Hypoxic exercise training causes erythrocyte senescence and rheological dysfunction by depressed Gardos channel activity

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Mao T, Fu L, Wang J. Hypoxic exercise training causes erythrocyte senescence and rheological dysfunction by depressed Gardos channel activity. J Appl Physiol 111: 382–391, 2011. First published May 5, 2011; doi:10.1152/japplphysiol.00096.2011.—Despite enhancing cardiopulmonary and muscular fitness, the effect of hypoxic exercise training (HE) on hemorheological regulation remains unclear. This study investigates how HE modulates erythrocyte rheological properties and further explores the underlying mechanisms in the hemorheological alterations. Twenty-four sedentary males were randomly divided into hypoxic (HE; n = 12) and normoxic (NE; n = 12) exercise training groups. The subjects were trained on 60% of maximum work rate under 15% (HE) or 21% (NE) O2 condition for 30 min daily, 5 days weekly for 5 wk. The results demonstrated that HE 1) downregulated CD47 and CD147 expressions on erythrocytes, 2) decreased actin and spectrin contents in erythrocytes, 3) reduced erythrocyte deformability under shear flow, and 4) diminished erythrocyte volume changed by hypotonic stress. Treatment of erythrocytes with H2O2 that mimicked in vivo oxidative stress resulted in the cell shrinkage, rigidity, and phosphatidylserine exposure, whereas HE enhanced the eryptotic responses to H2O2. However, HE decreased the degrees of clotrimazole to blunt ionomycin-induced shrinkage, rigidity, and cytoskeleton breakdown of erythrocytes, referred to as Gardos effects. Reduced erythrocyte deformability by H2O2 was inversely related to the erythrocyte Gardos effect on the rheological function. Conversely, NE intervention did not significantly change resting and exercise erythrocyte rheological properties. Therefore, we conclude that HE rather than NE reduces erythrocyte deformability and volume regulation, accompanied by an increase in the eryptotic response to oxidative stress. Simultaneously, this intervention depresses Gardos channel-modulated erythrocyte rheological functions. Results of this study provide further insight into erythrocyte senescence induced by HE.

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erythrocyte senescence declined Gardos channel activity (32), despite the increases in the activities of nonselective cation channels observed previously (5, 19, 30). Whether exercise training under hypoxic exposure influences erythrocyte aging process, eryptotic response to oxidative stress, and rheological properties remains unclear. Hence, we hypothesize that hypoxic exercise training impairs erythrocyte rheological functions by suppressing Gardos channel activity.

To answer the above questions, this study compared how normoxic and hypoxic exercise training programs for 5 wk affected erythrocyte senescence in blood, oxidative stress-induced eryptosis, and Gardos channel-modulated erythrocyte rheological properties.

METHODS

Subjects and Interventions

The Ethics Committee of Chang Gung Memorial Hospital approved the study protocol, which was consistent with institutional guidelines. Twenty-four healthy subjects who were nonsmokers, nonusers of medication/vitamins, and cardiopulmonary/hematological risk-free were recruited from Chang Gung University, Taiwan. No subject had engaged in any regular physical activity (i.e., exercise frequency ≤1 time weekly, duration <20 min) or had been exposed to high altitudes (≥altitude of 3, 000 m) for ≥1 yr before the experiment. All subjects gave informed consent after the experimental procedures had been explained to them. Subjects were randomly divided into hypoxic exercise training (HE; n = 12) and normoxic
exercise training (NE; \(n = 12\)) groups. The two groups did not significantly vary in terms of anthropometric data. Subjects were then instructed to fast for \(\geq 8\) h and to refrain from exercise for \(\geq 24\) h before the testing sessions. All subjects arrived at the testing center at 9:00 AM to eliminate any possible diurnal effect.

The subjects were trained on a bicycle ergometer (Corvial 400; Lode) at 60% of maximal work rate under 15% \(\text{O}_2\) (HE) or 21% \(\text{O}_2\) (NE) for 30 min daily, 5 days weekly for 5 wk in an air-conditioned normobaric hypoxia chamber (36). The \(\text{O}_2\) concentrations of 15 and 21% correspond to altitudes of \(-2,733\) m and sea level, respectively. The hypoxia chamber was maintained at a temperature of \(22 (\pm 0.5)\degree \text{C}\) and a relative humidity of \(60 (\pm 5)%\); a \(\text{CO}_2\) scrubber eliminated \(\text{CO}_2\) from the air (\(\leq 3,500\) ppm; Colorado Mountain Room; Ref. 33).

Graded Exercise Test

Each subject carried out graded exercise tests (GXTs) 48 h before and 48 h after the intervention. The GXT was conducted using a bicycle ergometer (Corvial 400; Lode; Ref. 36). The test comprised 2 min of unloaded pedaling; loading was then increased by 20–30 W every 3 min until exhaustion [i.e., progressive exercise to maximal \(\text{O}_2\) consumption (\(\text{VO}_2_{\text{max}}\))]. Heart rate (HR), blood pressure (BP), minute ventilation (\(\text{VE}\)), oxygen consumption (\(\text{VO}_2\)), and \(\text{CO}_2\) production (\(\text{VCO}_2\)) were measured using an automated system (MasterScreen CPX; Cardinal Health; Ref. 36). Arterial \(\text{O}_2\) saturation was monitored by finger pulse-oximetry (model 9500; Nonin Onyx); blood lactate (\(\text{Lac}_a\)) was determined using an automated analyser (i-STAT). The \(\text{VO}_2\)peak was defined by the following criteria: (1) the level of \(\text{VO}_2\) increased by 2 ml kg\(^{-1}\) min\(^{-1}\) (over \(\geq 2\) min; 2) HR exceeded its predicted maximum; 3) the respiratory exchange ratio exceeded 1.2; and 4) the venous lactate concentration exceeded 8 mM (1).

Hypoxic Exercise Test and Blood Sample Collection

Each subject performed a hypoxic exercise test 24 h before and on 24 h after intervention. The hypoxic exercise test (HET) on a bicycle ergometer (Corvial 400; Lode) required 50 W of warm-up for 3 min, increase of work rate to 100 W of continuous exercise for 20 min, and then recovery to 50 W of cool-down for 3 min. During the test, the \(\text{O}_2\) concentration was set to 12%, which corresponds to altitudes of \(-4,460\) m (36).

Initially before and immediately after HET, blood samples were collected from an antecubital vein using a clean venipuncture (21-gauge needle) under controlled venous stasis at 40 Torr. The first 2 ml of blood were discarded, and the remaining blood was used to evaluate hematological parameters and erythrocyte functions. Hematologic parameters, i.e., erythrocyte count, hemoglobin, and hematocrit (Hct), were determined using an automatic blood cell counter (Sysmax SF-3000; GMI).

Erythrocyte Isolation

Twenty milliliters of blood samples were transferred to polystyrene tubes that contained sodium citrate (3.8 g/dl; 1–9 vol blood; Sigma). Erythrocytes were isolated from whole blood by centrifugation (1,000 \(g\) for 15 min) at room temperature, followed by three washing steps in Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM MgSO\(_4\), 32 mM HEPES, 5 mM glucose, and 1 mM CaCl\(_2\) (osmolality = 300 mOsm/kg\(_2\)H\(_2\)O; pH 7.4; Sigma). Finally, the erythrocyte count was adjusted using Ringer solution to \(1 \times 10^8\) cells/\(\mu\)l.

Reticulocyte Count

The erythrocyte suspensions (\(1 \times 10^8\)cells/\(\mu\)l) were incubated with saturating concentrations of monoclonal anti-human CD71 antibody conjugated with phycoerythrin (DB Pharmingen) and Vybrant DyeCycle Green (Invitrogen) in the dark for 30 min at 37\degree\text{C} and then washed twice using Ringer solution. Finally, the count of CD71-positive reticulocytes obtained from 50,000 erythrocytes was determined using a two-color FACScan flow cytometer (Becton Dickinson; Ref. 7).

Senescence-Related Molecules on Erythrocyte

Erythrocyte suspensions (\(1 \times 10^4\) cells/\(\mu\)l) were incubated with saturating concentrations (10 \(\mu\)g/ml) of monoclonal anti-human CD47 antibody (BioLegend) or monoclonal anti-human CD147 antibody (eBioscience) that was conjugated with FITC in the dark for 30 min at 37\degree\text{C}. Erythrocytes that had been treated with FITC-conjugated anti-rabbit IgG control antibody were utilized to correct for background fluorescence (eBioscience). Finally, the mean fluorescence intensity (MFI) of FITC was determined using a two-color FACScan flow cytometer (Becton Dickinson; Ref. 7).
intensity (MFI) obtained from 10,000 erythrocytes was measured using FACScan flow cytometry (Becton Dickinson).

**Rheological Properties of Gardos Channel-Modulated Erythrocytes**

**Erythrocyte volume.** Erythrocyte volumes under isotonic (300 mOsm/kgH$_2$O) and hypotonic (150 mOsm/kgH$_2$O) solutions were determined by FACScan flow cytometry (Becton Dickinson), as described elsewhere (Fig. 2A) (37). Briefly, a time/forward scatter (FSC) acquisition plot, in which 10 identical regions by interval of 10 s, was created to acquire events based on time over a period of 100 s. This dot plot revealed dots moving from left to right, assuming time is the X parameter. The sample tube containing 0.5 ml of erythrocyte suspension ($1 \times 10^8$ cells/$\mu$L) was installed at the sample injection port. The sample tube containing 0.5 ml of erythrocyte suspension ($1 \times 10^8$ cells/$\mu$L) was installed at the sample injection port. The first region recorded erythrocyte volume, by calculated geo mean in FSC, in isotonic Ringer solution. After the region elapsed during acquisition, the sample tube was removed without ending acquisition, and then 0.5 ml of distilled water was spiked to the erythrocyte suspension, leading to the change of the solution’s osmolality from 300 to 150 mOsm/kgH$_2$O. Thereafter, the sample tube with hypotonic solution was reinstalled at the port, and acquisition was again continued up to the 10th region. Finally, the FSC geo mean of erythrocyte in hypotonic solution was recorded in the least region. Results were expressed as the percentage of erythrocyte volume changed by hypotonic stress by using the following formula: 

$$\text{EI} = \frac{\text{FSC geo mean of erythrocyte treated with ionomycin}}{\text{FSC geo mean of erythrocyte treated with 0.1% DMSO}} \times 100.$$ 

For examined Gardos channel activity, 5 $\mu$M clotrimazole [as a Gardos K$^+$ channel blocker (18) dissolved in solvent DMSO; Sigma] or 0.1% DMSO (as a vehicle; Sigma) was added to the erythrocyte suspension, which was then warmed to 37°C for 15 min. Following incubation, the reagent-treated erythrocytes were treated again with 0.1 $\mu$M ionomycin (as a Ca$^{2+}$ ionophore; Sigma) for 1 h in 37°C. The degrees of erythrocyte volume modulated by Gardos channel were then estimated by using the following formula: 

$$\text{EI} = \frac{\text{FSC geo mean of erythrocyte treated with clotrimazole plus ionomycin subtracted by FSC geo mean of erythrocyte treated with ionomycin}}{\text{FSC geo mean of erythrocyte treated with 0.1% DMSO subtracted by FSC geo mean of erythrocyte treated with ionomycin}} \times 100.$$ 

**Erythrocyte deformability.** Erythrocyte deformability was determined at various fluid shear stresses (0–35 Pa) by laser diffraction analysis using a slit-flow ektacytometer (Fig. 2B) (RheoScan-D system; InCyto; Refs. 3, 31). The basic apparatus consisted with a laser, CCD video, screen, and pressure-driven slit rheometry. The diode laser (650 nm, 5 mM) and a CCD camera combined with a frame grabber were used to obtain a laser-diffraction pattern. In this experiment, the washed erythrocytes were resuspended in a solution of 0.14 mM polyvinylpyrrolidone (molecular weight = 360,000; Sigma) at the optimal Hct of 1.0. When the erythrocyte sample flowed through the slit-flow chamber, a laser beam emitted from the laser diode traversed the cell, and then the diffraction pattern projected on the screen, which was captured by CCD video camera. The elongation index (EI) as a measurement of erythrocyte deformability was determined from an iso-intensity curve in the diffraction pattern by using an ellipse-fitting program (31). The EI is defined as 

$$\text{EI} = \frac{L - W}{L + W},$$ 

where L and W are the length and width of the diffraction pattern.

The degrees of erythrocyte deformability modulated by Gardos channel were calculated by using the following formula: 

$$\text{EI} = \frac{\text{EI of erythrocyte treated with clotrimazole plus ionomycin at 3 Pa subtracted by EI of erythrocyte treated with ionomycin at 3 Pa}}{\text{EI of erythrocyte treated with 0.1% DMSO at 3 Pa subtracted by EI of erythrocyte treated with ionomycin at 3 Pa}} \times 100.$$
Cytoskeletal proteins in erythrocytes. One milliliter of commercial fixation buffer (eBioscience) was added to 1 × 10⁷ purified erythrocytes in darkness for 30 min at room temperature. Following fixation, the cells were twice washed and permeabilized using a commercial permeabilization wash buffer (eBioscience); they were then incubated in darkness for 1 h at 37°C with a saturation concentration (10 μg/ml) of anti-spectrin α + β (Sigma) monoclonal antibody. After being washed again with the permeabilization wash buffer two times, the fixed and intracellularly labeled erythrocytes were reincubated with goat anti-rabbit IgG FITC conjugated secondary antibody (eBioscience) in 1% BSA in PBS (Sigma) for 1 h at 37°C. The sample of secondary antibodies given alone was used as negative controls. Additionally, Alexa 488-conjugated phalloidin (Sigma) was used to stain F-actin filaments in erythrocytes. Finally, the MFI obtained from 10,000 erythrocytes was measured using FACSscan flow cytometry (Becton Dickinson).

The degree of spectrin α + β or F-actin expression in erythrocyte modulated by Gardos channel was calculated by using the following formula: [(MFI of spectrin α + β or F-actin in erythrocyte treated with clotrimazole plus ionomycin subtracted by MFI of spectrin α + β or F-actin in erythrocyte treated with ionomycin) divided by (MFI of spectrin α + β or F-actin in erythrocyte treated with 0.1% DMSO subtracted by MFI of spectrin α + β or F-actin in erythrocyte treated with ionomycin)] multiplied by 100.

H₂O₂-Induced Eryptosis and Rheological Dysfunction

Erythrocyte samples (1 × 10⁴ cells/μl) obtained from resting and immediately after HET were exposed to 0.5 and 5 mM H₂O₂ (Sigma) in Ringer solution (Sigma) for 1 h at 37°C, respectively. Eryptosis was analyzed by exposure of PS. This assay is performed based on changes in the cell surface exposure of PS during the early stages of eryptosis and the selective affinity of annexin V for the phospholipids (9). Erythrocyte PS exposure was detected by FACSscan flow cytometry, using commercial annexin V Cy5 (eBioscience). Additionally, the changes in erythrocyte volume and deformability induced by H₂O₂ were determined using FACSscan cytometer (Becton Dickinson) and slit-flow ectacytometer (RheoScan-D system; InCyto), respectively.

Plasma Erythropoietin

Five milliliters of blood samples were transferred into polypropylene tubes containing EDTA (final concentration of 4 mM). Cell-free plasma was obtained by centrifugation at 3,000 g for 10 min at 4°C. Plasma erythropoietin (Epo) concentrations were determined by ELISA by using a commercially available kit (IBL).

<table>
<thead>
<tr>
<th></th>
<th>HE Pre</th>
<th>HE Post</th>
<th>NE Pre</th>
<th>NE Post</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>22 ± 1</td>
<td>22 ± 1</td>
<td>22 ± 1</td>
<td>22 ± 1</td>
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<tr>
<td>Height, cm</td>
<td>173 ± 1</td>
<td>172 ± 1</td>
<td>172 ± 1</td>
<td>172 ± 1</td>
</tr>
<tr>
<td>Weight kg</td>
<td>65.9 ± 2.5</td>
<td>65.7 ± 2.5</td>
<td>68.1 ± 2.2</td>
<td>66.9 ± 2.2</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21.6 ± 0.4</td>
<td>22.0 ± 0.7</td>
<td>22.6 ± 0.6</td>
<td>22.1 ± 0.6</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>127 ± 2</td>
<td>131 ± 3</td>
<td>128 ± 3</td>
<td>129 ± 3</td>
</tr>
<tr>
<td>HR, beat/min</td>
<td>193 ± 1</td>
<td>194 ± 2</td>
<td>193 ± 2</td>
<td>194 ± 1</td>
</tr>
<tr>
<td>VO₂, ml·min⁻¹·kg⁻¹</td>
<td>50.3 ± 1.3</td>
<td>58.5 ± 0.9⁺</td>
<td>50.9 ± 2.5</td>
<td>55.9 ± 1.8⁺</td>
</tr>
<tr>
<td>Work rate, watt</td>
<td>193 ± 6</td>
<td>228 ± 5⁺</td>
<td>193 ± 5</td>
<td>216 ± 7⁺</td>
</tr>
</tbody>
</table>

Values are means ± SE. NE, normoxic exercise training group; HE, hypoxic exercise training group; Pre, pretraining; Post, posttraining. BMI, body mass index; HR, heart rate; MAP, mean arterial pressure; VO₂, minute ventilation; VO₂, oxygen consumption. Experimental results were analyzed by 2 (groups: HE, NE) × 2 (time sample points: Pre, Post) repeated-measures ANOVA and Bonferroni’s post hoc test. *P < 0.05, Pre vs. Post; †P < 0.05, HE vs. NE.

RESULTS

Cardiopulmonary Fitness

Table 1 summarizes anthropometry data and exercise performance before and after the intervention in the HE and NE groups. The two experimental groups did not vary significantly in cardiopulmonary parameters at the beginning of the study. Following 5 wk of interventions, the HE and NE subjects exhibited increased work rate, VE, and VO₂ at maximal exercise performance. However, the HE group revealed a greater improvement in aerobic capacity than the NE did (Table 1, P < 0.05).

Hematologic Parameters and Senescence-Related Molecules on Erythrocyte

A bout of HET significantly increased erythrocyte and reticulocyte counts, Hct value, and hemoglobin level in blood and was accompanied by an elevated plasma Epo concentration (Table 2, P < 0.05). However, 5 wk of either HE or NE did not alter the values of reticulocyte count and Epo concentration in responses to HET (Table 2). Additionally, HE and NE interventions did not significantly change in resting and exercise mean corpuscular hemoglobin concentration levels (Table 2).

At the beginning of this study, HET markedly decreased the levels of CD47 and CD147 on erythrocytes (Table 2; P < 0.05). After 5 wk of interventions, HE down-regulated CD47 and CD147 expressions on erythrocytes at rest (Table 2; P < 0.05) and unaltered the levels of the three senescence-related molecules on erythrocytes following HET (Table 2). In
Table 2. Effects of normoxic and hypoxic exercise trainings on erythrocyte characteristics and serum erythropoietin concentration

<table>
<thead>
<tr>
<th></th>
<th>HE PreR</th>
<th>HE PreE</th>
<th>HE PostR</th>
<th>HE PostE</th>
<th>NE PreR</th>
<th>NE PreE</th>
<th>NE PostR</th>
<th>NE PostE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocyte, 10^6/μl</td>
<td>4.61 ± 0.11</td>
<td>5.03 ± 0.11*</td>
<td>4.57 ± 0.08</td>
<td>4.83 ± 0.08*</td>
<td>4.65 ± 0.06</td>
<td>4.92 ± 0.07*</td>
<td>4.64 ± 0.07</td>
<td>4.84 ± 0.07*</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>13.9 ± 0.2</td>
<td>14.8 ± 0.2*</td>
<td>13.6 ± 0.2</td>
<td>14.3 ± 0.3*</td>
<td>14.1 ± 0.2</td>
<td>14.9 ± 0.2*</td>
<td>14.0 ± 0.2</td>
<td>14.7 ± 0.3*</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>40.8 ± 0.4</td>
<td>43.7 ± 0.4*</td>
<td>39.6 ± 0.5</td>
<td>42.2 ± 0.5*</td>
<td>41.2 ± 0.5</td>
<td>43.9 ± 0.6*</td>
<td>41.2 ± 0.6</td>
<td>43.3 ± 0.7*</td>
</tr>
<tr>
<td>MCHC, g/dl</td>
<td>34.1 ± 0.2</td>
<td>33.9 ± 0.2</td>
<td>34.3 ± 0.2</td>
<td>33.9 ± 0.1</td>
<td>34.2 ± 0.2</td>
<td>33.9 ± 0.2</td>
<td>34.0 ± 0.2</td>
<td>33.9 ± 0.1</td>
</tr>
<tr>
<td>Reticulocyte, 10^3/μl</td>
<td>4.12 ± 0.35</td>
<td>6.21 ± 0.54*</td>
<td>4.72 ± 0.35</td>
<td>5.45 ± 0.54</td>
<td>4.52 ± 0.45</td>
<td>6.71 ± 0.63*</td>
<td>5.01 ± 0.62</td>
<td>5.87 ± 0.47</td>
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Values are means ± SE. Experimental results were analyzed by 2 (groups: HE, NE) × 4 (time sample points: PreR, PreE, PostR, PostE) repeated-measures ANOVA and Bonferroni’s post hoc test. *P < 0.05, PreR or PreE vs. PostR or PostE; †P < 0.05, PreR or PreE vs. PostR or PostE; ‡P < 0.05, HE vs. NE.

Table 3. Effects of normoxic and hypoxic exercise trainings on erythrocyte volume changed by osmotic stress

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<thead>
<tr>
<th></th>
<th>HE</th>
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<th>HE</th>
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<th>HE</th>
<th>HE</th>
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</thead>
<tbody>
<tr>
<td>Cell volume in 300 mOs/m/kgH2O, geometric mean in forward scatter</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>405 ± 7</td>
<td>410 ± 8</td>
<td>385 ± 6†</td>
<td>380 ± 7†</td>
<td>406 ± 7</td>
<td>410 ± 8</td>
<td>400 ± 5</td>
<td>402 ± 6</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>93 ± 3</td>
<td>89 ± 3</td>
<td>91 ± 3</td>
<td>89 ± 4</td>
<td>92 ± 2</td>
<td>90 ± 2</td>
<td>92 ± 2</td>
<td>91 ± 3</td>
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<tr>
<td>Clotrimazole/ionomycin</td>
<td>323 ± 10</td>
<td>318 ± 11</td>
<td>278 ± 10†</td>
<td>276 ± 9†</td>
<td>326 ± 9</td>
<td>323 ± 10</td>
<td>327 ± 9</td>
<td>324 ± 10</td>
</tr>
<tr>
<td>Cell volume in 150 mOs/m/kgH2O, geometric mean in forward scatter</td>
<td></td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>550 ± 9</td>
<td>558 ± 8</td>
<td>496 ± 7†</td>
<td>492 ± 13†</td>
<td>550 ± 9</td>
<td>551 ± 5</td>
<td>547 ± 8</td>
<td>546 ± 7</td>
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<tr>
<td>Ionomycin</td>
<td>139 ± 4</td>
<td>136 ± 4</td>
<td>136 ± 3</td>
<td>137 ± 2</td>
<td>139 ± 4</td>
<td>140 ± 5</td>
<td>140 ± 5</td>
<td>138 ± 4</td>
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<tr>
<td>Clotrimazole/ionomycin</td>
<td>388 ± 12</td>
<td>383 ± 24</td>
<td>302 ± 21†</td>
<td>300 ± 13†</td>
<td>369 ± 8</td>
<td>376 ± 7</td>
<td>384 ± 12</td>
<td>386 ± 10</td>
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<tr>
<td>Changes of cell volume by hypotonic stress (osmolality from 300 to 150 mOs/m/kgH2O, %)</td>
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<tr>
<td></td>
<td>35.4 ± 1.9</td>
<td>36.2 ± 2.8</td>
<td>28.0 ± 2.8†</td>
<td>26.3 ± 3.8†</td>
<td>35.3 ± 2.3</td>
<td>34.8 ± 2.2</td>
<td>37.5 ± 1.8</td>
<td>36.7 ± 0.9</td>
</tr>
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</table>

Values are means ± SE. Experimental results were analyzed by 2 (groups: HE, NE) × 4 (time sample points: PreR, PreE, PostR, PostE) repeated-measures ANOVA and Bonferroni’s post hoc test. *P < 0.05, PreR or PreE vs. PostR or PostE; †P < 0.05, HE vs. NE.
Effects of normoxic and hypoxic exercise trainings on erythrocyte deformability under shear flow

Table 4. Effects of normoxic and hypoxic exercise trainings on erythrocyte deformability under shear flow

<table>
<thead>
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<th>NE</th>
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<tbody>
<tr>
<td></td>
<td>PreR</td>
<td>PreE</td>
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<tr>
<td>Elongation index at 3 Pa</td>
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<tr>
<td>Vehicle</td>
<td>0.266 ± 0.005</td>
<td>0.264 ± 0.004</td>
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<tr>
<td>Ionomycin</td>
<td>0.016 ± 0.001</td>
<td>0.015 ± 0.001</td>
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<tr>
<td>Clotrimazole/</td>
<td>0.105 ± 0.004</td>
<td>0.102 ± 0.005</td>
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<tr>
<td>ionomycin</td>
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</table>

Values are means ± SE. Experimental results were analyzed by 2 (groups: HE, NE) × 4 (time sample points: PreR, PreE, PostR, PostE) repeated-measures ANOVA and Bonferroni’s post hoc test. *P < 0.05, PreR or PreE vs. PostR or PostE; †P < 0.05, HE vs. NE.
and reticulocyte counts as well as Epo level (10, 11) are consistent with a portion of our results. Our results further demonstrate that HE causes erythrocyte senescence in blood. According to a recent study (20), the expression of CD47 was largely eliminated during reticulocyte maturation. Furthermore, both CD47 and CD147 expressions on erythrocytes decline with age, which can regulate erythrocyte turnover throughout the life span of erythrocytes (6, 15). The CD47 molecule regulates the susceptibility of erythrocytes to destruction by phagocytosis (15), whereas the CD147 molecule facilitates the recirculation of mature erythrocytes from spleen into a circulatory system (6). In this study, HE downregulates the CD47 and CD147 expressions on erythrocytes and is accompanied by the reductions in erythrocyte volume and deformability, which may reflect erythrocyte senescence caused by HE.

Impairment of rheological properties by altering Ca²⁺ handling is essential for a characterization of the erythrocyte senescence (30). Earlier studies (29, 30) have demonstrated that aged erythrocytes have a higher Ca²⁺ content than that of young erythrocytes. This Ca²⁺ increase in erythrocyte senescence is attributed mainly to the deficiencies in erythrocyte Ca²⁺ pumping (29, 30). Recent investigations (5, 19) have also demonstrated that the aging process of erythrocytes was accompanied by increases in the activities of nonselective cation channels. However, Gardos channel activity, as determined at the saturating Ca²⁺ load, declines sharply during erythrocyte

<table>
<thead>
<tr>
<th>Table 5. Effects of normoxic and hypoxic exercise trainings on erythrocyte volume changed by osmotic stress</th>
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<tr>
<td></td>
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<tr>
<td><strong>Spectrin, mean fluorescence intensity</strong></td>
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<tr>
<td><strong>Vehicle</strong></td>
</tr>
<tr>
<td>89.6 ± 2.8</td>
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<tr>
<td>89.3 ± 3.6</td>
</tr>
<tr>
<td>76.5 ± 4.7*†</td>
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<tr>
<td>76.8 ± 5.0*†</td>
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<tr>
<td><strong>Ionomycin</strong></td>
</tr>
<tr>
<td>69.0 ± 2.4</td>
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<tr>
<td>69.8 ± 2.3</td>
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<tr>
<td>65.8 ± 2.9</td>
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<tr>
<td>65.2 ± 2.8</td>
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<tr>
<td><strong>Clotrimazole/ionomycin</strong></td>
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<tr>
<td>88.2 ± 3.7</td>
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<tr>
<td>88.9 ± 3.8</td>
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<tr>
<td>73.8 ± 3.4*†</td>
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<td>72.1 ± 3.2*†</td>
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<tr>
<td><strong>F-actin, mean fluorescence intensity</strong></td>
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<tr>
<td><strong>Vehicle</strong></td>
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<tr>
<td>164.4 ± 9.7</td>
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<tr>
<td>172.5 ± 10.2</td>
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<tr>
<td>132.5 ± 8.0*†</td>
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<tr>
<td>135.1 ± 7.9*†</td>
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<tr>
<td><strong>Ionomycin</strong></td>
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<tr>
<td>104.4 ± 5.7</td>
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<tr>
<td>101.0 ± 7.8</td>
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<tr>
<td>102.6 ± 4.5</td>
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<td>99.3 ± 3.7</td>
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<tr>
<td><strong>Clotrimazole/ionomycin</strong></td>
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<tr>
<td>124.4 ± 4.7</td>
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<td>124.3 ± 5.1</td>
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<tr>
<td>106.7 ± 4.1*†</td>
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<td>104.5 ± 3.3*†</td>
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Values are means ± SE. Experimental results were analyzed by 2 (groups: HE, NE) × 4 (time sample points: PreR, PreE, PostR, PostE) repeated-measures ANOVA and Bonferroni’s post hoc test. *P < 0.05, PreR or PreE vs. PostR or PostE; †P < 0.05, HE vs. NE.

Fig. 4. Effects of hypoxic (A, C, and E) and normoxic (B, D, and F) exercise trainings on 0.5 and 5.0 mM H₂O₂-induced changes of phosphatidylserine exposure (A and B), volume (C and D), and deformability (E and F) of erythrocytes. Values are means ± SE. Experimental results were analyzed by 2 (groups: HE, NE) × 4 (time sample points: Pre-R, Pre-E, Post-R, Post-E) repeated-measures ANOVA and Bonferroni’s post hoc test. +P < 0.05, Pre-R or Pre-E vs. Post-R or Post-E.
aging (32). Results of this study demonstrate that HE diminishes the change of erythrocyte volume caused by hypotonic stress (i.e., osmolality from 300 to 150 mOsm/kgH2O). However, Ca2+ ionosphere “ionomycin” decreases erythrocyte volume in an isotonic or hypotonic solution, and the HE and NE groups do not differ in the erythrocyte shrinkage induced by Ca2+ influx. While blunting the Ca2+-mediated erythrocyte shrinkage by pretreatment with Gardos K+ channel blocker “clotrimazole” (18), the Gardos effect in the volume regulation of erythrocytes is desensitized by HE but not NE. Moreover, the change of erythrocyte volume by hypotonic stress is positively correlated with the Gardos effect in erythrocyte volume regulation. The above results suggest that HE decreases the Gardos channel-modulated volume, deformability, and cytoskeletal protein contents of erythrocytes. Additionally, hypoxia and physical exercise are independent of each other and highly potent metabolic stressors (36). Moreover, hypoxic exposure reduces the SaO2 level, whereas physical exercise elevates O2 consumption by working organs. Therefore, hypoxic exercise substantially lowers either the O2 concentration or saturation of working organs by simultaneously decreasing O2 supply and increasing O2 demand (36). Given this augmented hypoxemia by hypoxic exercise, the extent of erythrocyte rheological disturbance may be larger than that caused by normoxic exercise.

Limitations of This Study

A limitation of this study is lack of hypoxic (15% O2) intervention alone as control group. Our recent study (36) has demonstrated that the 15% O2 exposure alone for 4 wk was insufficient to trigger myocardial preconditioning against cardiac dysfunction when exercising in an extremely (12% O2) hypoxic environment. Although acute or chronic 12% O2 exposure enhanced thrombin generation (35), elevated oxidative stress (34), increased interleukin-6 production (33), or impaired endothelial function (34), these thrombo-inflammatory parameters (33–36) remained unchanged in responses to the 15% O2 intervention. Based on these previous findings (33–36), the present investigation excludes the 15% O2 intervention alone as a control group. However, exactly how chronic 15% O2 intervention affects erythrocyte rheological functions remains unclear and requires further investigation. Moreover, this study is also limited in that only young and sedentary males are analyzed. Further clinical evidence is

Fig. 5. Relationships between measurements with various erythrocyte rheological parameters. Changes of cell volume by hypotonic stress are directly related to changes of cell volume by Gardos channel (A), whereas the reductions of deformability by H2O2 are inversely related to the changes of deformability by Gardos channel (B). Furthermore, the deformability changes are positive associated with the cell volume changes in modulation of Gardos channel in erythrocytes (C). HE, Pre-R, resting before training in HE group; HE, Pre-E, HET before training in HE group; HE, Post-R, resting after training in HE group; HE, Post-E, HET after training in HE group; NE, Pre-R, resting before training in NE group; NE, Pre-E, HET before training in NE group; NE, Post-R, resting after training in NE group; NE, Post-E, HET after training in NE group. Associations of measurements with various erythrocyte rheological parameters were assessed by the Pearson correlation test.

A recent investigation (8) has demonstrated that a local membrane deformation of erythrocytes in microcirculation triggered a transient increase in Ca2+ permeability and subsequently activated Gardos and anion channels. Such responses may further induce erythrocyte dehydration to facilitate the cell flow through capillary. Our results demonstrate that the deformability changes are positively correlated with the volume changes in erythrocyte Gardos effects. The above findings suggest that HE suppresses the dynamic deformability of erythrocytes required for flow through capillaries, thus limiting blood oxygen delivery to the tissues.

In this study, NE does not significantly change basal and Gardos channel-modulated volume, deformability, and cytoskeletal protein contents of erythrocytes. Additionally, hypoxia and physical exercise are independent of each other and highly potent metabolic stressors (36). Moreover, hypoxic exposure reduces the SaO2 level, whereas physical exercise elevates O2 consumption by working organs. Therefore, hypoxic exercise substantially lowers either the O2 concentration or saturation of working organs by simultaneously decreasing O2 supply and increasing O2 demand (36). Given this augmented hypoxemia by hypoxic exercise, the extent of erythrocyte rheological disturbance may be larger than that caused by normoxic exercise.
required to extrapolate these results to the subjects acclimatized at high altitude.

It is possible that hemorheological perturbations induced by GXT while performing 24 h before HET preclude any conclusion regarding the baseline of blood parameters analyzed before training. According to a previous study (38), a single bout of heavy anaerobic exercise reduced erythrocyte deformability and aggregation, whereas the hemorheological deterioration caused by acute exercise returned to a normal range 24 h later. Our pilot study (n = 12) reveals that erythrocyte deformability remains unchanged immediately and 24 h after GXT (i.e., $E = 0.299 \pm 0.007, 0.302 \pm 0.005$, and $0.293 \pm 0.005$ at rest, immediately after GXT, and 24 h after GXT, respectively). Therefore, the physiological stress of the GXT performed 24 h earlier influences the HET minimally.

This study does not evaluate the changes of band 3 and Ca$^{2+}$ levels in erythrocytes caused by NE or HE intervention. Also, band 3 clustering generates an epitope on the senescent cell surface, leading to autologous IgG binding and consequent phagocytosis (14). This protein expression is commonly used to evaluate erythrocyte senescence (14). Therefore, band 3 expression and Ca$^{2+}$ concentration in erythrocytes modulated by normoxic and hypoxic exercise regimens must be evaluated further.

In conclusion, this study demonstrates that HE, not NE, for 5 wk downregulates erythrocyte CD47 and CD147 expressions, simultaneously promoting erythropoietin to oxidative stress. Furthermore, this HE intervention attenuates the extents of erythrocyte deformability and dehydration modulated by Gardos channel. The above experimental findings reflect a senescence-related erythrocyte rheological dysfunction following HE intervention, as well as provide a novel interpretation of why hemorheological dysregulation frequently occurs in acclimatization to hypoxic exposure.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


