Inhibition of Glutathione Synthesis Induced by Exhaustive Running Exercise via the Decreased Influx Rate of L-Cysteine in Rat Erythrocytes

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Key Words
Exhaustive exercise • L-cysteine • Erythrocyte • Glutathione synthesis

Abstract
Background/Aims: The main purpose of this study was to investigate the effect of exhaustive exercise on L-cysteine uptake and its effect on erythrocyte glutathione (GSH) synthesis and metabolism. Methods: Rats were divided into three groups: sedentary control (C), exhaustive running exercise (ERE) and moderate running exercise (MRE) (n=12 rats/group). We determined the L-cysteine efflux and influx in vitro in rat erythrocytes and its relationship with GSH synthesis. Total anti-oxidant potential of plasma was measured in terms of the ferric reducing ability of plasma (FRAP) values for each exercise group. In addition, the glucose metabolism enzyme activity of erythrocytes was also measured under in vitro incubation conditions. Results: Biochemical studies confirmed that exhaustive running exercise significantly increased oxidative damage parameters in thiobarbituric acid reactive substances (TBARS) and methemoglobin levels. Pearson correlation analysis suggested that L-cysteine influx was positively correlated with erythrocyte GSH synthesis and FRAP values in both the control and exercise groups. In vitro oxidation incubation significantly decreased the level of glucose metabolism enzyme activity in the control group. Conclusion: We presented evidence of the exhaustive exercise-induced inhibition of GSH synthesis due to a dysfunction in L-cysteine transport. In addition, oxidative stress-induced changes in glucose metabolism were the driving force underlying decreased L-cysteine uptake in the exhaustive exercise group.

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Published by S. Karger AG, Basel
Introduction

Reactive oxygen species (ROS) have been proposed as the main agents responsible for exercise-induced physiological and pathological dysfunction [1, 2]. The continuous generation of superoxide radicals during exercise results in the accumulation of the oxidation products of lipids, nucleic acids, and proteins and ultimately culminates in cellular dysfunction, making the body prone to external deleterious agents [3, 4]. Glutathione (GSH) is an important redox active biomolecule that is critical in the maintenance of cellular and organism homeostasis [5-7]. Previous studies have suggested that abnormal metabolism of GSH is closely related with various pathological and physiological conditions [8, 9]. However, whether the rate of GSH synthesis is affected by exhaustive exercise-induced oxidative stress remains unknown.

Exhaustive exercise-induced oxidant stress injury has been previously recognized as an important factor to elicit multiple changes at metabolic and physiological levels [10-13]. Erythrocyte GSH plays a vital role in mitigating the detrimental effects of reactive oxygen species (ROS) encountered in the circulation and produced by the continuous oxidation of hemoglobin within the cytosol of the erythrocyte [14]. Reduced GSH reacts with superoxide reaction products and degrades hydrogen peroxide and lipid peroxides by GSH peroxidase. In addition, GSH covalently modifies toxic xenobiotics and endogenous electrophiles to form water-soluble conjugates that are exported from the erythrocyte for excretion [15]. In diseases associated with an increased production of ROS that results in GSH depletion, restoration of the normal erythrocyte GSH concentration has been shown to have positive therapeutic effects [16-20].

Although there is no protein synthesis system in erythrocytes, GSH can be synthesized by glutamic acid, cysteine, and glycine [6, 21]. Although three amino acids are required for GSH synthesis, L-cysteine availability is the rate-limiting step of GSH synthesis [22]. In addition, it is the only amino acid that possesses the important functional free sulphydryl (–SH) group, which plays an important role in the elimination of free radicals and maintenance of proper intracellular redox status in red blood cells (RBCs) [23, 24]. A recent study by D. Yildiz demonstrated that L-cysteine efflux plays a vital role in maintaining a harmonious redox status in plasma [23]. Decreased influx of L-cysteine is considered an important factor contributing to the development of oxidative stress in human erythrocytes during the aging process [8]. However, the L-cysteine transport capacity varies under different exercise conditions, and its effect on the antioxidant capacity of erythrocytes remains unclear. The transport of L-cysteine in erythrocytes is mediated by sodium- and adenosine triphosphate (ATP)-dependent systems [25, 26]. A previous study by M Güven demonstrated that oxidative stress induces a significant decrease in glucose transport and utilization efficiency [27]. Herein, we propose that exhaustive exercise-induced oxidant stress results in decreased levels of glucose metabolism and ATP production in RBCs, which may further induce the inhibition of GSH synthesis in erythrocytes due to a dysfunction in ATP-dependent L-cysteine transport.

To address this issue, we investigated the effects of exhaustive exercise on changes in L-cysteine uptake-induced erythrocyte GSH synthesis. In addition, the energy metabolism process was also investigated under in vitro incubation conditions to examine the underlying molecular mechanisms of L-cysteine transport changes in different exercise groups.

Materials and Methods

Animal care

Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996), and all procedures were approved by the Institutional Review Board of the Institute of Health Sciences, Chongqing Institutes for Biological Sciences, Chinese Academy of Sciences and Chongqing University School of Medicine (Chongqing, China).
Animals, exercise protocol, and blood samples

Thirty-six adult male Wistar rats (8 weeks, 200–250 g) were used. Animals were reared at room temperature (22 ± 3°C), relative humidity of 40–60% and an illumination time from 07:30 to 20:00, having free access to standard rodent chow and softened tap water. The standard rodent chow contained 14% fat, 64% carbohydrate, and 22% protein (per 100 g: 6.9 g fat, 69.3 g carbohydrate, 23.8 g protein), which was provided by the Laboratory Animal Center of Chongqing University mentioned above.

After 2 weeks adaptive training (30 min run exercise), rats were initially divided into three groups: sedentary control (C), exhaustive running exercise (ERE) and moderate running exercise (MRE) (n = 12 rats/group). ET group rats were exercised according to a modification of the protocol designed by Davies et al. [28]. Briefly, all running exercise groups were introduced to running on a motor-driven rodent treadmill. The treadmill was equipped with an electric shocking grid on the rear barrier to provide exercise motivation to the animals. Animals in the ERE group started treadmill running at a speed of 25 m/min speed with a 5% gradient, and reached a 15% gradient in 20 min. Running was continued until exhaustion, and rats were protect from dehydration through intermittent drinking during exhaustive exercise. Exhaustion was defined as the inability of a rat to right itself if laid on its side. As a control group of exhaustive exercise, animals in the MRE group started treadmill running at a speed of 25 m/min with a 5% gradient, and reached a 15% gradient in 20 min. Each rat ran for 40 min. The sedentary group rats were placed on the treadmill but remained sedentary for the same period of time as the exercise-trained rats. Rats were anesthetized (50 mg/kg sodium pentobarbital, i.p.) immediately after exercise. Blood samples were obtained from the abdominal aorta of the rats under light ether anesthesia and were treated with the anticoagulant, heparin sodium (15 U/ml) (Fig. 1).

Plasma sodium, plasma potassium were tested using Gem Premier 3000 (Instrumentation Laboratory, Lexington, MA). Blood parameters including mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) were tested using LH 780 hematology analyzer (Beckman Coulter; Fullerton, CA).

Preparation of RBC suspensions and ghosts

Fresh heparinized blood was centrifuged at 900× g at 4°C for 10 min. Plasma and the buffy coats were removed. After removal of plasma, cells were filtered through cellulose to remove leucocytes and platelets, as described in [29]. Packed RBCs were washed thrice in isotonic Hepes buffer (145 mM NaCl, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, osmotic pressure 300mOsm) and was further used in the experiments at 20% hematocrit [15]. PBS-glucose contained 8 mM of glucose in the PBS.

For the preparation of RBC ghosts, washed packed RBCs were lysed with ice-cold 5 mM sodium phosphate buffer (pH 8.0) containing 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer) and incubated at 4°C for 10 min. RBC ghosts were washed thrice (13,500 rpm for 20 min at 4°C) to obtain "white" ghosts.

Measurements of methemoglobin levels

Blood was centrifuged at 2,000 rpm at 4°C to remove plasma, passed through cotton to eliminate white cells, and washed three times with choline wash solution (180 mM choline, 1 mM MgCl2, 10 mM Tris-Mops, pH 7.4 at 4°C, 320–340 mOsm). Red cell MetHb levels were determined as described by Kohn et al. [30]. Measurement of methemoglobin concentration was based on the absorbance of methemoglobin at 630 nm, which is characterized by εM 630 = 4.4 mM cm⁻¹.

Determination of SH-radicals in erythrocyte membranes

SH-radicals of erythrocyte membrane proteins were quantified according to Anderson [31] and Yamaguchi et al. [32]. SH-radicals levels were estimated by measuring absorbance at 415 nm after incubation with lysis buffer and incubation at 4°C for 10 minutes. The absorbance was measured at 415 nm.

Fig. 1. Timeline of study protocol.
bation at 37°C in water bath for 15 min, the contents of SH-radical were determined using GSH as a standard.

Membranous peroxide (thiobarbituric acid reactive substances, TBARS) level detection

The extent of lipid peroxidation of RBC membranes was estimated by measuring TBARS levels according to the method of Stocks and Dormandy [30]. TBARS levels were estimated by measuring absorbance at 532 nm after a reaction with thiobarbituric acid. Trichloroacetate-acid extracts of RBC samples were used to circumvent the interference of proteins with TBARS determinations. Results were expressed as nanomoles per gram hemoglobin (Hb) [33].

L-Cysteine influx studies

A total of 0.25 ml of washed erythrocytes was suspended in 1 ml of PBS-glucose containing 1.4 and 10 mM concentrations of L-cysteine and incubated for 1 hr at 37°C in a water bath. At the end of incubation erythrocytes were removed, centrifuged and the supernatants were discarded. The free -SH concentrations in erythrocytes were then determined as described by Yildiz et al. [23]. Briefly 100 ml of erythrocytes were lysed in 100 ml of 10% TCA prepared in sodium phosphate-EDTA buffer (0.01 M sodium phosphate/0.005 M EDTA). The erythrocyte lysates were then centrifuged at 12,000 g for 5 min. At the end of centrifugation 100 ml of the supernatant was mixed with 1.9 ml of Tris-EDTA buffer containing 0.6 mM/ml DTNB (262 mM Tris base, 13 mM EDTA, pH 8.9). Samples were allowed to stand for 5 min to develop color. The absorbances of the samples were then measured at 412 nm and the concentrations of free -SH were calculated by using the mM extinction coefficient of 13.6. Uptake rate where indicated was calculated from the following equation.

Influx rate = Free -SH concentration obtained following treatment with L-cysteine - Control Free -SH.

L-Cysteine efflux studies

A total of 0.25 ml of washed erythrocytes was resuspended in 1 ml of PBS-glucose in the presence of different concentrations of L-cysteine. Erythrocytes were incubated at 37°C in a water bath for 1 hr to allow the uptake process. At the end of incubation erythrocytes were centrifuged and the supernatants were discarded. The erythrocytes were then resuspended in 1 ml of fresh PBS-glucose and incubated at 37°C for indicated times to allow the efflux process. At the end of incubation erythrocytes were centrifuged and the supernatants were transferred to fresh tubes. The free -SH concentrations in the supernatant was then measured as described above.

Calculation of intracellular concentration for GSH and GSSG in RBCs

For each sample, the amount (mole) of GSH and GSSG per RBC was determined via the standard curve specific to each run. First, the GSH amount was divided by the number of RBCs associated with each sample giving a value of mole cell⁻¹. The mole cell⁻¹ value was then divided by the mean cell volume resulting in an intracellular concentration of mole L⁻¹ for GSH and GSSG. The median of the three independently processed samples was taken to reflect the intracellular GSH and GSSG concentration of an individual. From these molar concentrations the status of the GSSG/2GSH couple was calculated using the Nernst equation \( E_m = -255 - 30 \log ([GSH]^2/ [GSSG]) \) in mV [5], assuming an intracellular pH of 7.25 for RBCs and a temperature in vivo of 37°C [34]. Erythrocyte total free glutathione (TFG) was calculated as GSH + 2xGSSG.

Measurements of FRAP levels

The FRAP (ferric-reducing ability of plasma) values were determined following the method of Benzie et al. [35]. Working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 2, 4, 6- tri [2-pyridyl]-s-triazine (10 mM in 40 mM HCl) solution, and FeCl₃·6H₂O (20 mmol/L) solution in a 10:1:1 ratio, respectively. A total of 3 ml of FRAP reagent was mixed with 100 μL of plasma; the content was mixed vigorously to make a uniform solution. The absorbance was read at 593 nm at an interval of 30 sec for 4 min. The aqueous solution of known Fe³⁺ concentration in the range of 100-1,000 μmol/L was used for calibration. Using the regression equation, the FRAP values (μmol Fe (II) per liter) of the plasma were calculated. Relationships between various parameters were assessed using the Pearson correlation coefficient (r) and R squared. Statistical analyses were performed using GraphPad Prism version 4.0 for Windows, GraphPad Software (San Diego CA).
Measurements of ATP levels

ATP concentrations were determined enzymatically using a commercially available kit and controls (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). RBC samples were added to 10% trichloroacetic acid, vortexed, and placed on ice. The supernatants were combined with the substrates (glucose, and NAD\(^+\)) and enzymes (hexokinase and glucose-6-phosphate dehydrogenase) required for the enzymatic reaction to occur. The amount of NADH produced, which is proportional to the amount of ATP within the sample, was measured spectrophotometrically. The amount of ATP in the sample was calculated as \(\mu\text{mol/dl}\); this was further normalized using the total Hgb concentration (\(\mu\text{mol/g Hgb}\)) [36].

Measurements of Enzyme activity

Erythrocyte glucose 6-phosphatedehydrogenase (G-6-PD) activity was measured by the modified method of Zinkham by observing the conversion of NADP\(^+\) into NADPH after addition of glucose-6-phosphate. Briefly, 50\(\mu\)l hemolysate was mixed with 850\(\mu\)l of reaction mixture (2 mM NADP\(^+\), 0.1 M MgCl\(_2\), 6mM glucose-6-phosphate and Tris-HCl buffer (1 M, pH: 8)) and the absorbance at 340 nm was monitored for 10 min. G-6-PD activities were expressed as units/g of hemoglobin (one unit, U = 1 \(\mu\text{mol of NADPH formed/min}\)).

We determined hexokinase (HK, EC 2.7.1.1), lactate dehydrogenase (LDH, EC 1.1.1.27), pyruvate kinase (PK, EC2.7.1.40) activities in hemolysate essentially by Beutler’s methods [37].

Statistical analysis

All data from three or more than three groups of repeated experiments, and are expressed in the form of mean ± standard deviation (mean ± SD). Intergroup differences of different exercise groups were assessed by independent sample t-test. The intragroup comparisons between treated groups and their respective control values were performed using one-way ANOVA. Analyses were performed with SPSS PC version 19.0. \(P < 0.05\) means the difference was significant.

Results

Effect of exercise on in vitro quality parameters of RBCs

Table 1 describes the differences of in vitro quality parameters of RBC in the two tested exercise training conditions. The oxidative damage parameters of rats RBCs at different groups shows that the methemoglobin content and TBARS levels were significantly increased in exhaustive running exercise animals compared with the C group (\(P < 0.05\)). Meanwhile, free thiol groups in erythrocyte membranes significantly decreased in the ERE group (\(P <

Table 1. Quality parameters of RBCs in different groups. TBARS, thiobarbituric acid reactive substances; 2,3-DPG, 2,3-diphosphoglyceric; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration. Values are means ± SD. C, control groups; MRE, moderate running exercise; ERE, exhaustive running exercise. \(^*P<0.05\) and \(^{**}P<0.01\)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MRE</th>
<th>ERE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>261±9.2</td>
<td>256±5.6</td>
<td>243±6.8</td>
</tr>
<tr>
<td>Exhaustion time, min</td>
<td>0</td>
<td>40</td>
<td>243±9.6**</td>
</tr>
<tr>
<td>Methemoglobin, % of Hb</td>
<td>1.67±0.76</td>
<td>1.71±0.72</td>
<td>2.72±0.70*</td>
</tr>
<tr>
<td>TBARS, nmol/g Hb</td>
<td>12.43±5.6</td>
<td>13.8±6.3</td>
<td>33.35±6.4*</td>
</tr>
<tr>
<td>SH-radical, (\mu\text{mol/g protein})</td>
<td>84.2±4.9</td>
<td>81.7±5.9</td>
<td>73.9±6.1*</td>
</tr>
<tr>
<td>Plasma Na(^+), meq/l</td>
<td>150.2±1.7</td>
<td>149.6±1.9</td>
<td>147.5±1.8</td>
</tr>
<tr>
<td>Plasma K(^+), meq/l</td>
<td>4.82±0.31</td>
<td>4.89±0.39</td>
<td>4.96±0.54</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>19.6±1.7</td>
<td>19.8±1.8</td>
<td>19.1±1.6</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>64.5±5.9</td>
<td>65.1±6.4</td>
<td>62.5±5.9</td>
</tr>
<tr>
<td>MCHC, g/dl</td>
<td>29.9±0.5</td>
<td>30.1±0.6</td>
<td>31.9±0.5</td>
</tr>
</tbody>
</table>
Exhaustive running exercise did not result in significant changes in plasma Na⁺ and K⁺ value. In addition, no significant variations were observed in MCH, MCV and MCHC values.

**Effect of exercise on GSH, GSSG, GSH/GSSG and TFG levels of RBCs**

To explore the impact of exhaustive exercise on RBC glutathione parameters, the levels of GSH, GSSG, GSH/GSSG and TFG were analyzed and the results are presented in Fig. 2. Compared with C group and MRE group the GSH level significantly decreased (C group, 2.93 ± 0.12 μmol/g Hb; MRE group, 2.89 ± 0.14 μmol/g Hb vs. ERE group, 2.41 ± 0.23 μmol/g Hb; \( P < 0.05 \)) and GSSG level significantly increased (C group, 63.20 ± 4.30 nmol/g Hb; MRE group, 64.10 ± 5.21 nmol/g Hb vs. ERE group, 92.56 ± 7.32 nmol/g Hb; \( P < 0.01 \)) in the ERE group. Therefore, the GSH/GSSG ratio was found decline significantly after exhaustive running exercise (\( P < 0.01 \)). In addition, the TFG level of RBCs decreased significantly in ERE group compared with C and MRE groups (C group, 3.06 ± 0.16 μmol/g Hb; MRE group, 3.02 ± 0.18 μmol/g Hb vs. ERE group, 2.59 ± 0.25 μmol/g Hb; \( P < 0.01 \)).

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Effect of exercise on L-Cysteine influx and efflux rate of RBCs

L-cysteine is involved in the synthesis of glutathione, and the transport of L-cysteine was measured in different exercise groups. As shown in Fig. 3, the L-cysteine influx rate (C group, 3.06 ± 0.16 μmol/h/mL Hb; MRE group, 3.02 ± 0.18 μmol/h/mL Hb vs. ERE group, 2.59 ± 0.25 μmol/h/mL Hb; P < 0.05) and efflux rate (C group, 0.63 ± 0.05 μmol/h/mL Hb vs. ERE group, 0.16 ± 0.04 μmol/h/mL Hb; P < 0.01) decreased significantly in ERE group compared with C and MRE groups.

Furthermore, L-cysteine influx rate was positively correlated with TFG level in all tested groups (r = 0.8255*, 0.7221*, 0.7690** in C, MRE and ERE groups) between the L-cysteine influx rate and the TFG level in all tested groups (Fig. 4).

Table 2. Glucose metabolism parameters of RBCs in different groups. ATP, adenosine triphosphate; G6PDH, glucose-6-phosphate dehydrogenase; PK, phosphofructokinase; LDH, lactate dehydrogenase; HK, Hexokinase. Values are means ± SD. C, control groups; MRE, moderate running exercise; ERE, exhaustive running exercise. *P < 0.05 and **P < 0.01

Table 3. Glucose metabolism enzyme activity of RBCs under in vitro treatment. G6PDH, glucose-6-phosphate dehydrogenase; PK, phosphofructokinase; LDH, lactate dehydrogenase; HK, hexokinase. Values are means ± SD. C, control groups; ERE, exhaustive running exercise. Post incubation vs. C, *P < 0.05, **P < 0.01. Post incubation vs. ERE, †P < 0.05, ††P < 0.01

Fig. 5. FRAP value and correlation analysis with L-cysteine influx rate in RBCs from different exercise groups. (A) FRAP value in control and exercise groups. (B, C, D) Pearson correlation analysis between L-cysteine influx rate and FRAP value in different groups. Data are expressed as means ± SD with the significance level set at *P < 0.05 and **P < 0.01 for the exercise group compared with corresponding control group.
Effect of exercise on FRAP value of RBCs

As shown in Fig. 5A, FRAP value significantly decreased in ERE group compared with C and MRE groups (C group, 1730 ± 62 μmol/L; MRE group, 1680 ± 81 μmol/L vs. ERE group, 1150 ± 96 μmol/L; P < 0.05). Furthermore, Pearson correlation analysis suggested that L-cysteine influx rate and FRAP value in all tested groups (r = 0.7656*, 0.7979*, 0.8200** in C, MRE and ERE groups) (Fig. 5B).

Effect of exercise on glucose metabolism parameters of RBCs

As shown in Table 2, the lactate concentration in RBCs significantly elevated in ERE group (P < 0.01). Meanwhile, the glucose and ATP concentration of RBCs significantly decreased after exhaustive running exercise (P < 0.05).

The activity of glycolytic and pentose phosphate shuttle enzymes in three exercise groups was tested. G6PDH, PK, and LDH activities significantly lower in ERE group (P < 0.01).

Effect of in vitro treatment on glucose metabolism enzyme activity of RBCs

To evaluate the influence of oxidation on glucose metabolism of RBCs, erythrocyte suspensions were incubated for 30 min at 37°C in the presence and absence of HX 0.2 U/ml XO 1.5 mM (hypoxanthine/xanthine oxidase) and 1.0 mM dithiothreitol (DTT), and then were analyzed for enzyme activity in C and ERE groups. As shown in Table 3, activity of G6PDH, PK, LDH and HK decreased significantly after in vitro treatment of HX 0.2 U/ml XO 1.5 mM in C group. Meanwhile, activity of G6PDH, PK, LDH and HK increased significantly after in vitro reduction treatment with DTT in ERE groups.

Effect of in vitro treatment on influx rate of L-cysteine

To explore the influence of oxidation on L-cysteine transport of RBCs, L-cysteine influx rate was tested under in vitro oxidant condition. As shown in Fig. 6A, L-cysteine influx rate significantly decreased in C (pre incubation, 3.06 ± 0.16 μmol/h/mL Hb vs. post incubation, 2.34 ± 0.18 μmol/h/mL Hb, P < 0.01) and MRE (pre incubation, 3.02 ± 0.18 μmol/h/mL Hb vs. post incubation, 2.28 ± 0.25 μmol/h/mL Hb, P < 0.01) groups after in vitro treatment of HX 0.2 U/ml XO 1.5 mM. Meanwhile, L-cysteine influx rate significantly increased in ERE group (pre incubation, 2.59 ± 0.25 μmol/h/mL Hb vs. post incubation, 2.84 ± 0.41 μmol/h/mL Hb, P < 0.05) after the in vitro treatment of 1.0 mM DTT.

To determine the ATP induced L-Cysteine influx change in vitro, RBCs were portioned into aliquots and incubated with isotonic Hepes buffer (145 mM NaCl, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, osmotic pressure 300 mOsm) contained 0, 5, 10, 15, 20 μmol/l of ATP (A 3377, Sigma-Aldrich, St Louis, Missouri, MO, USA) and 10 mmol/l L-cysteine. Intracellular L-Cysteine concentration was measured at
predetermined time intervals to determine the rate of L-cysteine influx. As shown in Fig. 6B, L-cysteine influx rates significantly elevated with increased ATP concentration in control and exercise groups.

Discussion

Oxidative damage has long been implicated in the exercise process [1]. RBCs are continuously exposed to a large number of oxidants derived from various endogenous as well as exogenous sources during the exhaustive exercise process [38]. In this study, the inhibitory effect of GSH synthesis via the decrease influx rate of L-cysteine in rat RBCs was observed under exhaustive running exercise conditions. Based on in vitro observations, oxidant stress-induced glucose metabolism efficiency dysfunction contributes to the decrease in L-cysteine transport capacity after exhaustive running exercise.

As the oxygen transporter in circulation, mammalian erythrocytes are permanently vulnerable to oxidative damage throughout their lifespan due to the high cellular concentration of oxygen and hemoglobin, which accelerate the oxidative process. A previous study suggested that intra-cellular GSH levels protect cells from ROS-induced injury [39]. When RBCs are continuously exposed to oxidative stress, and their cellular systems are unable to counteract the ROS-mediated insults, the amount of free GSH is decreased, resulting in irreversible cell degeneration and death [40]. In a previous study, GSG/GSSG was considered an indicator of the oxidation/reduction state of erythrocytes [41]. Under various physiological and pathological conditions, a lower GSH/GSSH ratio was observed in erythrocytes, indicating a shift in the redox status of the erythrocytes. In the present study, we observed a significant reduction in the GSH and GSH/GSSH ratios in rat RBCs after exhaustive running exercise, which was consistent with the significant increase in methemoglobin content and TBARS levels. Increased oxidative injury induced by exhaustive exercise was reported in a previous study [38]. In this study, TFG levels of RBCs were significantly decreased, suggesting dysfunction of GSH synthesis after exhaustive exercise.

RBCs possess the ability to synthesize GSH by glutamic acid, cysteine, and glycine. As the main factor regulating the synthesis rate of glutathione, changes in L-cysteine uptake were studied under numerous physiological and pathological conditions [23, 41]. We found that the L-cysteine influx rate was significantly and positively correlated with erythrocyte GSH synthesis and total anti-oxidant potential in terms of FRAP value in both the control and exercise groups. Previous reports indicated that L-cysteine uptake in erythrocytes was predominantly mediated by the Na-dependent, ATP-dependent ASC and Na-independent systems [25, 42, 43]. In addition, the kinetic parameters of L-cysteine uptake were closely related with the intracellular L-cysteine requirements for GSH biosynthesis, which was determined by the intracellular GSH turnover rate [44]. In the present study, we observed a significant reduction in the ATP concentration of RBCs after exhaustive running exercise, while plasma Na+ remained unchanged. Given the significant decline in GSH concentrations in RBCs, the ATP-dependent transport dysfunction may represent the main factor responsible for decreased L-cysteine uptake capacity in the exhaustive exercise group.

Oxidative injury to erythrocytes has been widely studied and found to contribute to the destructive events observed in many hematological disorders [45-48]. In the present
study, we observed a significant decrease in glucose metabolism enzyme activity of rat RBCs after exhaustive exercise. There are two principal pathways for glucose metabolism: (i) the Embden-Meyerhof pathway (EMP), which generates adenosine triphosphate (ATP), NADH, and 2,3-diphosphoglycerate (2,3-DPG), and (ii) the hexose monophosphate pathway (HMP), which serves as the sole source of NADPH for erythrocytes, an important factor in the antioxidant status of the erythrocyte, and a necessary metabolite for the reduction of the disulfide form of glutathione (GSSG) to produce GSH. Relative flux through the EMP and HMP is modulated by $O_2$-linked transitions in Hb conformation due to competitive binding for the cytoplasmic domain of Band 3 between deoxyHb and key EMP enzymes (PFK, Aldo, G3PD, PK, and LDH). Our previous observations suggested that exhaustive running exercise results in elevated band-3 tyrosine phosphorylation and altered band-3 membrane organization [38]. Oxidative injury of Band-3 may result in a decrease in related enzymatic activity. In this study, in vitro incubation with HX/XO significantly decreased the levels of glucose metabolism enzyme activity in the control group. In addition, in vitro incubation with ATP significantly improved the L-cysteine influx rate in all tested groups. Taken together, our data suggested that the exercise related intracellular redox state could affect the glucose metabolism enzyme activity of RBCs, which further results in a decrease in ATP production, and the inhibition of L-cysteine uptake and GSH synthesis in RBCs (Fig. 7). This finding was also consistent with our findings of changes in the L-cysteine influx rate under in vitro oxidation or reduction treatment conditions.

Recently, our understanding of GSH metabolism in the erythrocyte has grown from information obtained by studies of the kinetics of isolated enzymes [22]. Recent studies have indicated that increased activity of the γ-glutamate-cysteine ligase (GCL) could increase the rate of glutathione synthesis [49]. In addition, the extent to which exhaustive exercise induces changes in GSH synthesis and the metabolic reactions of RBCs remain unclear. Thus, further studies are required to examine the role of the primary enzyme activity and steady-state kinetic parameter values of GSH synthesis and metabolism in exhaustive exercise regimens.

**Conclusion**

In summary, we presented the evidence that exhaustive exercise induced inhibition of GSH synthesis due to L-cysteine transport dysfunction. In addition, oxidant stress induced glucose metabolism change was the driving force of L-cysteine uptake decline after exhaustive exercise. These experimental findings reflected an underlying molecular mechanism by which decreased anti-oxidant potential via GSH leading to oxidative damage in exhaustive exercise.

**Abbreviation**

RBC (red blood cell); GSH (Glutathione); GSSG (oxidized glutathione); TFG (total free glutathione); FRAP (ferric-reducing ability of plasma); ATP (adenosine triphosphate); ROS (reactive oxygen species); ERE (exhaustive running exercise); MRE (moderate running exercise); Hb (hemoglobin); HK (hexokinase); PK (pyruvate kinase); LDH (lactate dehydrogenase); G-6-PD (glucose 6-phosphatedehydrogenase); EDTA (ethylenediaminetetraacetic acid); MCH (mean corpuscular hemoglobin); MCV (mean corpuscular volume); MCHC (mean corpuscular hemoglobin concentration).

**Acknowledgments**

Dr. Yanlian Xiong was supported by grants from the Initial Scientific Research Fund of Binzhou Medical University (Grant No. BY2014KYQD09) and Shandong Provincial Natural Science Foundation (Grant No. ZR2014HM009).

**Disclosure Statement**

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