015$, eta squared = 0.849) erythropoietin after 84 km compared with 21 km also suggests stimulation of the erythrocyte production. Unfortunately, we did not measure the reticulocyte percentage, which would undoubtedly further elucidate an increased production of new cells.

Hemolysis curves indicated that the erythrocyte membrane is more prone to hemolysis after 21, 42, and 84 km with a buildup effect from 21 to 42 km and less hemolysis during the subsequent phases of the race (Fig. 3). Densitometric scan of the SDS-PAGE-separated membrane proteins (Figs. 4 and 5) shows a maximum reduction of spectrins during the early part of the race ($P < 0.001$), consistent with the observed osmotic fragility and blood losses. The apparent recovery observed after 126 km ($P < 0.05$) and 216 km ($P < 0.001$) is indicative of the presence of more deformable cells. Shape and deformability of erythrocytes are maintained by spectrins; therefore, impaired deformability promotes removal of damaged and aged erythrocytes from circulation by spleen, forming a more homogenous distribution of erythrocytes with more younger erythrocytes (28).

The high negative correlation between spectrins and susceptibility to osmotic hemolysis (Fig. 6) suggests that the exercise-induced hemolysis seen in the present study reflects the structural changes of erythrocyte membrane related to membrane cytoskeletal proteins. The present exercise-induced changes in spectrin are similar to those found after a marathon run (18) and after 10 – 35 min of exercise in the severe intensity domain (2) and in inherited anemic diseases like Sphaerocytosis and Fanconi’s anemia and after in vitro oxidative stress (22, 29, 31, 32). Spectrins located on the inner surface of erythrocytes are most vulnerable to oxidative attacks by reactive oxygen species compared with membrane lipids, which form the integral part of the membrane bilayer. Treating whole erythrocyte with H$_2$O$_2$ in vitro showed alterations of the membrane spectrin complex, which could be inhibited by pretreatment of cells with carbon monoxide, which interferes with hemoglobin peroxidative reactions (32). Decrease in membrane spectrins and rapid degradation of the modified proteins could provide a sensitive marker of cellular reactive oxygen species activities during exercise. Oxidation of spectrin has been shown to decrease the overall thickness of erythrocyte membrane and increases with the age of the cells (7). There is evidence for an increase in oxidatively modified hemoglobin during endurance exercise, which initiates the free radical generation and subsequent protein oxidation (34). We are convinced that the observed decrease in membrane spectrins have taken place in vivo during the race and not by proteolytic activity of protease enzymes during treatments ex vivo because the ghost membranes were treated with PMSF to inhibit endogenous proteases enzymes and prevent any further proteolytic modification. Other noncytoskeletal membrane proteins such as band 3, which involves in the anion exchange and cell functions, may also be oxidatively modified during a prolonged exercise and thus warrant further investigation.

The losses in erythrocytes during the initial 84 km suggest acceleration of the erythrocyte ageing process and selective degradation of modified membrane proteins by intracellular proteinases, proteases, and peptidases (9). Rapid removal of damaged erythrocytes is combined with renewal by young cells. New blood cells are more robust, more deformable under shear stress, and less susceptible to hemolysis than older cells (23). Apparent recovery observed during the later part of the race seems to reflect a higher level of antioxidant enzyme activity boosted by an increased fraction of younger erythrocytes in the circulation (26, 28).

In conclusion, the present study is the first to demonstrate that exercise-induced hemolysis occurs particularly in the early phase of an ultraendurance race because of a relatively older cell population. The old and damaged cells are preferentially removed from the circulation by spleen filtration rather than

---

**Fig. 5.** Image analyzer measurements of the percentage loss of membrane spectrin bands separated using the SDS-PAGE system ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

**Fig. 6.** Correlation between percentage of spectrins during the race and hemolysis $H_{so}$. 

---

*J Appl Physiol • VOL 102 • FEBRUARY 2007 • www.jap.org*
hemolyzed intravascularly. It supports recent suggestions that exercise-induced hemolysis reflects in vivo alterations in erythrocyte membrane proteins, especially in the α- and β-spectrins, similar to those found after severe relatively short lasting exercise and in hereditary anemic diseases like Sphaeroctytosis and Fanconi’s anemia, resulting in an increased susceptibility of the cells to physical and/or chemical stress. Decrease in membrane spectrins and rapid degradation of the modified proteins may not only provide a sensitive marker of cellular reactive oxygen species activities during exercise but may also serve as a signal for red blood cell renewal and thus exercise-induced erythropoiesis. (30)

ACKNOWLEDGMENTS

We gratefully acknowledge all runners participating in this study; Lawrence A. Golding and Jane Conway-Klaassen, University of Las Vegas, Las Vegas, NV, for generous support with equipment and laboratory access; Brandon Reeder, University of Essex, United Kingdom, for running the HPLC system, and all members of the runex123-team.

REFERENCES