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Elevated Glutathione Accelerates Oxidative Damage to Erythrocytes Produced by Aromatic Disulfides

By Yoshimitsu Maede, Mikanori Kuwabara, Akira Sasaki, Mutsumi Inaba, and Wakako Hiraoka

It has been shown that certain dogs have erythrocytes characterized by an inherited high concentration of reduced glutathione (GSH), five to seven times the normal level (high-GSH RBCs). We examined whether increased GSH in dog erythrocytes leads to increased protection against oxidative damage induced by acetylphenylhydrazine (APH) and/or 4-aminophenyl disulfide (4-AD). When erythrocytes were incubated with 30 mmol/L APH, the Heinz body count was appreciably higher in normal RBCs than in high-GSH RBCs, while there was no difference in the increase of the methemoglobin (metHb) concentration in both RBCs. In contrast, both the Heinz body count and metHb production were much higher in high-GSH RBCs than in normal RBCs when erythrocytes were incubated with 4-AD. Furthermore, the generation of the superoxide in erythrocytes treated with 4-AD, which was measured by spin trapping combined with electron spin resonance (ESR), was obviously higher in high-GSH RBCs than in normal RBCs. These results clearly indicate that erythrocyte GSH is an important defense against oxidative damage induced by certain compounds such as APH, but that, in contrast, elevated GSH appears to accelerate oxidative damage to erythrocytes produced by aromatic disulfides, such as 4-AD, which generated a superoxide in erythrocytes via its redox reaction with GSH.

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RBCs were demonstrated with vital stain using 2% crystal violet, and the Heinz body count was determined as the percentage of the cells that had five or more Heinz bodies by examination of 500 erythrocytes. Another quantitative estimate of Heinz bodies in erythrocytes was also made by the turbidimetric method of Winterbourn,\(^{13}\) in which the decrease in absorbance at 700 nm (A\(_{700}\)) of erythrocyte lysates following centrifugation was determined.

**RBC enzyme assays.** The activity of NADH-methemoglobin reductase was determined as described by Board\(^{12}\) and Choury et al.\(^{11}\) Catalase and superoxide dismutase (SOD) were assayed according to Beutler.\(^{14}\)

**Measurement of superoxide production.** A generation of active oxygen was made within erythrocytes incubated with 4-AD was determined by spin trapping combined with electron spin resonance (ESR), similarly as the manner applied to neutrophils by Britgan et al.\(^{15}\) Washed RBCs were resuspended in phosphate-buffered saline (PBS) (pH 7.4) to yield hematocrit values of about 30%. One volume of the cell suspension was mixed with one volume of 70 mmol/L 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and kept at 4°C for one hour and centrifuged. After centrifugation, all of the supernatant was discarded and the cells were resuspended to yield hematocrit values of 60%. After 5 μL ethanol solution of 2.5 mmol/L 4-AD was added to 195 μL of the cell suspension, the reaction mixture was transferred to a flat quartz ESR cell, fitted into the cavity of the ESR spectrometer (Varian Associates Model E-9, CA), and the spectrum was obtained at room temperature with sequential six-to-eight-minute scans.

**Measurement of H\(_2\)O\(_2\) production.** The generation of H\(_2\)O\(_2\) within erythrocytes incubated with 4-AD was determined by the method of Cohen and Hochstein\(^{16}\) with a slight modification. Washed RBCs were suspended in an isotonic phosphate buffer (pH 7.4) containing 30 mmol/L 3-amino-1,2,4-triazole to yield a hematocrit value of about 30% at 4°C for one hour. After that, 4-AD was added to the cell suspension to a final concentration of 300 μmol/L, and then incubated at 37°C. Aliquots of the suspension were withdrawn at appropriate times, and catalase activity was assayed.

**Chemicals.** 4-AD and 4-aminophenol were purchased from the Aldrich Chemical Co, Milwaukee. APH and all other reagents used in this experiment were from the Wako Pure Chemical Co, Osaka, Japan.

**RESULTS**

**Erythrocyte enzymes.** The activities of SOD and catalase in high-GSH erythrocytes were significantly higher (\(P < .01\)) than those in normal GSH cells (Table 1). There was no significant difference between the total activity of NADH-methb reductase in high- and normal GSH cells, although membrane-bound activity of the enzyme in high-GSH cells was about twofold that in normal cells. In our previous study,\(^3\) the activities of GSH-peroxidase and glutathione reductase (GR) in high-GSH cells were almost the same as those in normal cells, while GR activity in high-GSH cells was slightly higher than that in normal cells when flavin adenine dinucleotide was added to the reaction system. These results suggest that high-GSH cells may have a higher ability to resist oxidative stress than normal cells.

**Effects of APH on dog erythrocytes with a high or normal level of GSH.** Both high- and normal GSH erythrocytes showed a linear increase of metHb concentration as soon as both cells were incubated with 6 mmol/L APH either in the presence or absence of glucose. There was no difference in the increase of the metHb concentration in high- and normal GSH cells during the incubation (Fig 1).

The concentration of GSH was not significantly changed during the incubation with glucose (Fig 1), but it gradually decreased in the absence of glucose in both cells (data not shown). When erythrocytes were incubated with 6 mmol/L APH in the absence of glucose, the Heinz body count and turbidity of the erythrocyte lysate rose concomitantly and were significantly (\(P < .05\)) higher in normal cells than in high GSH cells. In the presence of glucose, however, there were almost no changes in the Heinz body count or the turbidity in either erythrocyte during the incubation. This result seemed to indicate that both cells could completely protect themselves from the oxidative insult by 6 mmol/L APH. Instead of 6 mmol/L APH, therefore, the RBC suspensions were incubated with 30 mmol/L APH in the presence of glucose. Under these conditions, both the Heinz body count and the turbidity of the hemolysate were appreciably higher in normal cells than in high-GSH cells (Fig 2).

That is, the turbidity of the erythrocyte lysate was increased to 2.19 ± 0.56 (A\(_{700}\)) in normal cells, while it was only 0.63 ± 0.26 in high-GSH cells at six hours of incubation with 30 mmol/L APH. Likewise, the Heinz body count ranged from 30% to 80% in normal cells, whereas it was only about 1% in high-GSH cells at six hours of incubation.

**Effects of 4-AD.** When erythrocytes were incubated with 150 μmol/L 4-AD, the concentration of GSH fell to about 15% of the initial level in high-GSH cells and to about 30% in normal cells within 30 minutes after the onset of the incubation with 4-AD in the presence of glucose. After that, however, GSH levels in both cells were gradually increased and returned to 30% of the initial level in high-GSH cells and to 50% in normal cells at 180 minutes. In contrast, the GSH level fell to 7% of its initial level in high-GSH cells and to

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<th>Table 1. MethHb Concentration, Methemoglobin Reductase, SOD, and Catalase Activities in High- and Normal GSH Erythrocytes</th>
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<td><strong>High GSH RBCs</strong></td>
</tr>
<tr>
<td>MetHb (%)</td>
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<tr>
<td>MetHb reductase (μmol/min/g Hb)</td>
</tr>
<tr>
<td>Total activity</td>
</tr>
<tr>
<td>Soluble enzyme</td>
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<tr>
<td>Membrane-bound enzyme</td>
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<td>SOD (IU/g Hb)</td>
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Values are mean ± SD (n = 5).
Abbreviation: Hb, hemoglobin.
*P < .01 according to the Student's t test when compared with normal RBCS.
The metHb concentration increased rapidly along with the fall of the GSH level and was virtually complete after 30 minutes, although a little additional increase was observed after this time in both cells (Fig 3). The rate of metHb formation in high-GSH cells was significantly higher than that in normal cells when both cells were incubated with 4-AD either in the presence or absence of glucose. When erythrocytes were incubated with 4-AD, both the Heinz body counts and the turbidity of the hemolysate were increased earlier and were appreciably higher in high-GSH cells than in normal cells either in the presence or absence of glucose. However, the Heinz body count of both cells incubated in the presence of glucose were much higher than those in the cells incubated in the absence of glucose (Fig 4). This was more apparent in normal cells than in high-GSH cells; i.e., the Heinz body counts were 94% in high-GSH cells and 80% in normal cells when both cells were incubated for 360 minutes in the presence of glucose. In the absence of glucose, the Heinz body counts were 95% and 40% in high- and normal GSH cells, respectively.

Effects of 4-aminothiophenol. The changes in the Heinz body count, metHb formations, and GSH reduction observed in both cells incubated with 4-aminothiophenol were similar to those observed when both cells were incubated with 4-AD. That is, the Heinz body count and metHb formation were significantly higher in high GSH cells than in normal cells (data not shown).

Generation of $O_2^-$ within erythrocytes treated with 4-AD. Erythrocyte suspensions mixed with 35 mmol/L DMPO were treated with 4-AD and examined by ESR (Fig 5). The resulting spectra showed typical DMPO-OH adducts ($a_N = a_H = 1.49$ mT). DMPO-OH can arise as a consequence of the degradation of the superoxide adduct (DMPO-$O_2^-$ or DMPO-OOH) or direct addition of the OH radicals generated by the reaction of $O_2^-$ with $H_2O_2$ through an iron-catalyzed process as outlined below:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$
$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^- + Fe^{3+}$$

In either case, DMPO-OH adduct formation provided evidence for $O_2^-$ generation in erythrocytes treated with 4-AD, although we could not directly detect DMPO-OOH. This is probably due to the fact that rapid reduction of DMPO-OOH to DMPO-OH occurred within the erythrocytes. Comparison of the spectra shown in Fig 5 with each other clearly demonstrated that the generation of $O_2^-$ in high-GSH cells was obviously higher than that in normal cells.

Generation of $H_2O_2$ in erythrocytes incubated with 4-AD. Endogenous catalase in high-GSH erythrocytes was more rapidly and intensively inhibited by aminotriazole than that in normal cells, although the inhibition of catalase...
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In this reaction, it is possible to consider that the generation

reached almost the same level in both erythrocytes at 60 minutes after incubation (Fig 6). In this experiment, H₂O₂ formation is reflected by the inhibition of endogenous catalase in the presence of aminotriazole, and the rate of inhibition of the catalase is proportional to the rate of H₂O₂ formation within the cells. Thus, the results obtained indicate that H₂O₂ was more readily produced within high-GSH cells than normal cells when both cells were incubated with 4-AD.

DISCUSSION

Recently, Munday demonstrated that the aromatic thiol, thiophenol, generated a superoxide radical and hydrogen peroxide by its autoxidation and by its reaction with oxyhemoglobin at neutral pH, and that the oxidation product, diphenyl disulfide, was reduced back to thiophenol by GSH. Thus, it was shown that, in the presence of an excess of GSH, the disulfide generated active oxygen species via the redox reaction with GSH. In the present experiments, the intracellular GSH concentration was rapidly depleted with increasing methHb concentration in both high and normal GSH erythrocytes when both of the cells were incubated with 4-AD, suggesting that the thiol exchange between the disulfide and GSH, as described by Munday, occurred:

\[
\text{NADPH} + \text{GSSG} + 4\text{-aminothiophenol} \rightarrow \text{NADP} + \text{GSH} + \text{HbO}_2 + \text{methHb} + O_2^-
\]

In this reaction, it is possible to consider that the generation

Fig 3. Effects of 4-AD on the concentration of GSH and metHb in high- and normal erythrocytes. High- (•) and normal (○) GSH cells were incubated with 150 μmol/L 4-AD in the absence of glucose at 37°C. Data are the mean ± SD of three separate experiments.

Fig 4. Effect of 4-AD on the formation of Heinz bodies in high- and normal GSH erythrocytes. High- (●) and normal (○) GSH cells were incubated with 150 μmol/L 4-AD in the presence (A) or absence (B) of glucose at 37°C. Heinz body count was measured as described in the legend of Fig. 2.

Fig 5. Superoxide formation in high- and normal GSH erythrocytes treated with 4-AD. The scans are sequential (six- to eight-minutes) ESR spectra obtained following incubation of high-GSH (A) and normal (B) cells with 4-AD in the presence of the spin trap DMPO.

Fig 6. Superoxide formation in high- and normal GSH erythrocytes treated with 4-AD. The scans are sequential (six- to eight-minutes) ESR spectra obtained following incubation of high-GSH (A) and normal (B) cells with 4-AD in the presence of the spin trap DMPO.
of O$_2^-$ during the reaction would be greater in high-GSH cells than in normal cells, because high-GSH cells have a higher concentration of GSH, indicating a higher ability of the cells to convert 4-AD into 4-aminothiophenol, which generates O$_2^-$ in erythrocytes. This would account for the high susceptibility of high-GSH cells to oxidative damage induced by 4-AD. This possibility is supported by the following results.

The generation of O$_2^-$ in high-GSH cells was shown to be greater than that in normal cells, as measured by spin trapping combined with ESR (Fig 5). This result suggested that the generation of H$_2$O$_2$ in erythrocytes incubated with 4-AD would also be higher in high-GSH cells than in normal cells, since the generated O$_2^-$ within erythrocytes is known to be converted to H$_2$O$_2$ by a catalytic function of SOD. This was demonstrated by the fact that the generation of H$_2$O$_2$ in high-GSH cells was increased more rapidly and abundantly than in normal cells when both cells were incubated with 4-AD (Fig 6). It is known that the O$_2^-$ and H$_2$O$_2$ induce oxidative damage in the erythrocyte, resulting in the formation of insoluble oxidative products that precipitate within the cell as Heinz bodies. Thus, it was not surprising that the numbers of Heinz bodies and metHb formations were significantly higher in the high-GSH cells than in normal cells when these cells were incubated with 4-AD and/or 4-aminomothiophenol. In addition, Heinz bodies were more prominent in both types of erythrocytes incubated with 4-AD in the presence of glucose than those cells incubated with it in the absence of glucose. This result seems to indicate that intracellular GSH concentration would be more abundant in the cells incubated with glucose than in those incubated without glucose. This was shown by the following result. The concentration of GSH rapidly fell as soon as the cells were incubated with 4-AD, but subsequent increase of it was observed in the cells incubated with glucose, but not in those incubated without glucose. This may be due to the depletion of NADPH in those cells incubated without glucose, because NADPH is known to be glucose dependent in erythrocytes.

The depletion of NADPH should result in a decreasing GSH level of erythrocytes, since NADPH is essential for the reduction of GSSG to GSH. These results clearly indicate that increased intracellular GSH concentration did not increase the erythrocyte's ability to protect itself from the oxidative damage induced by aminophenyl disulfide, but rather accelerated the damage to the cell. Similarly, Kramer et al observed previously that GSH accelerated methemoglobin (metHb) formation by an antimalarial drug, 4,4'-diaminodiphenylsulfone (Daspon), in solutions of purified hemoglobin, and that depletion of intracellular GSH reduced metHb formation by the drug in erythrocytes.

In contrast, APH is a well-known hemolytic agent that also generates O$_2^-$ and H$_2$O$_2$ in erythrocytes. The production of active oxygens and oxidative damage is thought to be initiated by the direct reaction of APH with oxyhemoglobin, in which metHb and O$_2^-$ are produced. The generated O$_2^-$ in erythrocytes is promptly converted to H$_2$O$_2$ by SOD, as described. The breakdown of H$_2$O$_2$ is catalyzed by both catalase and glutathione peroxidase (GSH-Px). The GSH is believed to play a major role in the elimination of H$_2$O$_2$ from the erythrocyte through its oxidation by H$_2$O$_2$, which is catalyzed by GSH-Px. In the study reported, the activities of both catalase and SOD in high-GSH erythrocytes were higher than those in normal GSH cells. Furthermore, there was no difference in the increase of the metHb concentration in high- and normal GSH cells during the incubation with APH. This result suggested that the production of O$_2^-$ in high-GSH erythrocytes might be at the same level as that in normal GSH cells when these cells were incubated with APH. If this were the case, it would be expected that the high-GSH cells would show increased protection against oxidative damage induced by APH as compared with normal cells, because a higher level of GSH was maintained during the incubation in high-GSH cells than in normal cells (Fig 1). In the present experiment, the formation of Heinz bodies in the high-GSH cells exposed to APH was significantly less than in normal cells, indicating that the elevated GSH level in high-GSH cells increased the cells' ability to withstand oxidative damage by APH, as was expected. This result is well compatible with the concept that GSH is involved in protecting biologic systems from oxidative stress.

In conclusion, the present study demonstrated that erythrocyte GSH is an important defense against oxidative damage induced by certain compounds such as APH, but in contrast, elevated GSH appeared to accelerate oxidative damage produced by aromatic disulfides, such as 4-AD. As described elsewhere, dogs with high-GSH erythrocytes showed a more severe hemolytic anemia after eating onion than did normal dogs. Onion contains n-propyl disulfide, which is thought to be a causative agent of hemolytic anemia. This may suggest that n-propyl disulfide also could generate active oxygens via the redox reaction with GSH in erythrocytes, resulting in more severe hemolytic anemia in the high-GSH dogs.
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