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RESEARCH ARTICLE | *Cardiovascular and Renal Integration*

H_2S-stimulated bioenergetics in chicken erythrocytes and the underlying mechanism

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[1]*Cardiovascular and Metabolic Research Unit, Laurentian University, Sudbury, Ontario, Canada;* [2]*School of Life Science, Shanxi University, Taiyuan, People's Republic of China;* [3]*College of Environment and Resources, Shanxi University, Taiyuan, People's Republic of China;* [4]*School of Human Kinetics, Laurentian University, Sudbury, Ontario, Canada;* [5]*Health Sciences North Research Institute, Sudbury, Ontario, Canada; and* [6]*Department of Biology, York University, Toronto, Ontario, Canada*

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Jin Z, Zhang Q, Wondimu E, Verma R, Fu M, Shuang T, Arif HM, Wu L, Wang R. H_2S-stimulated bioenergetics in chicken erythrocytes and the underlying mechanism. *Am J Physiol Regul Integr Comp Physiol* 319: R69–R78, 2020. First published May 20, 2020; doi:10.1152/ajpregu.00348.2019.—The production of H_2S and its effect on bioenergetics in mammalian cells may be evolutionarily preserved. Erythrocytes of birds, but not those of mammals, have a nucleus and mitochondria. In the present study, we report the endogenous production of H_2S in chicken erythrocytes, which was mainly catalyzed by 3-mercaptopyruvate sulfur transferase (MST). ATP content of erythrocytes was increased by MST-generated endogenous H_2S under normoxic, but not hypoxic, conditions. NaHS, a H_2S salt, increased ATP content under normoxic, but not hypoxic, conditions. ATP contents in the absence or presence of NaHS were eliminated by different inhibitors for mitochondrial electron transport chain in chicken erythrocytes. Succinate and glutamine, but not glucose, increased ATP content. NaHS treatment similarly increased ATP content in the presence of glucose, glutamine, or succinate, respectively. Furthermore, the expression and activity of sulfide:quinone oxidoreductase were enhanced by NaHS. The structural integrity of chicken erythrocytes was largely maintained during 2-wk NaHS treatment in vitro, whereas most of the erythrocytes without NaHS treatment were lysed. In conclusion, H_2S may regulate cellular bioenergetics as well as cell survival of chicken erythrocytes, in which the functionality of the electron transport chain is involved. H_2S may have different regulatory roles and mechanisms in bioenergetics of mammalian and bird cells.

ATP; avian cells; mitochondria; red blood cells; *S*-sulfhydration; 3-mercaptopyruvate sulfur transferase

INTRODUCTION

Mounting evidence connects metabolism of hydrogen sulfide (H_2S) to various physiological cellular and system functions (34, 45). Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) produce H_2S from L-cysteine, homocysteine, and cystathionine (37). Cysteine aminotransferase (CAT) catalyzes the interaction of L-cysteine with α-ketobutyrate to yield 3-mercaptopyruvate (3-MP). The conversion of 3-MP to pyruvate is catalyzed by 3-mercaptopyruvate sulfur transferase (MST), and eventually H_2S is released from the transformed persulfide (37). Given that sulfide at high concentration inhibits cytochrome-*c* oxidase as potently as cyanide (5), the ability of eukaryotic cells to synthesize adenosine triphosphate (ATP) through oxidation of sulfide is a surprisingly recent observation (7, 8). H_2S-driven bioenergetic processes in mitochondria have been observed in lugworms (33), chicken liver (44), rat liver (17), and mouse smooth muscle cells (SMCs) under hypoxia (7). These observations indicated that sulfide oxidation was localized in the mitochondrion and accompanied by ATP synthesis. Sulfide:quinone oxidoreductase (SQR) is bound to the inner mitochondrial membrane and intimately associated with the respiratory chain supercomplex (10, 17). It is involved in sulfide catabolism by oxidizing sulfide into thiosulfate or glutathione persulfide. H_2S oxidation-generated electrons may be transmitted to the ubiquinone, enhancing oxidative phosphorylation and ATP production.

The most prominent role of mature erythrocytes is to carry oxygen to all parts of the body (6). To accommodate this task and save space to carry as many oxygen molecules as possible, the nucleus and organelles gradually disappeared in mammalian erythrocytes over evolution. Whereas mammalian erythrocytes have ~120 days of life span, the life span of chicken erythrocytes is ~30 days (23). It has long been recognized that birds possess higher metabolic rates than other vertebrates (38a). Unlike mammalian erythrocytes, avian erythrocytes retain their nuclei and mitochondria, capable of aerobic metabolism via the tricarboxylic acid cycle. The functionality of mitochondria in these cells and its regulation, however, have been unknown (27). Furthermore, endogenous production of H_2S and its function in avian erythrocytes have not been studied to date.

We hypothesized that both endogenous production of H_2S and mitochondrion-driven ATP production are preserved in avian erythrocytes and that H_2S participates in the regulation of ATP content and cell survival. Since bioenergetics processes are different between avian and mammalian erythrocytes, this study will help us better understand the role of H_2S in regulating mitochondrial functions across species and under different metabolic conditions.

MATERIALS AND METHODS

Female broiler chickens (*Gallus gallus*) were obtained from Arkell Poultry and Equine Research Station of Guelph University (Guelph, ON, Canada) and housed at the Animal Care facility of Laurentian

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University in an air-conditioned environment (22 ± 1°C, 50 ± 5% relative humidity, 12:12-h light-dark cycle) with free access to standard chow diet. Chickens were allowed 5 days to acclimatize before the experiments, and chicken blood was withdrawn from brachial veins after the area around the ulnar-humoral joint was cleaned with 70% ethyl alcohol. Chicken blood was collected in heparin (1,000 U/mL) and kept on ice (21). The animal use protocol was in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Pub. No. 85-23, revised 1996) and approved by the Animal Care Committee of Laurentian University.

Separation and treatment of chicken erythrocytes. Chicken blood was collected on the day of use and centrifuged at 3,000 rpm for 3 min at 4°C. The supernatants and buffy coat were removed by aspiration. The remaining erythrocytes were resuspended in 10 volumes of phosphate-buffered saline solution (PBS, pH 7.4) and centrifuged at 3,000 rpm again for 3 min. The wash/centrifugation cycle was repeated three times. Then the erythrocytes were diluted 100 times with PBS.

For hypoxic experiments, erythrocytes diluted with PBS were incubated in an anaerobic chamber (Coy Laboratory Products) with continuous flow of a humidified gas mixture of 1% O_2, 94% N_2, and 5% CO_2 gas to mimic the hypoxic condition at 37°C. For normoxic experiments, the cells were incubated at 37°C in humidified room air (~20% O_2) containing 5% CO_2. For prolonged culture, chicken erythrocytes were incubated with 1640 medium from Roswell Park Memorial Institute (RPMI), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in humidified air containing 5% CO_2.

Measurement of ATP content. After different treatments, erythrocytes were sonicated on ice three times, each time for 10 s. ATP content was measured with an ATP Bioluminescent Assay Kit (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions. Briefly, the sonicated solutions with pH adjusted to 7.8 were allowed to react with ATP Assay Mix Reagent [1:1 (vol/vol)] containing luciferin and luciferase. ATP content was quantified by measuring the amount of light produced by the samples and fitting the results to a standard intensity vs. ATP content calibration curve. All experiments were performed at room temperature in the dark. The data are presented as percentage of control content for each group.

Measurement of H_2S production rate. H_2S production rate of the sonicated cells was measured as described previously (4, 7). Diluted erythrocytes were sonicated in ice-cold 50 mM phosphate-buffered saline (PBS, pH 6.8). Sonicated cells were then centrifuged at 10,000 rpm for 5 min, and the supernatant containing protein extract was isolated in a fresh tube. Protein content was measured with a BCA Kit (Thermo Fisher Scientific). Individual reaction flasks contained 0.6 mg/mL protein extract in 1 mL of 0.4 mM PBS (pH 7.4), to which L-cysteine, 3-mercaptopyruvate (3-MP), or pyridoxal 5′-phosphate (P5P) was selectively added in different experiment sets. The center well inside this reaction flask (25 mL in volume; Fisher Scientific, catalog no. S63268) contained a filter paper (2 × 2.5 cm) and 0.5 mL of trapping solution (0.05 M zinc acetate, 0.05 M Na_2EDTA; pH 12.8). In reactions examining the effect of inhibition of MST and CSE, 40 mM 2-ketobutyric acid (2-KA, a MST inhibitor) and/or 5 mM propargylglycine (PPG, a CSE inhibitor) was preincubated with 0.6 mg/mL protein extract at 37°C for 30 min before loading into reaction flasks. The flasks with center wells were flushed with nitrogen gas for 1 min after the reaction mixtures were loaded and then allowed to react in a water bath at 37°C for 90 min with continuous shaking at 50 rpm. The reaction was stopped by addition of 0.5 mL of 50% trichloroacetic acid, and the flasks were incubated at 37°C for another 60 min. The center well contents (filter paper and trapping solution) were transferred to test tubes, each containing 3.5 mL of water, into which 0.5 mL of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl and 0.5 mL of 30 mM $FeCl_3$ in 1.2 M HCl were added. The test tubes with all contents were kept in the dark for 20 min to form methylene blue. The absorbance of the resulting methylene blue at 670 nm was measured with a spectrophotometer (FLUOstar OPTIMA; BMG Labtech).

To overcome the low detection limit of the aforementioned spectrophotometry-based method for endogenous H_2S formation, we developed a new HPLC-based method for the experiments where 1 μM 3-MP was used as substrate to stimulate endogenous H_2S production. In this method, production of H_2S from the isolated and sonicated erythrocytes was trapped and reacted with *N*, *N*-dimethyl-*p*-phenylenediamine sulfate and $FeCl_3$ to yield methylene blue. Methylene blue formation was detected with Dionex Ultimate 3000 U-HPLC (Thermo Scientific) with acetonitrile and 0.1% trifluoroacetic acid in HPLC-grade water as mobile phase. A 30-min gradient was run with a C18 column (250 × 4.6, 5 μm) (Thermo Scientific Hypersil GOLD) with 20-μL injection volume, 0.5 mL/min flow rate, and 30°C column temperature. The detector was set at 660 nm.

Measurement of protein content. Protein content was determined with Pierce BCA Protein Assay Reagent (Thermo Fisher, Waltham, MA). The working reagents were prepared by mixing BCA Reagent A and Reagent B (50:1, Reagent A:B). To each well, 100 μL of the working reagents was added and then 20 μL of each standard or sample replicate was pipetted into the plate. The plate was covered and incubated at 37°C for 30 min and then cooled to room temperature for 3 min. A plate reader was used to measure the absorbance at 560 nm. Different concentrations of bovine serum albumin (BSA) were used to calibrate the standard protein concentration curve.

Western blotting. Erythrocytes were lysed with RIPA buffer in the presence of protease inhibitor cocktail (Sigma, St. Louis, MO). Equal amounts of proteins (100 $\mu g \cdot 20\ \mu L^{-1} \cdot well^{-1}$) were boiled and separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Pall Corporation, Pensacola, FL). The membranes were blocked with 5% milk and then incubated overnight at 4°C with appropriate primary antibodies. Peroxidase-conjugated secondary antibodies (1:2,000) were used and visualized with ECL (GE Healthcare, Amersham, UK). The dilutions of primary antibodies were as follows: rabbit polyclonal CBS antibody (1:2,000; Abnova, H00000875-D01P), mouse monoclonal CSE antibody (1:1,000; Abnova, H00001491-M01), rabbit polyclonal MST (1:1,000; Novus Biologicals, NBP1-82617), rabbit polyclonal SQRDL antibody (1:500; ProteinTech Group, 17256-1-AP), rabbit polyclonal α-tubulin antibody (1:1,000; Cell Signaling Technology, 2125S), and mouse monoclonal GAPDH antibody (1:1,000; Santa Cruz Biotechnology, sc-365062).

Biotin switch assay of S-sulfhydration. S-sulfhydration of proteins was detected by biotin switch assay as described previously (19, 42). In brief, erythrocytes were isolated and kept at −80°C for 10 min and then centrifuged and washed with HEN buffer at 14,000 rpm for 10 min at 4°C five times, until the pellet became white. Thereafter, pellets were homogenized in HEN buffer [in mM: 250 HEPES-NaOH (pH 7.7), 1 EDTA, 0.1 neocuproine], supplemented with 100 μM deferoxamine and 1% protease inhibitor cocktail, and centrifuged at 14,000 rpm for 10 min at 4°C. Protein content was measured, and 1 mg of protein in 100 μL of HEN buffer was taken with the addition of the same volume of HEN-NaHS solution (100 μM). The sample was incubated at 37°C for 1 h with shaking and then in blocking buffer (HEN buffer adjusted to 2.5% SDS and 20 mM methyl methanethiosulfonate) at 50°C for 20 min. The samples were precipitated with acetone at −20°C for 1 h. Then the protein pellets were resuspended in blocking buffer, and 30 mM biotin-HPDP (Thermo Scientific, catalog no. 21341) and 1% SDS were supplemented for another 3-h incubation at 25°C. The biotinylated proteins were precipitated with streptavidin-agarose beads (Sigma, catalog no. S1638) and washed with HEN buffer and PBS. SDS-PAGE was performed to separate the biotinylated proteins, and anti-SQR antibody (SQRDL) (1:500; ProteinTech Group, 17256-1-AP) was used for Western blotting analysis.

SQR activity assay. SQR activity was detected by thioredoxin-dependent activity assay under air at room temperature, as described

previously (30). The reaction solution (1 mL) contained 50 mM potassium phosphate (pH 8.2), 100 μM decylubiquinone (Sigma), 15 μM thioredoxin (from *Escherichia coli*; Sigma), 0.2 U of thioredoxin-reductase (from *E. coli*; Sigma), 1 mM NADPH, and protein sample. The reaction was started with the addition of NaHS at the final concentration of 1 μM (prepared freshly with N_2-flushed H_2O), and the decrease in absorption at 275 nm was followed for 1.5 min.

Blood smear and Giemsa staining. A glass microscope slide (25 × 75 × 1 mm; VWR, catalog no. 48312-705) was placed on a horizontal surface, and one drop (30–40 μL) of isolated chicken erythrocytes was added onto one end of the slide. A 6-in. wood applicator stick (Fisherbrand, catalog no. 23400119) was used, lying across the glass slide and keeping the applicator in contact with the blood and glass. The stick was moved down the glass slide to the opposite end. Then air-dried film was fixed in absolute methanol and air-dried. The slide was stained with diluted Giemsa stain [1:20 (vol/vol) with water buffered to pH 7.2; Sigma, catalog no. GS500]. The slide with film was washed with PBS (pH 7.2) and water three times. Finally, the film was air-dried in a vertical position.

Survival of chicken erythrocytes in vitro. Chicken erythrocytes were isolated by differential centrifugation of whole blood at 3,000 rpm for 3 min at 4°C. The top plasma and buffy coat layers were discarded. The packed erythrocytes were then washed three times by resuspension in sterile PBS (pH 7.4) and centrifuged for 3 min at 3,000 rpm and 4°C. Cell density was counted with a hemocytometer, and 1.25×10^6 cells were plated and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Oakville, ON, Canada) supplemented with sodium bicarbonate, BSA [0.1% (wt/vol)], 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin and adjusted to pH 7.4. The control group was treated with PBS, whereas the other groups were treated with 1 μM NaHS and incubated in a humidified atmosphere containing 5% CO_2 and 95% air. The culture medium was partially replaced with fresh medium and NaHS or PBS every 2 days. Cells were stained with Trypan blue solution [0.4% (wt/vol)], and cell number was counted with a hemocytometer.

Transmission electron microscopy. The collected blood was fixed in 1% osmium tetroxide and 1.25% potassium ferrocyanide in cacodylate buffer for 1.5 h, immediately after sampling. It was then washed with cacodylate buffer three times and with water two times. Dehydration was done through a graded series of ethanol (20 min in 70%, 20 min in 90%, 30 min in 100%, and 30 min in propylene

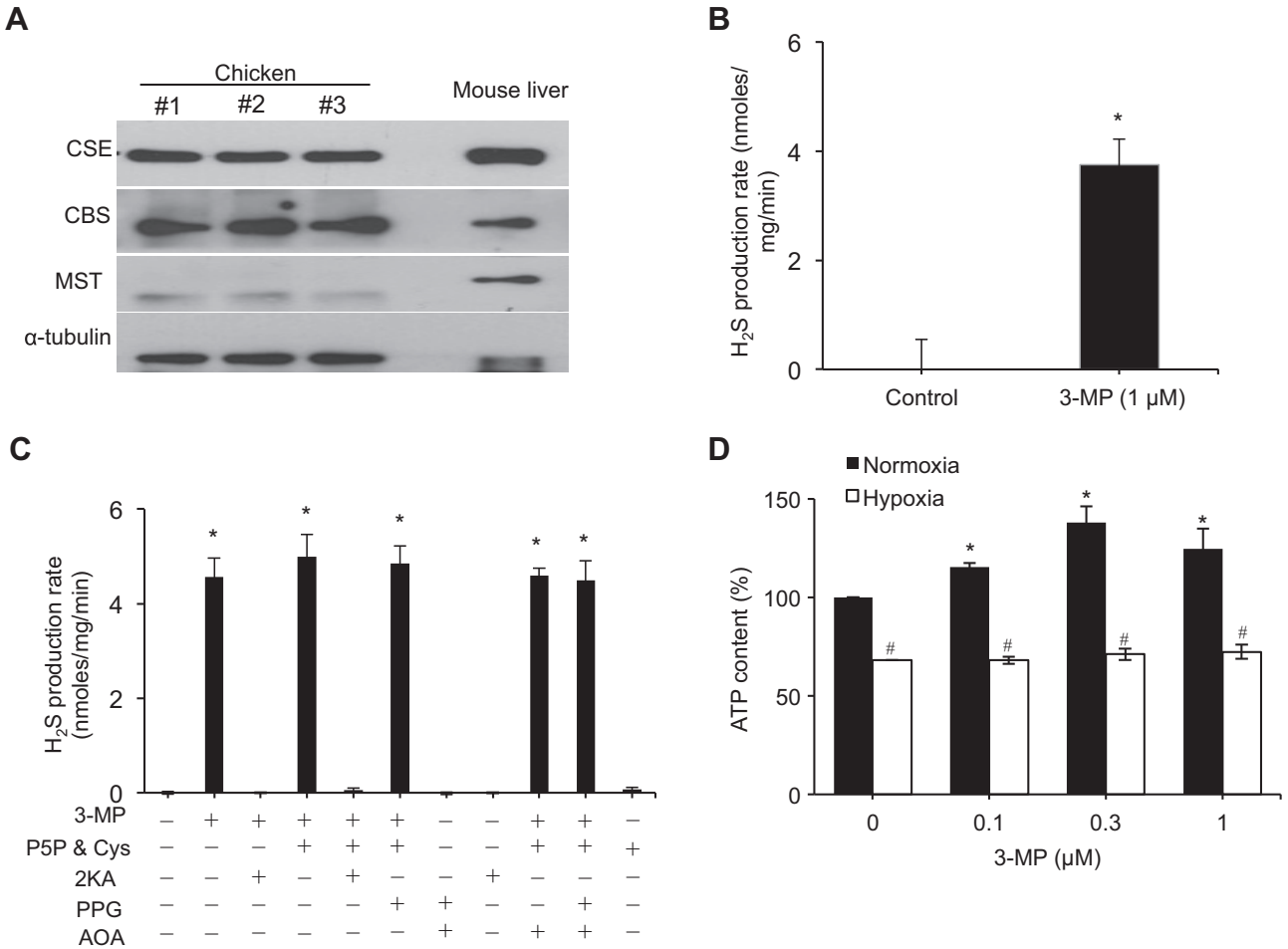

Fig. 1. H_2S production in chicken erythrocytes. *A*: expression of cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfur transferase (MST) in chicken erythrocytes, detected by Western blot analysis. α-Tubulin was used as load control. Example Western blot lanes are from chicken erythrocytes from 3 different chickens. *B*: H_2S production rate of chicken erythrocytes in the presence or absence (Control) of 3-mercaptopyruvate (3-MP, final concentration at 1 μM), detected by HPLC-based methylene blue detection. $n = 4$. *C*: roles of H_2S-producing enzymes in H_2S production in chicken erythrocytes, detected with spectrophotometry-based methylene blue detection. The final concentration of 3-MP was 1 mM. AOA, aminooxyacetic acid; Cys, L-cysteine; 2KA, 2-ketobutyric acid; P5P, pyridoxal 5′-phosphate; PPG, propargylglycine. *$P < 0.01$ vs. the group without any treatment. $n = 4$. *D*: the effect of 3-MP on ATP content in chicken erythrocytes under normoxia (5% CO_2 in room air) or hypoxia (1% O_2, 94% N_2, and 5% CO_2) conditions. Chicken erythrocytes were incubated with 3-MP at different concentrations for 1 h at 37°C in the absence of glucose ($n = 6$). *$P < 0.05$ vs. control groups (0 3-MP); #$P < 0.05$ vs. normoxia conditions. *n*, number of chickens.

oxide). Finally, the blood samples were infiltrated with a series of exposure to propylene oxide and Epon and embedded with epoxy resins at 70°C overnight. Ultrathin sections (80 nm) were obtained by using a Leica Ultracut UTC ultramicrotome. The sections were placed onto copper grids, stained with uranyl acetate and lead citrate, and examined under a Hitachi H-7500 transmission electron microscope at the Centre for the Neurobiology of Stress of University of Toronto. The images were captured with an Olympus SIS Megaview II digital camera.

Statistical analysis. Each experiment was performed with at least five biological replicates. All data are presented as means ± SE. Statistical analyses were carried out by one-way ANOVA with Dunnett's multiple-comparisons post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

MST-dependent H_2S production in chicken erythrocytes. The expressions of CSE, CBS, and MST were detected by Western blot in chicken erythrocytes (Fig. 1*A*). In the presence of 3-MP (the substrate of MST), the erythrocytes produced a significant amount of H_2S (Fig. 1*B*), which was not further enhanced by 10 mM L-cysteine (the substrate of CSE and CBS) and 2 mM pyridoxal 5′-phosphate (P5P) (Fig. 1*C*). Propargylglycine (PPG, inhibitor of CSE) at 5 mM and aminooxyacetic acid (AOA, inhibitor of CBS as well as CSE) at 1 mM failed to affect 3-MP-based H_2S production, but 40 mM 2-ketobutyric acid (2-KA, inhibitor of MST) completely abolished it (Fig. 1*C*). Although the reaction kinetics with L-cysteine alone without 3-MP was not measured, the blockade of 3-MP-based H_2S production by 2-KA, rather than by PPG or AOA, strongly suggests that MST is the main H_2S-producing enzyme in chicken erythrocytes.

Effect of H_2S on ATP content is oxygen dependent. To explore the relationship of endogenous H_2S production and ATP content, chicken erythrocytes were incubated with 3-MP. ATP content was significantly increased in the presence of 3-MP at concentrations from 0.1 to 1 μM under normoxic conditions (Fig. 1*D*). The failure of 3-MP to affect ATP contents under hypoxic conditions (Fig. 1*D*) suggests the need of oxidative phosphorylation for ATP content in chicken erythrocytes. NaHS (a H_2S salt) significantly increased ATP content at low concentrations (1–3 μM) (Fig. 2*A*). Hypoxia decreased the ATP content of chicken erythrocytes by 35% in comparison with the normoxic condition. NaHS did not affect ATP content under hypoxic conditions (Fig. 2*A*). We further tested the time-dependent effect of NaHS (1 μM) on ATP content. NaHS increased ATP content 10 min after the addition of NaHS (Fig. 2*B*). Therefore, treatment with 1 μM of NaHS for 10 min was used in the following experiments.

Succinate is the most effective substrate for ATP production in chicken erythrocytes. ATP production in chicken erythrocytes requires substrates different from those for mammalian erythrocytes (15). In the present study, chicken erythrocytes were incubated with glucose, glutamine, and succinate for 50 min, respectively. Thereafter, the erythrocytes were washed with PBS three times for 3 min for each time before being subjected to ATP content assay. Whereas succinate increased ATP content most effectively and glutamine had a slight stimulatory effect at 25 mM, glucose at 10 or 25 mM had no effect on ATP content (Fig. 3*A*). NaHS treatment similarly increased ATP contents regardless of whether glucose, glutamine, or succinate was included as the substrate (Fig. 3*B*).

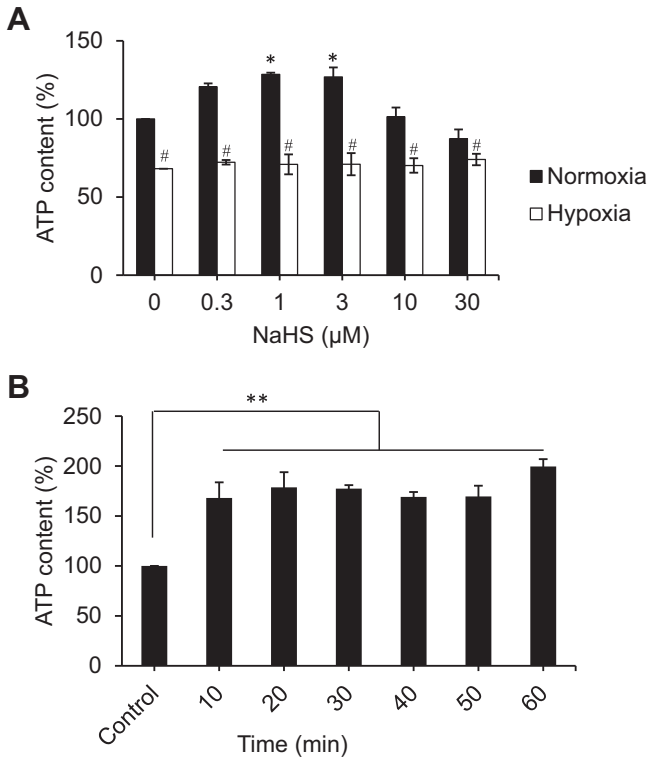

Fig. 2. The effect of H_2S on ATP content in chicken erythrocytes under normoxic and hypoxic conditions. *A*: chicken erythrocytes were exposed to normoxia (5% CO_2 in room air) or hypoxia (1% O_2, 94% N_2, and 5% CO_2) and treated with NaHS at different concentrations at 37°C for 1 h. *$P < 0.05$ vs. control groups (0 NaHS); #$P < 0.05$ vs. normoxia conditions. $n = 6$. *B*: chicken erythrocytes, under normoxia conditions, were treated with NaHS (1 μM) at 37°C for different times (0–60 min). **$P < 0.01$. $n = 6$. Chicken erythrocytes in all groups were incubated with glucose-free PBS. *n*, number of chickens.

Insulin stimulates glucose uptake by insulin-sensitive cells (18). Neither insulin and glucose alone, nor coapplication of insulin and glucose, affected basal ATP content of the erythrocytes (Fig. 3*C*), consistent with a previous report (2). However, coapplication of insulin and glucose decreased the stimulatory effect of NaHS on ATP content in comparison to the effects of NaHS in the presence of insulin alone or glucose alone (Fig. 3*C*). It is likely that a low level of endogenous glucose in cellular plasma would not be sufficient to consume ATP. The effects of insulin/glucose to lower NaHS-increased ATP content may result from increased glucose influx that then boosts ATP consumption.

H_2S-increased ATP content involves the functionality of electron transport chain in mitochondria. To investigate the target of H_2S affecting ATP content in erythrocytes, inhibitors of various complexes in the electron transport chain (ETC) were used. In the presence of rotenone (inhibitor of Complex I), atpenin A5 (inhibitor of Complex II), and antimycin A (inhibitor of Complex III), not only was ATP content virtually depleted but also NaHS failed to affect ATP content (Fig. 3*D*).

After incubation of the erythrocytes with low concentration of NaHS (3 μM) for 6–24 h, the expression of SQR was upregulated compared with the control level in the absence of NaHS treatment (Fig. 4*A*). H_2S may regulate cellular functions by inducing *S*-sulfhydration of specific cysteine residue(s) of targeted proteins (19, 42). In this study, we found that SQR

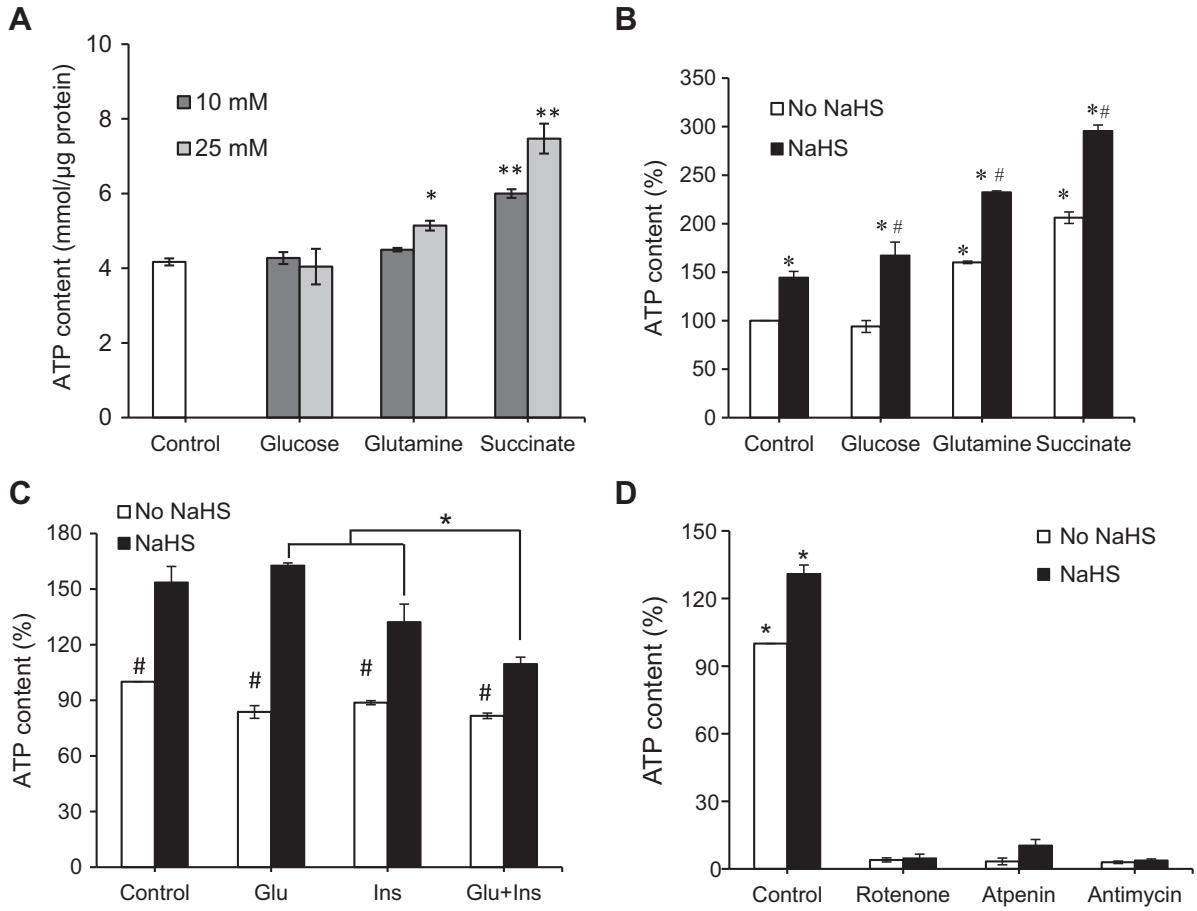

Fig. 3. ATP content with different substrates in chicken erythrocytes. *A*: contributions of different substrates to ATP content in chicken erythrocytes. $*P < 0.05$, $**P < 0.01$ vs. Control (glucose-free PBS incubation). $n = 4$ for each group. *B*: the effect of H_2S on ATP content with different substrates. Chicken erythrocytes were treated with 25 mM glucose, glutamine, or succinate for 50 min and then NaHS (1 μM) for 10 min at 37°C. $*P < 0.05$ vs. control without NaHS; $\#P < 0.05$ vs. control with NaHS. $n = 4$ for each group. *C*: the effect of H_2S on ATP content in the presence of glucose (Glu, 25 mM) and/or insulin (Ins, 0.5 ng/mL) for 50 min. NaHS (1 μM) was added for 10 min at 37°C. $\#P < 0.05$ vs. NaHS treatment in the same group; $*P < 0.05$ vs. other groups as marked. $n = 4$ for each group. *D*: effects of different inhibitors for mitochondrial electron transport chain. Chicken erythrocytes were diluted 100 times and treated with rotenone (1 μM), atpenin A5 (0.037 μM), and antimycin A (100 μM) for 1 h and then with 100 μM NaHS for 10 min at 37°C. $*P < 0.05$ vs. all other groups. $n = 4$. *n*, number of chickens.

was significantly *S*-sulfhydrated in native erythrocytes. The baseline *S*-sulfhydration was significantly decreased by MST inhibitor 2-KA treatment (Fig. 4*B*). Moreover, the activity of SQR was significantly increased and sustained by NaHS treatment (Fig. 4*C*).

Structural integrity of chicken erythrocytes was preserved by NaHS in vitro. To observe the effect of NaHS on the morphological changes of chicken erythrocytes, the cells were collected and incubated in vitro and the culture media containing 3 μM NaHS were changed every other day. At the beginning of incubation, mature erythrocytes were oval in general shape, with clear membrane contour and nuclei (Fig. 5*A*). As the incubation continued, plasma membrane of untreated cells began to degrade (Fig. 5*B*) and eventually disappeared after 1 wk, whereas most of the NaHS-treated erythrocytes appeared with normal shape with condensed and smaller nucleus (Fig. 5*B*). Two weeks after in vitro incubation, the untreated cells showed aberrant morphology, and their membranes swelled or were destroyed with obscure nuclei (Fig. 5*B*). In contrast, NaHS-treated cells appeared with much more intact contour and regular shape (Fig. 5*B*). These observations suggested that exogenous H_2S protected the integrity of chicken erythrocytes.

We further incubated the isolated chicken erythrocytes in vitro with RPMI 1640 at 37°C and then counted the numbers of living cells under a dissecting microscope. Within 2 wk of incubation, all the cells were dead under this condition. With the addition of NaHS to RPMI 1640 medium, however, there were significantly more viable cells during the 2-wk incubation (Fig. 6*A*). Electromicroscopic examination clearly showed the presence of nuclei and mitochondria in chicken erythrocytes (Fig. 6, *B* and *C*). The cells with 1-week in vitro incubation with PBS exhibited more degraded nuclei and fewer mitochondria, whereas those treated with NaHS for the same period largely maintained intact nuclei and more mitochondria (Fig. 6*B*). Two-week incubation damaged the cell membrane and exhibited degraded nuclei and even fewer mitochondria, and these damages were more obvious in nontreated erythrocytes than in NaHS-treated cells (Fig. 6*C*).

DISCUSSION

H_2S was the "energy fuel" for some of the earliest forms of life on Earth (35). This bioenergetic role has been reserved over billions of years of life evolution, from single-cell organ-

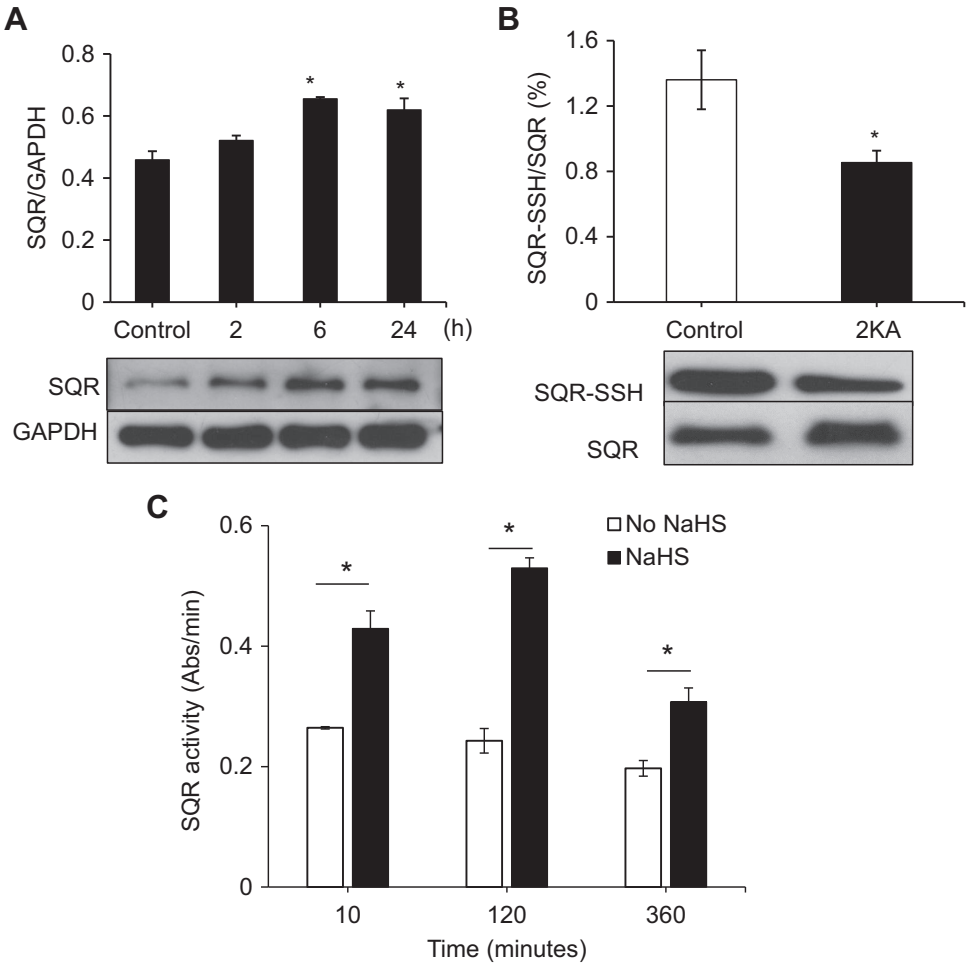

Fig. 4. Effects of H_2S on sulfide:quinone oxidoreductase (SQR) expression and activities. *A*: NaHS-induced upregulation of SQR. Chicken erythrocytes were incubated with 1 μM NaHS at 37°C for different periods. GAPDH was used as loading control. $n = 6$. *B*: *S*-sulfhydration (SSH) of SQR. Chicken erythrocytes were incubated with PBS or 40 mM 2-ketobutyric acid (2-KA) at 37°C for 2 h, and total proteins were obtained from hemoglobin-free lysate. $n = 4$. *C*: NaHS-enhanced activity of SQR. Chicken erythrocytes were incubated with 1 μM NaHS at 37°C. Samples were obtained after 10-min, 120-min (2 h), or 360-min (6 h) treatment. Absorption at 275 nm after the reaction in presence of thioredoxin was followed for 1.5 min. $^*P < 0.05$ vs. Control groups. $n = 4$ for each group. *n*, number of chickens.

isms to sophisticated human beings. Belonging to different lineages, the convergent evolution of birds and mammals is manifested with many similar signaling mechanisms, bioenergetic processes, and functionalities. The shared bird-mammal characteristics are considered to contribute to the increased sustained activity and enhanced parental care during evolution for these two lineages (40). Although H_2S signaling pathway and bioenergetic process regulated by this pathway have been extensively studied in mammals, endogenous H_2S production and its physiological functions have been unknown in birds to date. Erythrocytes from both birds and mammals perform the same function as the oxygen carrier for the body, with hemoglobin as the main vehicle. The bioenergetic processes in red blood cells from these two groups, however, are very different. Bird red blood cells contain the mitochondrion, which is the place where oxidative phosphorylation-dependent ATP production occurs. Mammalian red blood cells do not have the mitochondrion or nucleus, and obviously ATP production in these cells is not mitochondrion dependent. We also know little about endogenous metabolism of H_2S in erythrocytes from mammals, even less in those from birds. H_2S production in erythrocytes from brown bears results likely from L-Cys-based CSE activity, but no attempt was made to detect the expression of H_2S-producing enzymes in these red blood cells (22). The comparison between wild-type mice and CSE-knockout mice shows no difference in H_2S production, with L-cysteine as substrate and P5P as cofactor, in erythrocytes from these two strains of mice. This observation suggests that the expression and/or activity of CSE do not contribute to the endogenous production of H_2S in mouse erythrocytes (43). Human erythrocytes produce endogenous H_2S in the presence of MST substrate (3-MP) but not the substrates of CSE/CBS (L-cysteine and homocysteine). Furthermore, the expression of MST protein, but not of CSE/CBS, was detected with Western blot analysis in human erythrocyte lysates (32). The expression of CSE and CBS in the erythrocytes from chickens, but not those from humans are not needed for the endogenous H_2S production because only MST is functionally responsible for endogenous H_2S production in both human and chicken cells. CSE and CBS are likely needed for mitochondrion-related cysteine and homocysteine metabolism in mitochondrion-containing cells such as chicken erythrocytes (7, 39).

The present study was designed to first characterize endogenous H_2S production and its enzymatic foundation in chicken erythrocytes and then investigate H_2S-mediated bioenergetic processes in these bird cells. We detected the expression of all three known H_2S-producing enzymes (CSE, CBS, and MST) in chicken erythrocytes (Fig. 1*A*). On the basis of our data, we cannot conclude as to which enzyme is expressed the most abundantly because of the differences in respective antibody affinities and the lack of calibration of the same. Interestingly,

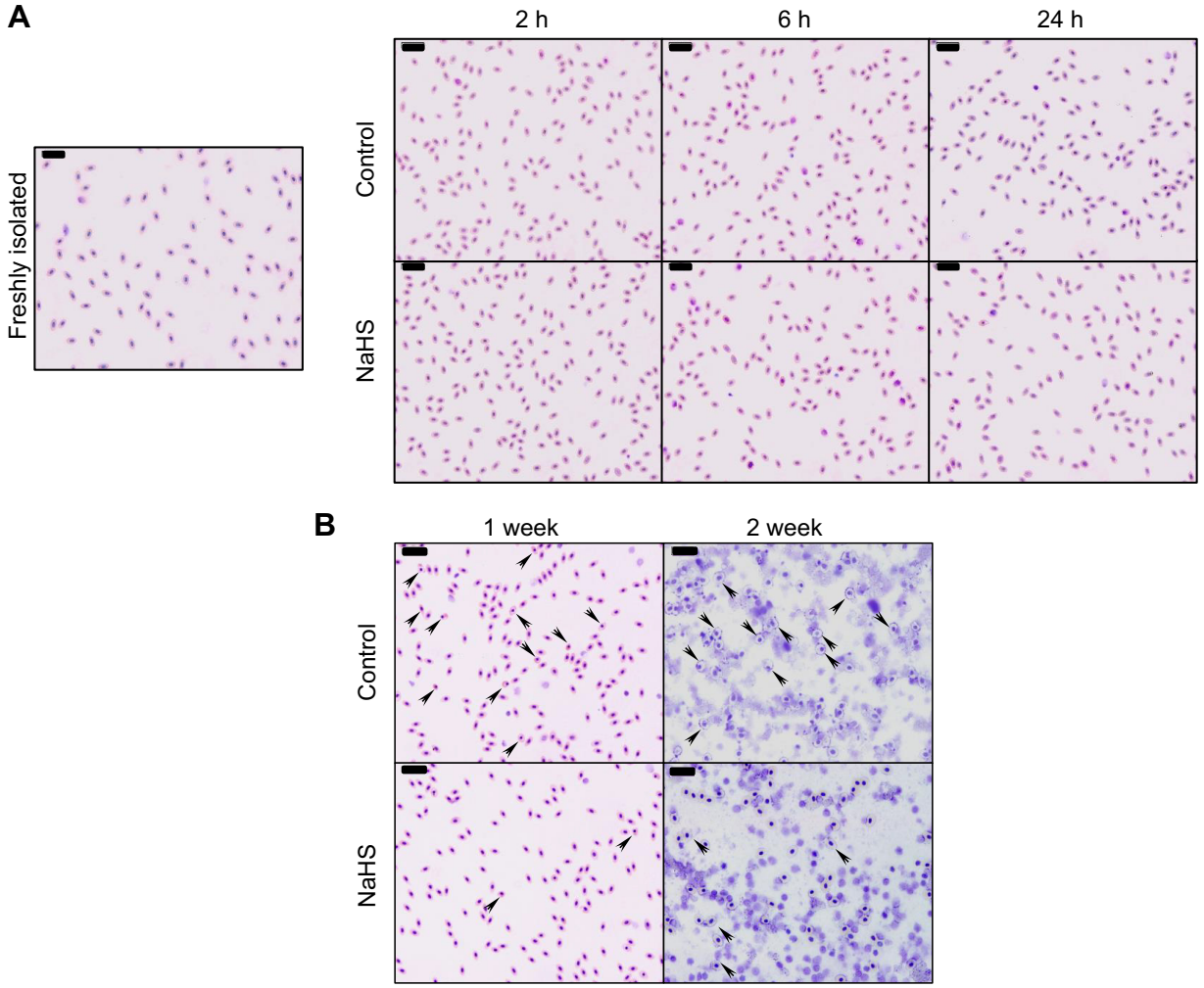

Fig. 5. Morphology of cultured chicken erythrocytes. The cells were incubated with PBS (control) or PBS plus NaHS (1 μM) in vitro for different periods at 37°C. The erythrocytes (×640) were stained with Giemsa. The nucleus was stained blue and the cytoplasm pink. Scale bars, 20 μm. *A*: morphological changes in erythrocytes with short term of incubation with or without NaHS. Freshly isolated chicken erythrocytes without NaHS treatment are shown on *left*. *B*: morphological changes of erythrocytes with longer term of incubation with or without NaHS. The arrows point to examples of lysed cell membrane, more in the Control groups than in NaHS-treated groups.

we found that these red blood cells produced H_2S in the presence of 3-MP, which was blocked by 2-KA (Fig. 1, *B* and *C*). This 3-MP-induced H_2S production directly linked to 3-MP increased ATP content (Fig. 1*D*). The functionality of MST in chicken erythrocytes is thus established. On the other hand, the stimulatory effect of 3-MP on H_2S production was not further enhanced by L-cysteine and P5P (Fig. 1*C*) and neither blocked by PPG and AOA.

The major conduit of ATP production in eukaryotes is the citric acid cycle/oxidative phosphorylation in the mitochondrion, which relies on sufficient oxygen supply. Mature mammalian erythrocytes are among the few exceptions for eukaryotes in which ATP production relies on oxygen-independent glycolysis because of the lack of the mitochondrion. Having the same mission of carrying oxygen in the circulation as that of mammalian erythrocytes, mature avian erythrocytes have both the nucleus and the mitochondrion. The regulation of ATP production in avian erythrocytes, however, has been unknown. In the present study, we found that ATP content was decreased greatly under hypoxic conditions (Fig. 2*A*) and blocked totally by inhibitors of ETC complexes (Fig. 3*D*). It appears that the main contributor of ATP generation in chicken erythrocytes is oxidative phosphorylation in the mitochondrion.

H_2S participates in the regulation of mitochondrial ATP generation in different types of eukaryotes (19, 33, 44). In mouse smooth muscle cells (SMCs), NaHS decreased mitochondrial ATP production under normoxia but stimulated ATP production under hypoxia (7). It is likely that H_2S has two action modes in mitochondrial bioenergetics in mammalian eukaryotes. By inhibiting cytochrome-*c* oxidase (34), H_2S inhibits the oxidative phosphorylation-dependent ATP production at high concentration. By acting on SQR, on the other hand, H_2S stimulates oxygen-independent ATP production at low concentration (28). Under hypoxia, basal ATP level is already low, and the oxidative phosphorylation process ceases to be functional and the role of cytochrome-*c* oxidase is very much weakened. The direct stimulatory effect of H_2S on SQR-related ATP production may emerge with this low basal ATP background.

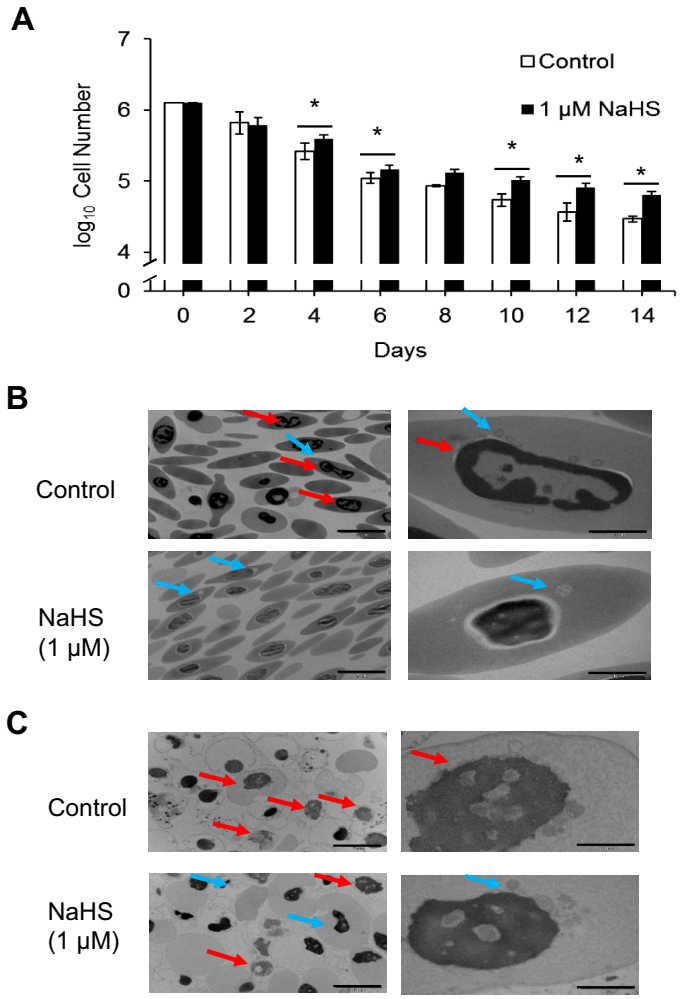

Fig. 6. The viability of chicken erythrocytes incubated in vitro at 37°C. *A*: effect of NaHS on the viability of chicken erythrocytes. *Day 0* was when the cells were freshly obtained. Cell numbers were counted under a dissecting microscope in the presence of 0.1% Trypan blue. $*P < 0.05$. $n = 4$ for each group. *n*, number of chickens. *B*: morphologies of chicken erythrocytes incubated in vitro for 1 wk. *C*: morphologies of chicken erythrocytes incubated in vitro for 2 wk. Scale bars, 5 μm (*left*) and 1 μm (*right*) for *B* and *C*. Red arrows point to smeared and degenerated nuclei, and blue arrows point to mitochondria.

The present study revealed that MST-catalyzed production of H_2S (Fig. 1*C*) elevated ATP content in chicken erythrocytes (Fig. 1*D*). Exogenously applied H_2S salt (NaHS) increased ATP content under normoxic condition (Fig. 2*A*). Under hypoxic conditions, on the other hand, neither 3-MP nor NaHS has any effect on ATP content in chicken erythrocytes (Fig. 1*D* and Fig. 2*A*). These findings indicate that H_2S regulates the oxygen-related bioenergetics of chicken erythrocytes in opposite fashion as that for mammalian eukaryotes, such as SMCs (7). One explanation of this phenomenon is that the oxygen dependence of the bioenergetics of chicken erythrocytes is significantly lower than that of mammalian eukaryotes, although both are dependent on the mitochondrial ETC. Hypoxia decreased basal ATP content by ~30% in chicken erythrocytes but by >90% in mammalian SMCs (7). It is known that flight is the most important function for birds, which is among the most energy-consuming activities (25). About 95% of energy in avian species is provided by the mitochondrion (26). It appears that adaptive evolution in avian cells leads to bioenergetics processes as well as the role of H_2S different from those of mammalian eukaryotes.

It has been suggested that sulfide oxidation via SQR directly donates electrons to quinone, bypassing Complexes I and II of ETC in the mitochondrion (5, 9, 11, 14, 17, 28). The donated electrons are eventually shuttled from Complexes III and IV via cytochrome *c*, leading to ATP production. Accordingly, SQR-mediated electron transfer in ETC is not sensitive to inhibition of Complex I or Complex II (succinate dehydrogenase). The results shown in Fig. 3*D* that the bioenergetic effects of H_2S were blocked by the inhibitors of Complexes I and II carry two connotations. First, ETC functionality is required for both the basal and H_2S-enhanced ATP content in chicken erythrocytes. Hypoxia would decrease electron acceptance at the level of Complex IV, which would explain the lack of effects of 3-MP and NaHS on ATP content under hypoxia (Fig. 1*D* and Fig. 2*A*). Second, it seems unlikely that a SQR-driven electron transfer in ETC can explain the stimulatory effect of H_2S on ATP content in chicken erythrocytes. The presence of SQR in the mitochondrion of chicken erythrocytes and its role in sulfide oxidation have been unknown. In the present study, we detected the expression of SQR in chicken erythrocytes (Fig. 4*A*). The posttranslational modification (i.e., *S*-sulfhydration) and activity of SQR were increased by H_2S (Fig. 4, *B* and *C*). Increased total activities of SQR may decrease the availability of H_2S by increasing sulfide oxidation. By doing so, SQR may indirectly compensate the effect of H_2S on ATP content in avian erythrocytes.

Another major difference between avian erythrocytes and other types of mitochondrion-containing cells is the role played by glucose in bioenergetics. The plasma membrane of avian erythrocytes is much less permeable to glucose (13). Glucose transport in avian erythrocytes also does not respond to multiple modulatory factors that are effective in avian muscle cells, e.g., insulin or alterations in intracellular Na^+ content (2). Our study shows that basal glucose level in chicken erythrocytes is not affected by extracellular concentrations of glucose, being glucose free or at 10–25 mM (Fig. 3). This observation is consistent with a previous report that glucose is not the primary energy substrate for chicken erythrocytes (15). Different from glucose, nucleosides and glutamine are primary energy substrates for chicken erythrocytes (15). This was also confirmed in our study, in which glutamine and, more significantly, succinate increased ATP content in chicken erythrocytes (Fig. 3, *A* and *B*). Succinate, but not glutamine, is the substrate of Complex II in ETC. NaHS increased ATP content regardless of the presence or absence of different ATP substrates: glucose, glutamine, or succinate (control groups in Fig. 3*B*). Therefore, the stimulatory effect of NaHS on ATP content may not target on the availability of ATP substrates.

In recent years, low micromolar to high nanomolar concentrations of H_2S in circulation were reported under physiological conditions. For example, plasma free sulfide levels are reported at ~0.2 μM in humans and 0.8 μM in mice (24), 0.2–2.5 μM in mice and pigs (16, 36), and 1.08–2.00 μM in brown bears (22). Furthermore, free sulfide is interchangeable with sulfane-sulfur and acid-labile sulfide under different conditions (36). H_2S salt in aqueous state is in the form of 3 units of HS^- and 1 unit of free H_2S at pH of 7.4 (34). One needs also

to consider rapid evaporation of free H_2S from liquid in in vitro open systems (petri dishes, test tubes, organ baths, etc.), whereas under physiological conditions H_2S in systemic circulation is retained in a relatively closed vessel system (34). The present study used NaHS as the source of H_2S to examine bioenergetics of chicken erythrocytes. In our study, NaHS at the concentrations of 1–3 μM increased ATP content of chicken erythrocytes. We consider this effect of NaHS on chicken erythrocytes as being physiologically relevant on the basis of the aforementioned literature and our present experimental conditions.

We used NaHS to treat erythrocytes for 10–60 min. Not surprisingly, ATP level was increased by NaHS rapidly and continually (Fig. 2*B*). H_2S and HS^- rapidly permeate the plasma membrane of human red blood cells, with a H_2S permeability coefficient at 37°C of 0.01 cm/s (12). This is much faster than most published information on H_2S and HS^- transport to bacteria, algae, and marine invertebrates, only slower than to artificial lipid bilayers. Whether the rapid passage of H_2S and HS^- across erythrocyte membranes leads to efficient trapping or consumption of endogenous H_2S in these cells has been unclear. The answer to this question rests in future studies to examine the formation of sulfur-bound proteins, such as sulfhemoglobin or oxidized ferric hemoglobin (metHb), and the speed and extent of sulfur oxidation in red blood cells. The rapid effect of NaHS, on the other hand, may partially explain the fast upregulation of SQR expression induced by H_2S in as short as 6 h (Fig. 4*A*). Rapid upregulation of SQR expression and increased SQR activity also suggest SQR being a direct target of H_2S signal, which is consistent with the previous conclusion that H_2S oxidation begins with its binding to SQR (20).

The function of erythrocytes requires these cells to have a flexible shape for easy transportation in the circulation and effective volume control for optimizing oxygen-carrying hemoglobin content. NaHS has been reported to increase the volume of human erythrocytes in vitro at the concentrations of 2.5 and 10 μM (3). In vitro incubation of chicken erythrocytes with NaHS for a prolonged period offers protection for the integrity and survival of these cells (Figs. 5 and 6). These effects can be explained by direct interaction of exogenous H_2S with various cellular proteins or H_2S-caused decrease in oxidative stress. Consequently, erythrocyte membrane integrity is reserved to a certain extent. There is also an alternate explanation. ATP content is one of the most critical metabolic parameters for erythrocyte viability. ATP depletion leads to malfunctioned biochemical reactions, followed by deteriorated biophysical/mechanical properties of erythrocytes. Providing ATP content maintains at physiological level, the chance for erythrocytes to function normal and survive is high (1). In this context, the ability of H_2S to increase erythrocyte ATP content may strengthen ATP-dependent structural and functional integrity of erythrocytes. For example, activation of calcium-activated potassium (K_{Ca}) channels in human red blood cells decreases cell volume (3). The viscosity and rigidity of erythrocyte membranes depend on Na^+-K^+-ATPase activity (31). By increasing ATP content in erythrocytes, H_2S may augment the activities of K_{Ca} channels (38) as well as Na^+-K^+-ATPase.

Perspectives and Significance

We discovered the expression of three H_2S-producing enzymes in chicken erythrocytes, which contain mitochondria. Endogenous H_2S is produced mainly by MST in chicken erythrocytes. Bioenergetics of chicken erythrocytes is mitochondrion and oxygen dependent but not significantly affected by glucose levels. Exogenous NaHS at physiologically relevant concentrations (1–3 μM) elevates ATP level under normoxic conditions. Upregulated expression and increased activity of SQR by H_2S may increase sulfide oxidation to compensate the effect of H_2S on ATP content. Furthermore, incubation of chicken erythrocytes with NaHS for a prolonged period in vitro enhances cell integrity (Fig. 5) and viability (Fig. 6). One of the potential mechanisms for this NaHS protection is the increased ATP content in these cells. The in vitro incubation conditions with NaHS for chicken erythrocytes are not physiological. The protective effect of NaHS incubation, however, offers a clue for long-term preservation of red blood cells in vitro. These findings may widen and deepen our understanding of the role of H_2S in regulating different cellular bioenergetics processes, oxygen dependent or independent, in avian as well as mammalian cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Z.J., Q.Z., L.W. and R.W. conceived and designed research; Z.J., Q.Z., E.W., R.V., M.F., T.S., and H.M.A. performed experiments; Z.J., Q.Z., E.W., M.F., T.S., H.M.A., L.W., and R.W. analyzed data; Z.J., Q.Z., E.W., M.F., T.S., H.M.A., L.W., and R.W. interpreted results of experiments; Z.J., Q.Z., E.W., M.F., T.S., and H.M.A. prepared figures; M.F., L.W., and R.W. drafted, edited and revised manuscript; L.W. and R.W. approved final version of manuscript.

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