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Hemin-mediated Hemolysis in Erythrocytes: Effects of Ascorbic Acid and Glutathione

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Abstract In the present work, we investigated the effect of ascorbic acid and glutathione on hemolysis induced by hemin in erythrocytes. Ascorbic acid not only enhanced hemolysis, but also induced formation of thiobarbituric acid-reactive substances in the presence of hemin. It has been shown that glutathione inhibits hemin-induced hemolysis by mediating hemin degradation. Erythrocytes depleted of glutathione became very sensitive to oxidative stress induced by hemin and ascorbic acid. H_2O_2 was involved in hemin-mediated hemolysis in the presence of ascorbic acid. However, a combination of glutathione and ascorbic acid was more effective in inhibiting hemolysis induced by hemin than glutathione alone. Extracellular and intracellular ascorbic acid exhibited a similar effect on hemin-induced hemolysis or inhibition of hemin-induced hemolysis by glutathione. The current study indicates that ascorbic acid might function as an antioxidant or prooxidant in hemin-mediated hemolysis, depending on whether glutathione is available.

Key words erythrocyte; ascorbic acid; hemin; glutathione; H_2O_2

Heme (iron protoporphyrin IX) is a functional group of various proteins, including hemoglobin, myoglobin, cytochromes, nitric oxide synthases and cystathionine β-synthase [1,2]. Under some pathological conditions, such as β-thalassemia, sickle cell anemia, glucose 6-phosphate dehydrogenase deficiency, hemorrhage, and muscle injury, hemin (ferric protoporphyrin IX) might be released [3–5]. An excess of hemin can then interact with the cell membrane, resulting in formation of reactive oxygen species, cellular injury [6]. Hemin also functions as a catalyst for the oxidation of low-density lipoprotein [7]. The toxic effects of hemin on erythrocytes include inhibition of erythrocyte enzymes, and dissociation of erythrocyte membrane skeletal proteins [8,9]. Hemin might cause hemolysis by a colloid-osmotic mechanism, that is, hemolysis is preceded by the loss of potassium from erythrocytes [10].

Glutathione (GSH), the most abundant cellular antioxidant, can protect erythrocytes against hemin-induced hemolysis [11]. Furthermore, it has been reported that GSH itself could mediate hemin degradation by an oxygen-dependent mechanism [12]. Ascorbic acid, another cellular antioxidant, has been shown to inhibit oxidation of human low-density lipoprotein induced by hemin and H_2O_2 and to suppress Ca^{2+}-Mg^{2+}-ATPase inactivation in pink or white ghosts by t-butyl hydroperoxide and hemin [13,14]. However, ascorbic acid can react with iron or iron-containing compounds to produce hydroxyl radical or H_2O_2 [15–17]. In sickle ghosts exposed to H_2O_2, ascorbate at low concentrations (<20 μM) inhibits thiobarbituric acid reactive substrates (TBARS) production, whereas it increases TBARS production at high concentrations (>50 μM) [18].

It has been shown that GSH and ascorbic acid have synergistic antioxidant effects on hemin-mediated cellular toxicity. The combination of GSH and ascorbic acid inhibits lipid peroxidation in rat liver microsomes and the degradation of folic acid induced by hemin and H_2O_2 [19, 20]. Our previous results demonstrated that ascorbic acid

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could enhance GSH-dependent hemin degradation by increasing the production of H_2O_2 [21].

In the present work, we studied the effect of ascorbic acid and GSH on hemolysis induced by hemin. Our results indicated that ascorbic acid function as an antioxidant or prooxidant in hemin-mediated hemolysis.

Materials and Methods

Materials and reagents

Hemin was purchased from Biobasic Inc. (Markham, Canada). 3-amino-1,2,4-triazole (3-AT), catalase, 5,5′-dithiobis (2-nitrobenzoic acid), diethylmaleate, 2,4-dinitrophenylhydrazine, ascorbic acid, and dehydroascorbic acid (DHA) were purchased from Sigma Chemical (St. Louis, USA). GSH was purchased from Shanghai Dongfeng Biochemical (Shanghai, China) and thiobarbituric acid (TBA) from Shanghai Hengxin Chemical Reagent Co. (Shanghai, China). All other reagents were of analytic grade.

Blood was obtained from healthy human volunteers by venipuncture into heparinized tubes and centrifuged at 2000 *g* for 10 min at 4 °C. The plasma was removed and packed erythrocytes were washed three times with phosphate-buffered saline (PBS; 10 mM sodium phosphate, 135 mM NaCl, pH 7.4). The buffy coat of white cells was removed. The washed erythrocytes were suspended in PBS in a final hematocrit of 5%.

Hemin was freshly prepared at the beginning of each experiment as a stock solution of approximately 1–2 mM in 5 mM NaOH and was kept in the dark on ice. Hemin concentration was determined in 5 mM NaOH using an extinction coefficient of 5.84×10^4 $cm^{-1}\cdot M^{-1}$ at 385 nm.

Hemolysis assay

Erythrocyte suspension was incubated with various reagents at 37 °C. One milliliter of aliquots was removed and centrifuged for 3 min at 3000 *g*. The degree of hemolysis was determined by the absorbance of hemoglobin at 540 nm in the supernatant. Absorbance at 100% hemolysis was determined by adding 10 μl of Triton X-100 (10%, *V*/*V*) to 1 ml of the erythrocyte suspension.

Measurement of TBARS

TBARS was assayed as described by Stocks and Dormandy [22]. Briefly, 0.4 ml of 28% (*W*/*V*) trichloroacetic acid and 0.1 M sodium arsenite solution was added to 0.8 ml of erythrocyte suspension. Samples were mixed and centrifuged at 3400 *g* for 5 min. We took 0.8 ml of supernatant from each of these samples and combined them with 0.2 ml of 1% (*W*/*V*) TBA in 0.05 M NaOH to a final volume of 1.0 ml. Samples were boiled for 15 min in capped microcentrifuge tubes and cooled on ice. The absorbance at 532 nm was determined with quantification based upon an extinction coefficient of 1.56×10^5 $cm^{-1}\cdot M^{-1}$.

Measurement of GSH

GSH in erythrocytes was assayed according to the colorimetric method by evaluating the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) [23]. Briefly, 1 ml of 5% erythrocyte suspension was added to 1 ml of distilled water. Three milliliters of precipitating solution [1.67% (*W*/*V*) glacial metaphosphoric acid, 0.2% (*W*/*V*) EDTA and 30% (*W*/*V*) NaCl] was mixed with the hemolysate. The mixture was allowed to stand for 5 min then filtered. We added 0.2 ml of filtrate to 0.8 ml of 0.3 M Na_2HPO_4 solution in a cuvette, then 0.1 ml of 0.04% (*W*/*V*) 5,5′-dithiobis(2-nitrobenzoic acid) in 1% (*W*/*V*) sodium citrate solution was added. The absorbance was measured at 412 nm.

Catalase inactivation

Catalase inhibition in erythrocytes exposed to 3-AT *in vitro* is a function of H_2O_2 flux within the cells [16]. In our experiments, 5% erythrocytes were incubated with PBS containing 15 μM hemin, 0.2 mM ascorbic acid, 40 mM 3-AT and 1 mM diethylenetriaminepentaacetic acid at 37 °C. At various time intervals, 1 ml of each sample was removed to microcentrifuge tubes. Erythrocytes in these tubes were washed three times with PBS, then lysed in 1 ml of lysis buffer (sodium phosphate buffer, 5 mM, pH 8.0). Ten microliters of lysate was added to 990 μl of 6 mM H_2O_2. Decomposition of H_2O_2 was followed by the absorbance at 236 nm. Uninhibited catalase activity was calculated from the mixture only containing erythrocytes and 3-AT.

Measurement of ascorbic acid

Ascorbic acid in erythrocytes was assayed as described by Omaye *et al*. [24]. Briefly, 1 ml of erythrocyte suspension was added to 1 ml of ice-cold 10% trichloroacetic acid, mixed well, and centrifuged at 3500 *g* for 15 min. Then 0.5 ml of supernatant was mixed with 0.1 ml of 2,4-dinitrophenylhydrazine/thiourea/copper solution (3.0 g 2,4-dinitrophenyl-hydrazine, 0.4 g thiourea, 0.05 g $CuSO_4\cdot5H_2O$ in 100 ml 65% H_2SO_4) and incubated for 3 h at 37 °C. The mixture was added to 0.75 ml of ice-cold 65% H_2SO_4, mixed well, and kept at room

temperature for 30 min. The absorbance was determined at 520 nm.

Statistics

Data represent mean±SD of five separate experiments. The paired *t*-test was used to compare mean values of two groups (with and without ascorbic acid). Multiple comparisons between groups were made by one-way ANOVA. $P<0.05$ was considered statistically significant.

Results and Discussion

Extracellular ascorbic acid induces oxidative stress in the presence of hemin

Hemin is a potential hemolytic agent [10]. Approximately 27% hemolysis was induced by 50 μM hemin after 60 min incubation with human erythrocytes. However, the addition of ascorbic acid to erythrocytes caused an increase in hemolysis induced by hemin, and 0.2 mM ascorbic acid is sufficient to achieve maximal effect (data not shown). Fifty micromoles of hemin caused 37%, 43%, and 58% of hemolysis in the presence of 0.05, 0.1, and 0.2 mM ascorbic acid, respectively [**Fig. 1(A)**]. In the current study, ascorbic acid alone did not elicit hemolysis (data not shown).

The possibility that ascorbic acid might stimulate lipid peroxidation of erythrocytes in the presence of hemin was assessed by measuring the formation of TBARS. As shown in **Fig. 1(B)**, hemin (50 μM) alone caused minimal TBARS formation but greatly promoted TBARS formation in the presence of ascorbic acid. The level of TBARS was increased with increasing concentration of ascorbic acid [**Fig. 1(B)**]. TBARS formation was not detected in the presence of ascorbic acid alone. It has not been clearly established whether hemin promotes lipid peroxidation [25, 26]. Schmitt *et al*. suggested that this depends on the state of hemin [27]. At lower concentrations (<1 μM), hemin mainly exists as monomers. Hemin aggregates with increased concentration [28]. Monomers are effective in promoting lipid peroxidation, whereas aggregates are responsible for the increase in permeability and membrane damage. According to Schmitt *et al*., there is a lack of correlation between the permeability changes and lipid peroxidation induced by hemin [27]. The present results are consistent with this view, as hemin itself causes hemolysis but is unable to induce apparent formation of TBARS. Thus, the effects of hemin alone on hemolysis are associated with the dissociation of erythrocyte

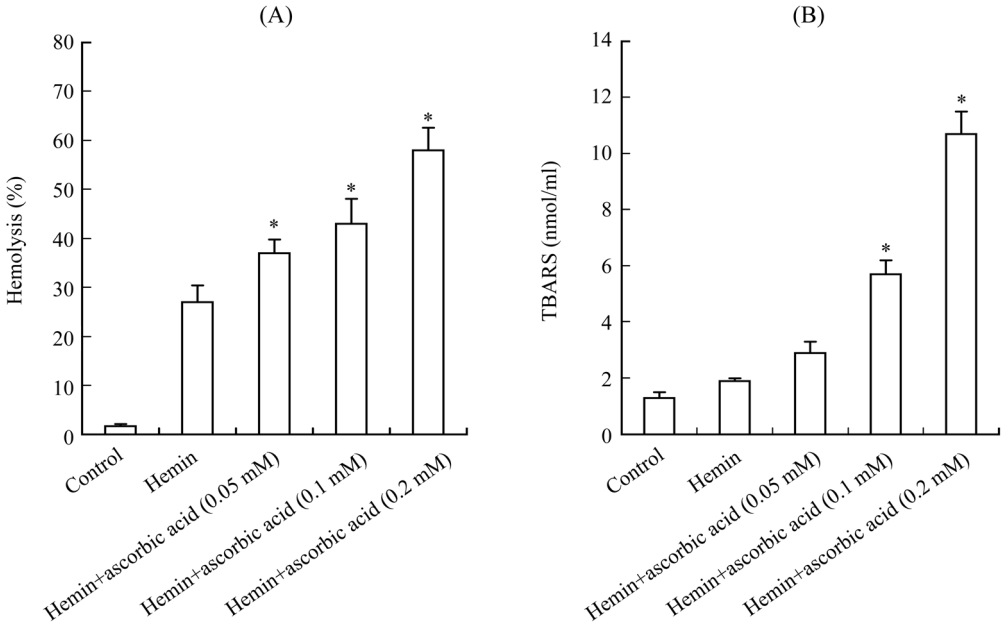

Fig. 1 Ascorbic acid stimulates hemolysis and formation of thiobarbituric acid-reactive substances (TBARS) induced by hemin
Erythrocyte suspensions (5%) were incubated with 50 μM hemin in the presence or absence of ascorbic acid at 37 °C for 60 min. (A) Hemolysis. (B) TBARS. * $P<0.05$ compared with hemin alone. Data represent mean±SD of five separate experiments.

membrane proteins, which disturbed the two-layer phospholipid membrane [8,9].

H_2O_2 is involved in the oxidative stress induced by hemin and ascorbic acid

It has been shown that ascorbic acid can react with iron or iron-containing compounds to produce H_2O_2 [15–17]. We therefore studied whether H_2O_2 is generated by hemin and ascorbic acid. Catalase inhibition in the presence of 3-aminotriazole has been used to demonstrate H_2O_2 influx in erythrocytes [16]. Incubation of erythrocytes with 15 μM hemin and 0.2 mM ascorbic acid for 30 min resulted in 34% inactivation of catalase (**Fig. 2**), indicating H_2O_2, generated from hemin and ascorbic acid at an extracelluar site, reaches catalase within the cell. To further confirm whether H_2O_2 is involved in the oxidative stress by hemin and ascorbic acid, the effect of catalase on hemolysis and formation of TBARS was investigated. Although catalase had no effect on hemolysis induced by hemin alone, it effectively abolished the enhanced hemolysis as well as TBARS formation in the presence of hemin and ascorbic acid (data not shown). These data indicate that H_2O_2 becomes the primary source of oxidant stress for hemolysis and lipid peroxidation. The net reaction leading to formation of H_2O_2 proceeds as follows (**Reaction 1**):

$$\text{Ascorbic acid}+O_2 \rightarrow H_2O_2+\text{dehydroascorbic acid} \quad 1$$

This reaction is catalyzed by hemin. Thus, H_2O_2 might participate in a Fenton reaction leading to the formation of hydroxyl free radical (**Reaction 2**):

$$\text{Heme-}Fe^{2+}+H_2O_2 \rightarrow \text{Heme-}Fe^{3+}+HO\cdot \quad 2$$

In the current study, it is speculated that HO· is the primary cause of hemolysis induced by hemin and ascorbic acid. However, more experiments need to be done to prove this.

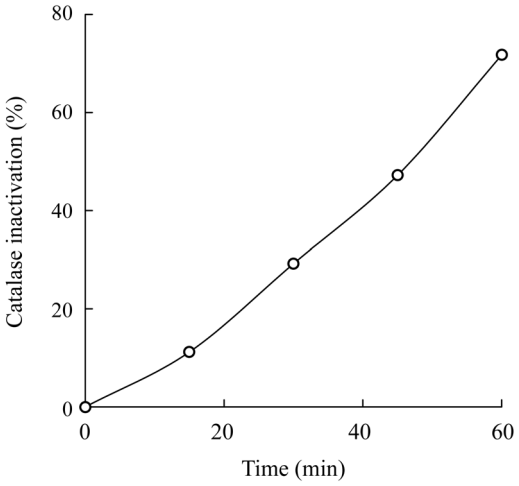

Fig. 2 Time-course of erythrocyte catalase (CAT) inactivation by hemin and ascorbic acid

Erythrocyte suspensions (5%) were incubated with 15 μM hemin, 0.2 mM ascorbic acid and 40 mM 3-amino-1,2,4-triazole at 37 °C for 60 min.

Effect of intracellular GSH on oxidative stress mediated by hemin and ascorbic acid

To assess the effect of intracellular GSH on oxidative stress induced by hemin and ascorbic acid, erythrocytes were depleted of GSH by preincubation with 1 mM diethylmaleate for 60 min. Diethylmaleate depletes GSH in a conjugation reaction catalyzed by GSH-S-transferase [29]. At this concentration of diethylmaleate, the level of intracellular GSH in erythrocytes decreased from 2.3 mM to 0.25 mM. This treatment did not cause further oxidative stress in erythrocytes [29]. However, erythrocytes depleted of GSH became very sensitive to oxidative stress induced by hemin and ascorbic acid. Compared with normal erythrocytes, an increase in hemolysis and TBARS formation induced by 50 μM hemin and 0.2 mM ascorbic acid was observed in erythrocytes depleted of GSH (**Table 1**).

We further evaluated whether replenishment of GSH by *D*-glucose metabolism in the pentose cycle provides more protection of cells against oxidative stress by hemin and ascorbic acid. As shown in **Table 1**, TBARS formation induced by hemin and ascorbic acid was much lower in cells in the presence of 0.5 mM *D*-glucose than in the absence of *D*-glucose. GSH levels in the absence and presence of 0.5 mM *D*-glucose were 0.31 mM and 1.5 mM, respectively, after erythrocytes were incubated with 50 μM hemin and 0.2 mM ascorbic acid for 60 min. These results indicate that intracellular GSH plays an important

Table 1 Effect of intracellular glutathione on oxidative stress by hemin and ascorbic acid

Sample	TBARS (nM/ml cells)	Hemolysis (%)
Normal erythrocytes	9.5±1.3	53±6.7
Erythrocytes−GSH	16.7±0.9*	77±6.1*
Erythrocytes+*D*-glucose	5.2±0.4*	43±3.1

Erythrocyte suspensions (5%) were incubated with 50 μM hemin and 0.2 mM ascorbic acid in the presence or absence of 5 mM *D*-glucose at 37 °C for 60 min. Erythrocytes were depleted of GSH by preincubation with 1 mM diethylmaleate for 60 min. *P<0.05 compared with normal erythrocytes. Data were represented as mean±SD (n=5). TBARS, thiobarbituric acid-reactive substances; GSH, glutathione.

role in protecting the cell against oxidative stress induced by hemin and ascorbic acid.

GSH and ascorbic acid have synergistic antioxidant effects on hemin-mediated hemolysis

Our previous results have demonstrated that ascorbic acid enhances GSH-dependent hemin degradation, due to an increase in the production of H_2O_2 by hemin and GSH [21]. In the current study, our data indicated that the combination of GSH and ascorbic acid was more effective in inhibiting hemolysis induced by hemin than GSH alone (**Table 2**). Thus, ascorbic acid might protect erythrocytes against hemin-induced damage if enough GSH is available. According to previous results, hemin might initially react with GSH and produce $O_2\cdot^-$. H_2O_2 is generated by ascorbic acid and $O_2\cdot^-$ if ascorbic acid exists in the system, whereas H_2O_2 is generated from dismutation of $O_2\cdot^-$ in the absence of ascorbic acid. Thus, ascorbic acid enhances GSH-mediated inhibition of hemolysis induced by hemin through the production of H_2O_2. To test this hypothesis, the effect of catalase on the inhibition of hemolysis by GSH and ascorbic acid was investigated. Preincubation of catalase (100 U/ml) with erythrocytes suppressed the inhibitory effect of GSH and ascorbic acid on hemolysis induced by hemin (**Table 2**). These results imply that H_2O_2 is also involved in hemin-mediated hemolysis inhibition by GSH and ascorbic acid.

Table 2 Effect of extracellular glutathione (GSH) and ascorbic acid on hemolysis induced by hemin

Sample	Hemolysis
Normal erythrocytes	29±3.1
Erythrocytes+GSH	17±1.8*
Erythrocytes+AA+GSH	7.2±1.2*
Erythrocytes+AA+GSH+catalase	22±2.2$^{\#}$

Erythrocyte suspensions (5%) were incubated with 50 μM hemin at 37 °C for 60 min. The concentrations of GSH, ascorbic acid, and catalase were 1 mM, 0.2 mM, and 100 U/ml, respectively. *P<0.05 compared with normal erythrocytes; $^{\#}P$<0.05 compared with Erythrocytes+AA+GSH. Data represent mean±SD (n=5). GSH, glutathione; AA, ascorbic acid.

Effect of intracellular ascorbic acid on hemin-mediated hemolysis

Although ascorbic acid is transported across erythrocyte membranes with a much lower efficiency, incubation of erythrocytes with DHA results in marked accumulation of intracellular ascorbic acid [30]. We examined the effect of intracellular ascorbic acid on hemolysis by hemin. After incubation of erythrocytes (normal cells) with 0.5 mM DHA in the presence of 5 mM glucose for 30 min, the level of intracellular ascorbic acid rose from 71 μM to 1.2 mM. The level of intracellular GSH remained constant (2.1 mM). The erythrocytes loaded by DHA (cells with high ascorbic acid) and normal cells were further used to prepare GSH-depleted erythrocytes. The cells were incubated with 1 mM diethylmaleate for 60 min. As demonstrated by May *et al*., diethylmaleate treatment has no effect on concentration of intracellular ascorbic acid [29]. In our experiments, the level of ascorbic acid was 1.0 mM in the DHA-loaded cells after diethylmaleate treatment. The level of GSH in the erythrocytes treated with diethylmaleate (GSH-depleted cells with high ascorbic acid) decreased to 0.29 mM. The level of GSH in normal cells was 0.19 mM when treated with diethylmaleate (GSH-depleted cells).

We next evaluated the effect of hemin on hemolysis and formation of TBARS in four types of erythrocytes (normal cells, GSH-depleted cells, cells with high ascorbic acid, GSH-depleted cells with high ascorbic acid) in the presence of *D*-glucose. As shown in **Table 3**, 50 μM hemin caused 22%, 35%, 10%, and 54% hemolysis in these four types of erythrocytes, respectively. In the presence of *D*-glucose, GSH was replenished by the metabolism of *D*-glucose in the pentose cycle. Of these four types of erythrocytes, hemolysis was lowest in cells with high ascorbic acid, and highest in GSH-depleted cells. Hemin alone interacts with the erythrocyte membrane, resulting

Table 3 Effect of intracellular ascorbic acid on hemolysis and thiobarbituric acid-reactive substances (TBARS) by hemin

Sample	Hemolysis (%)	TBARS (nM/ml cells)
Normal erythrocytes	22±1.8	1.1±0.1
Erythrocytes−GSH	35±3.7	2.7±0.4*
Erythrocytes+AA	10±1.1*	3.9±0.3*
Erythrocytes−GSH+AA	54±4.2*$^{\#¢}$	14.9±0.9*$^{\#¢}$

Erythrocyte suspensions (5%) were incubated with 50 μM hemin at 37 °C for 60 min. Cells with high ascorbic acid were generated by treatment of 0.2 mM dehydroascorbic acid (+AA) with erythrocytes in the presence of 5 mM *D*-glucose at 37 °C for 30 min. Erythrocytes were depleted of glutathione (−GSH) by preincubation with 1 mM diethylmaleate for 60 min. *P<0.05 compared with normal erythrocytes; $^{\#}P$<0.05 compared with erythrocytes−GSH; $^{¢}P$<0.05 compared with erythrocytes+AA . Data were represented as mean±SD (n=5). TBARS, thiobarbituric acid-reactive substances; GSH, glutathione; AA, ascorbic acid.

in membrane damage and hemolysis. However, hemin easily diffuses into erythrocytes through the cell membrane [12]. Thus, hemin can react with intracellular ascorbic acid. The reaction might be enhanced with time as more extracellular hemin and intracellular ascorbic acid would have a chance to react as permeability enhancement proceeds. According to our previous results, hemin reacts first with GSH in a system, where both GSH and ascorbic acid exist [21]. In normal cells with high ascorbic acid, ascorbic acid enhances the hemin degradation mediated by GSH, as there is enough GSH available in the presence of *D*-glucose. Thus, of these four types of erythrocytes, hemolysis is lowest in normal cells with high ascorbic acid (**Table 3**). In contrast, hemin might react with ascorbic acid to generate H_2O_2 in cells depleted of GSH with high ascorbic acid. This might explain why hemolysis is highest in this type of erythrocyte in our experiments.

In the current study, the formation of TBARS was not consistent with hemolysis in these four types of erythrocytes. Although TBARS was highest in GSH-depleted cells, it was lowest in normal cells (**Table 3**). According to the present data, lipid peroxidation *per se* is unlikely to contribute to hemolysis induced by hemin.

In conclusion, we showed that ascorbic acid alone elicits hemolysis and induces lipid peroxidation in the presence of hemin. We also demonstrated that H_2O_2 is more likely to serve as an oxidant to induce further hemolysis and formation of TBARS. However, the combination of GSH and ascorbic acid is more effective in inhibiting hemolysis by hemin. Although ascorbic acid itself does not induce hemin degradation, it significantly stimulates the hemin degradation mediated by GSH [21]. These results could provide a ready explanation for the ascorbic acid enhancement of GSH-dependent inhibition of hemolysis. The current study also demonstrated that extracellular and intracellular ascorbic acid exhibit similar effects on hemin-induced hemolysis or inhibition of hemin-induced hemolysis by GSH. Thus, ascorbic acid might function as an antioxidant or prooxidant in hemin-mediated hemolysis, depending on whether GSH is available.

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