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Research Communication

Red Blood Cell Extrudes Nucleus and Mitochondria Against Oxidative Stress

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Summary

Mammal red blood cells (erythrocytes) contain neither nucleus nor mitochondria. Traditional theory suggests that the presence of a nucleus would prevent big nucleated erythrocytes to squeeze through these small capillaries. However, nucleus is too small to hinder erythrocyte deformation. And, there is no sound reason to abandon mitochondria for the living cells. Here, we found that mammal erythrocyte reactive oxygen species (ROS) levels kept stable under diabetes, ischemia reperfusion, and malaria conditions or *in vitro* sugar/heme treatments, whereas bird erythrocyte ROS levels increased dramatically in these circumstances. Nuclear and mitochondrial extrusion may help mammal erythrocytes to better adapt to high-sugar and high-heme conditions by limiting ROS generation. © 2011 IUBMB

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Keywords erythrocytes; mitochondria; nucleus; reactive oxygen species.

INTRODUCTION

Red blood cells (RBCs) or erythrocytes are continually formed in the bone marrow. RBCs originate from nucleated stem cells, which mature into nucleated erythroblasts, then differentiate into anuclear reticulocytes, and finally into RBC. RBCs are terminally differentiated cells (they cannot divide anymore) and are shed from the bone marrow into the blood circulation. In contrast to mammals, RBCs in birds, reptiles, and other "lower" vertebrates have nuclei. The anucleated erythrocyte, as it is seen in mammals, is considered more evolutionarily "advanced" (*1*). In addition to the differences in the circulatory system, mammals have smaller end-blood-vessels (capillaries of about 3 μm in diameter) than birds. To squeeze through these small blood capillaries and make maximum surface for hemoglobin, RBC (about 8 μm in diameter for human and 10–15 μm in diameter for birds) must be very flexible (*1–3*). The presence of a nucleus may prevent big nucleated RBC to squeeze through these small capillaries. Therefore, during the evolutionary development, nature has found that it was better to extrude the nucleus and also other cell organelles, such as endoplasmic reticulum for protein synthesis, which were not needed for their actual function as oxygen carrier (*1*). However, nucleus in the erythroblast is about 2 μm in diameter (*2*). And, if it was distributed at peripherally inflated region of the erythrocyte, it would neither hinder erythrocyte deformation nor its entrance into end-blood-vessels. Regulation of cytoskeleton for deformation into the biconcave shape may not necessarily need nuclear extrusion. Treatment of sickle cell anemia mouse with pluripotent stem (iPS) cells changed the sickled deformed erythrocytes to the normally biconcave shape (*4, 5*), which was not related with nuclear extrusion. Mitochondria are known as the powerhouses (batteries) of the cell, although are not related with oxygen carrier. There is no sound reason to abandon mitochondria for the living cells either.

To explain nuclear and mitochondrial extruding in mammal RBCs, we should pay attention to their living conditions—blood. Blood sugar and heme levels fluctuate diurnally. Stress-induced hyperglycemia and diabetes increase blood glucose substantially. Then, the glucose 6-phosphate (Glc-6-P) accumulation leads to great increases in reactive oxygen species (ROS) generation (*6, 7*). Severe hemolysis or myolysis occurring during pathological

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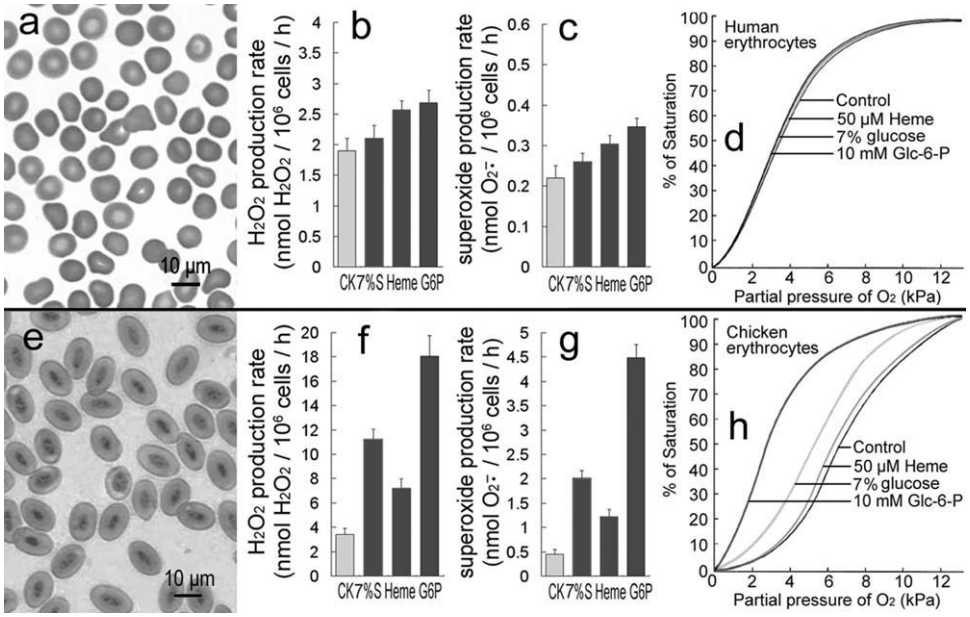

Figure 1. (a) Wright's staining (for untreated cells, CK), (b) H_2O_2, (c) superoxide production rates, and (d) O_2 dissociation curves of 7% glucose-, 50 μM heme-, or 10 mM Glc-6-P (G6P)-treated (2 h) human erythrocytes. (e) Wright's staining (for CK), (f) H_2O_2, (g) superoxide production rates, and (h) O_2 dissociation curves of 7% glucose (7% S)-, 50 μM heme-, or 10 mM Glc-6-P-treated (2 h) chicken erythrocytes. Error bars show standard deviations ($n = 3$).

states, such as sickle cell disease, ischemia reperfusion (IR), and malaria, results in high levels of free heme, also causing severely ROS accumulation (*8*). Here, we find that lacking of nucleus and mitochondria benefits mammal erythrocytes against sugar/heme-induced oxidative stress.

MATERIALS AND METHODS

Materials

Human red cells were obtained from normal volunteers (West China Hospital, Sichuan University, China), and chicken red cells were obtained from a local market. The erythrocytes were washed twice in phosphate-buffered saline (145 mM NaCl, 5 mM NaPi, and 1 mM EDTA, pH 7.4) and white cells were removed by filtration through cellulose (*9*). Adult Sprague-Dawley rats (average body weight, 350 ± 50 g) were purchased from Li-Nuo Biotechnology Comp. (China). Adult chickens were purchased from a local market.

Chemical Treatment and Porphyrin Feeding

For erythrocytes, sterile glucose (7%), heme (50 μM), and 10 mM Glc-6-P were applied to the cell suspension [in Hanks' balanced salt solution (HBSS)] directly.

Superoxide and H_2O_2 Detection

A total of 0.5 mL of isolated cells in HBSS (about 5×10^6 cells per milliliter) were added to the tubes containing 1.5 mM cytochrome *c* or ferricytochrome *c*. The formation of H_2O_2 in RBCs was followed by measuring the oxidation of acetylated ferrocytochrome *c* catalyzed by cytochrome *c* peroxidase at the room temperature (*10*). This method was based on the measurement of the oxidation rate of reduced cytochrome *c* H_2O_2, when the H_2O_2 generation rate was higher than 50 nM min^{-1} (*11*). While the formation of superoxide in the animal cells was monitored by the reduction of acetylated ferricytochrome *c* (*12*).

Blood Oxygen Dissociation Curve Measurement

The O_2 dissociation curve was determined in the last aliquot of red cell suspension with a micropbotometric reaction apparatus equilibrating a microsample of sample with gas mixtures of known PO_2 and PCO_2 and measuring blood O_2 saturation (SO_2) microphotometrically (*13*).

Renal Ischemia and Reperfusion Surgery, Diabetes Experiments, and Malaria Infection

Rat renal ischemia (IR) was induced by nontraumatic vascular clamps over the pedicles for 20 min (*14*). Because birds tolerate hypoxia much more than mammals (*15*), chicken renal ischemia (IR) was induced for 40 min to get the physiological status. After clamps were released, the incision was closed in two layers with 2-0 sutures. Two hours after renal ischemia, rats were placed in metabolic cages at 22 °C for 24 h and allowed free access to water. Individual 24-h urine samples were collected. Urinary protein excretion was measured by a

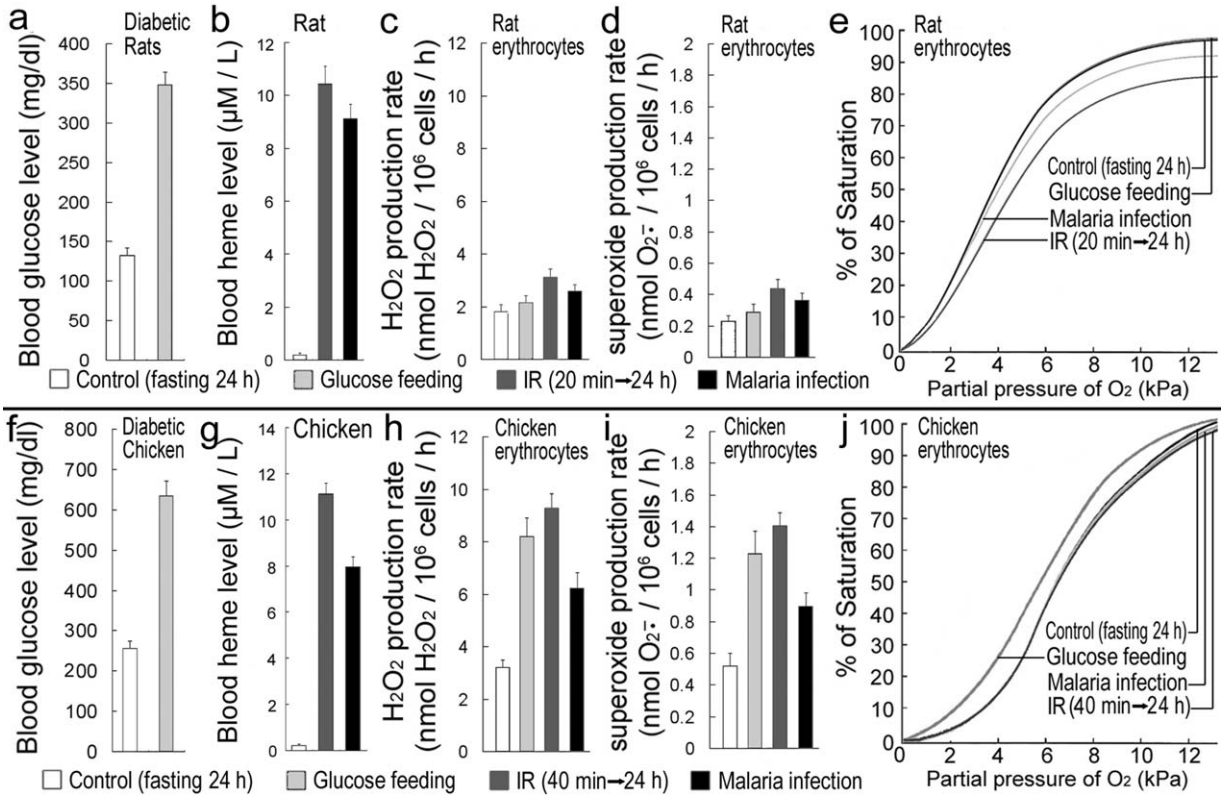

Figure 2. Erythrocyte ROS levels and O_2 dissociation curves after diabetes, ischemia reperfusion (IR), and malaria infection. The animals were fasted for 24 h (Control) and then 30% glucose solution was administered orally (to diabetic animals, every 8 h, three times total), or IR or malaria infection (to normal animals) was performed. Blood samples were taken at 24 h after the first glucose administration or 24 h after reperfusion, or 8 days after malaria infection. (a, f) Blood glucose level, (b, g) free heme level, (c, h) erythrocyte H_2O_2, (d, i) superoxide production rates, and (e, j) O_2 dissociation curves in diabetic rats and chickens were measured. Error bars show standard deviations ($n = 3$).

TCA turbidimetric method (*14*), and *N*-acetyl-β glucosaminidase was measured spectrophotometrically (*14*). Diabetic rats were acquired by streptozotocin treatment (*16*). While persistent hypoglycemia in chickens (diabetic chickens) were induced by tolbutamide administration (three times per day for 5 days, 200 mg tolbutamide/kg body weight) (*17*). Oral glucose feeding to diabetic animals (30% glucose solution, every 8 h, three times total) and blood glucose level determination were performed as described previously (*16*). Twenty-four hours after the first glucose feeding, kidney blood samples were collected. Malaria infection was applied with 10^6 *Plasmodium berghei* to normal rats (*18*) or 10^6 *Plasmodium gallinaceum* to normal chickens (*19*). The experimental procedures were approved by the local Animal Welfare Committee in accordance with the guidelines issued by the China Council on Animal Care.

Blood Free Heme Detection

Hemolysates were prepared by the method of Garrick et al. (*20*). Then, the free heme in lysates was separated by running through a 0.5 cm $\times$ 4 cm column (0.8 mL) of Dowex 1-X8 resin equilibrated in 2 M NaCl and 5 mM $NaPO_4$, pH 7.4. The column was rinsed with five times the bed volume of 2 M NaCl and 5 mM $NaPO_4$, pH 7.4, to assure that all the hemoglobin had passed through, and then with 10 times the bed volume of 5 mM $NaPO_4$, pH 7.4. Free heme was then eluted from the column with 10% SDS. Fractions of 900 μL were collected. Ninety microliters of 100 mM KCN was added to each fraction. A_{405} and A_{540} were read for each fraction. The amount of free heme was calculated from the A_{540} using an extinction coefficient of 11.1 or $A_{405} = A_{540} \times 8.15$ (*21*).

Statistical Analysis

An independent (unpaired) Student's *t*-test (two-tailed) was chosen to test the significance of differences among means of small "*n*" sample sets.

RESULTS

In Vitro Sugar and Heme Treatments

When human erythrocytes (without nuclei and mitochondria, Fig. 1a) were treated with 7% glucose or 50 μM heme, their

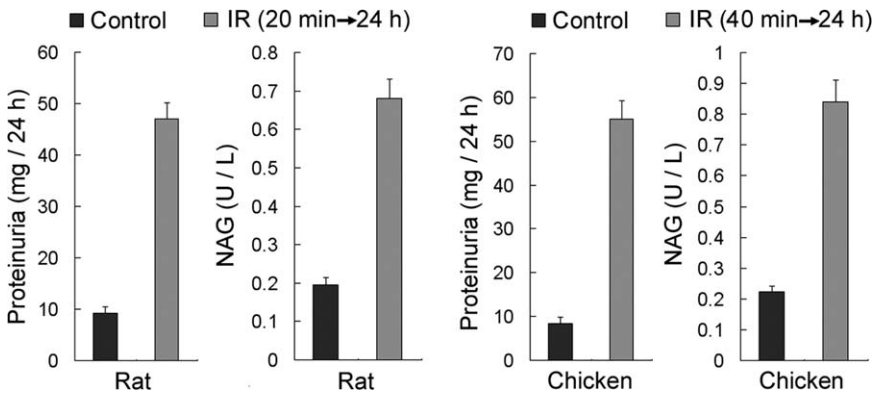

Figure 3. Ischemia reperfusion injuries to rat and chicken kidneys. Renal ischemia (IR) was induced by nontraumatic vascular clamps over the pedicles for 20 or 40 min for rats and chickens, respectively. After clamps were released, the incision was closed in two layers with 2-0 sutures. Two hours after renal ischemia, rats were placed in metabolic cages at 22 °C with a 12:12-h light–dark cycle and allowed free access to water. Individual 24-h urine samples were collected. Urinary protein excretion was measured by a TCA turbidimetric method, and *N*-acetyl-β glucosaminidase (NAG) was measured spectrophotometrically. Error bars show standard deviations ($n = 3$).

ROS levels were enhanced by less than 50% (Figs. 1b and 1c). Correspondingly, human blood oxygen dissociation curve was slightly affected by sugar/heme (Fig. 1d). Contrastingly, chicken (birds) erythrocytes with intact nucleus and mitochondria (Fig. 1e) accumulated much higher levels of ROS under the sugar/heme treatments, especially under the Glc-6-P treatment (Figs. 1f and 1g). Left shifts of blood oxygen dissociation curve were observed for chicken erythrocytes, especially for the Glc-6-P treatment (Fig. 1h), corresponding to their much elevated ROS levels.

In Vivo Diabetic IR or Malaria Experiments

In vivo experiments also indicated the sensibility of chicken erythrocytes to high-glucose and free-heme conditions. When diabetic rats or chickens were fed with 30% glucose solution (every 8 h, three times total), their blood glucose levels doubled similarly, although chickens have higher basic blood glucose levels (Figs. 2a and 2f). Renal ischemia (20 or 40 min for rats and chickens, respectively) and 24-h reperfusion caused similarly great damages to rat and chicken kidneys (indicated by greatly increased urinary protein excretion and *N*-acetyl-β glucosaminidase activity, Fig. 3) and large free-heme accumulation (Figs. 2b and 2g). Eight-day *Plasmodium* infections resulted in hemolysis and erythrocyte parasitism (30% for both rats and chickens, Fig. 4) and great free-heme accumulation (Figs. 2b and 2g). Similar to the above *in vivo* data of human erythrocytes, rat erythrocyte's redox state was insensitive to diabetics, IR, or malaria infection. They accumulated much lower levels of ROS under these circumstances (Figs. 2c and 2d) compared with chicken erythrocytes (Figs. 2h and 2i).

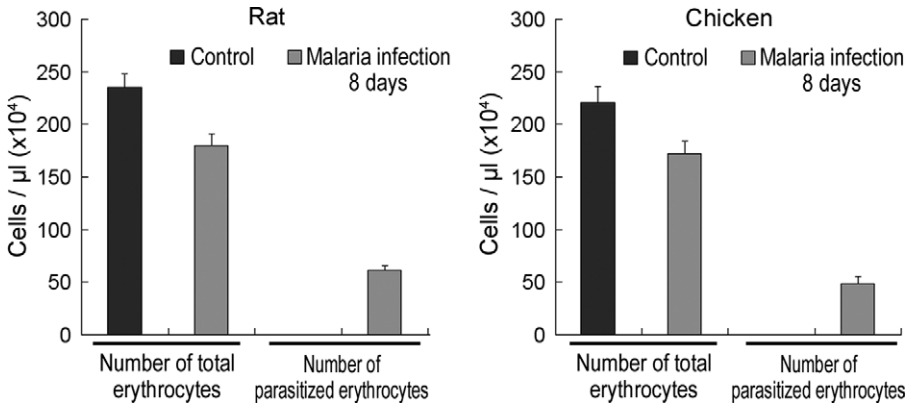

Figure 4. Severity of malaria infection to rats and chickens. Malaria infection was applied with 10^6 *Plasmodium berghei* to normal rats or 10^6 *Plasmodium gallinaceum* to normal chickens. Eight days later, the number of erythrocytes per microliter of renal blood ($\times 10^4$) and the number of parasitized erythrocytes per microliter of renal blood ($\times 10^4$) were examined by a microscopy. Error bars show standard deviations ($n = 3$).

Glucose feeding caused an apparent left shift of O_2 dissociation curve for diabetic chickens (Fig. 2j). However, diabetic rat O_2 dissociation curve could not be affected by the glucose feeding (Fig. 2e). Ischemia induced a shift of oxyhemoglobin dissociation curve to the right, which could not be reversed by reperfusion (Fig. 2e) (*22*). Severe malaria infection to mammals also induces a right shift of O_2 dissociation curve (Fig. 2e) (*23*). However, ROS themselves caused left shifts of O_2 dissociation curves (opposite to above shifts) (*13*). Therefore, IR or malaria-induced right shifts of blood O_2 dissociation curves were counteracted by ROS in chicken erythrocytes (Fig. 2j), but not in rat erythrocytes (Fig. 2e). Thus, nuclear and mitochondrial extrusion may help mammal erythrocytes to better adapt to hyperglycemia, ischemia, and reperfusion or malaria infection by reducing ROS accumulation.

DISCUSSION

Stress-induced hyperglycemia (such as head injury or acute stroke) and diabetes increase blood glucose substantially. Then, the Glc-6-P accumulation leads to great increases in mitochondrial membrane potential and ROS generation (*6, 7*). Mammal erythrocytes do not have mitochondria and, therefore, are insensitive to high levels of sugars.

While free heme generates ROS independent of mitochondria (*8*). Free heme damages lipid, protein, and DNA through the generation of ROS. Free heme catalyzes the oxidation, covalent crosslinking, and aggregate formation of protein and its degradation to small peptides. It also catalyzes the formation of cytotoxic lipid peroxide via lipid peroxidation and damages DNA through oxidative stress (*8*). However, not many membranes could be oxidized by free heme in mammal erythrocytes, and therefore, oxidative damages were less in mammal erythrocytes than in bird erythrocytes.

Mitochondria are connected with nucleus by the endomembrane system, such as endoplasmic reticulum. Mammalian erythroid cells undergo enucleation (nuclear extruding), an asymmetric cell division (*2, 3*) may involving extrusion of the endomembrane system including nuclear membranes and mitochondrial membranes. Therefore, the nucleus and mitochondria are distributed to the small daughter cell and disappear latterly. Rac GTPase-, mDia2-, and adenosine 5′-triphosphate-mediated actin filament movement may participate in this process (*2, 3, 24*). Enucleation may facilitate erythrocyte deformation into the biconcave shape and becoming soft and elastic (*3, 25*). On the other hand, nuclear and mitochondrial extrusion may help mammal erythrocytes to better adapt to high-sugar and high-heme conditions, where they live.

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