Elevated Glutathione Accelerates Oxidative Damage to Erythrocytes Produced by Aromatic Disulfide

By Yoshimitsu Maede, Mikinori 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.

REDUCED glutathione (GSH) in erythrocytes has been thought to participate in intracellular redox reactions to protect hemoglobin and some thiol-dependent enzymes or membrane proteins from oxidative damage. The depletion of intracellular GSH is thought to be a cause of hemolysis induced by oxidative stress. A number of hereditary deficiencies of GSH have been reported, and these are often associated with the occurrence of hemolytic anemia.[1,2] However, current knowledge concerning the role of glutathione in the erythrocyte is not yet complete.

Recently, we have shown that certain dogs have erythrocytes characterized by inherited high Na,K-ATPase activity and a high concentration of GSH, whereas normal canine erythrocytes completely lack Na,K-ATPase activity.[3,4] The Na gradient-dependent L-glutamate and L-aspartate transport in these cells was greatly increased by the high gradient of Na and K across the cell membrane induced by the Na,K-ATPase, resulting in a high concentration of these amino acids in the erythrocytes.[5] Thus, the increased level of GSH, five to seven times the normal level, could be explained by the fact that the feedback inhibition of glutamyl cysteine synthetase by GSH was released by the high level of glutamate in the cells.[3]

Interestingly, those dogs with high-GSH erythrocytes showed no clinical manifestations under normal circumstances, but they showed severe hemolytic anemia after eating a small amount of onion.[6] Onions, wild and domestic, contain n-propyl disulfide, which produces Heinz body hemolytic anemia in dogs as well as in other domestic animals when they eat large amounts.[7,8] Since GSH is a major reducing substance in the erythrocyte and is believed to play an important role in protecting the cells against hemolytic damage by oxidative stress, such as that produced by onion feeding, it seems a curious fact that the dogs showed severe anemia after eating onion in spite of their high level of erythrocyte GSH. Therefore, this phenomenon makes us reconsider the present theory concerning the role of GSH in the erythrocyte metabolism.

In the study reported, we examined whether increased GSH in dog erythrocytes leads to increased protection against oxidative damage.

MATERIALS AND METHODS

Preparation of erythrocytes. Erythrocytes were obtained from six dogs of one family, including three dogs with an inherited high concentration of red cell GSH. Since this abnormality is inherited as an autosomal recessive,[3] the erythrocyte GSH metabolism in the dogs with a high GSH concentration (homozygotes for this trait) was compared with that in dogs with a normal level of GSH (heterozygotes) in this experiment. Blood was collected into heparinized tubes from the saphena vein of each dog. The blood was centrifuged and the plasma and buffy coat removed by aspiration. The blood was then washed three times at 4°C with isotonic phosphate buffer consisting of 7.1 mmol/L Na_2HPO_4, 2.9 mmol/L NaH_2PO_4, 154 mmol/L NaCl, and/or 5.5 mmol/L glucose (pH 7.4), before being resuspended in the same medium to yield hematocrit values of about 50%.

Incubation of erythrocytes with oxidants. In these experiments, freshly prepared suspensions of dog RBCs were diluted to 10% hematocrit with the isotonic buffer and incubated with each of the oxidants, 6 mmol/L acetylphenyl hydrazine (APH), 150 μmol/L 4-aminophenyl disulfide (4-AD), and 300 μmol/L 4-amino-thiophenol in the presence or absence of 5.5 mmol/L glucose, in a shaking waterbath at 37°C. APH was added to the isotonic buffer as a solution, while ethanol was employed as a solvent for the other two oxidants. Control erythrocyte suspension was added to the solvent alone. Aliquots of the suspension were withdrawn for analysis after various time intervals.

Measurements of GSH, methemoglobin, and Heinz bodies. RBC GSH concentration was determined by the 5,5-dithiobis-(2-nitrobenzoate) derivative.[9] Methemoglobin (metHb) concentration was measured as described by Hegesh et al.[10] Heinz bodies in

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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,[11] 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.[13] Catalase and superoxide dismutase (SOD) were assayed according to Beutler.[14]

Measurement of superoxide production. A generation of active oxygens within erythrocytes incubated with 4-AD was measured by spin trapping combined with electron spin resonance (ESR), similarly as the manner applied to neutrophils by Britigan 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_2O_2 production. The generation of H_2O_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 50 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-aminothiophenol 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

Table 1. MetHb Concentration, Methemoglobin Reductase, SOD, and Catalase Activities in High- and Normal GSH Erythrocytes

	High GSH RBCs	Normal RBCs
MetHb (%)	0.35 ± 0.09	0.34 ± 0.21
MetHb reductase (μmol/min/g Hb)		
Total activity	13.08 ± 0.99	11.60 ± 0.98
Soluble enzyme	10.08 ± 1.11	10.46 ± 0.99
Membrane-bound enzyme	3.31 ± 0.49*	1.15 ± 0.47
SOD (IU/g Hb)	2,696 ± 232*	1,533 ± 285
Catalase (IU/g Hb)	10,120 ± 2,335*	6,747 ± 2,098

Values are mean ± SD (n = 5).

Abbreviation: Hb, hemoglobin.

*$P < .01$ according to the Student's t test when compared with normal RBCS.

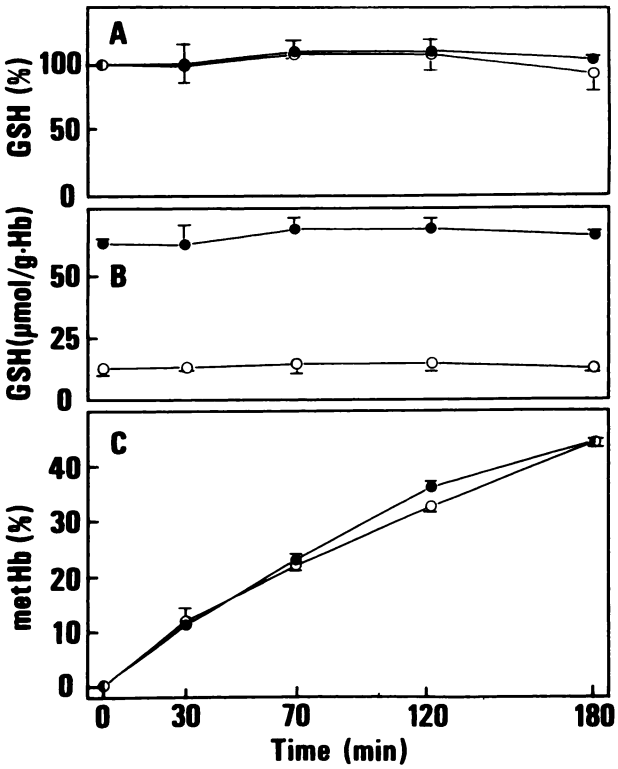


Fig 1. Effects of APH on the concentration of GSH and methemoglobin in high- and normal GSH erythrocytes. High- (●) and normal (○) GSH cells were incubated with 6 mmol/L APH in the presence of glucose at 37°C. Data represent the mean ± SD of three independent experiments.

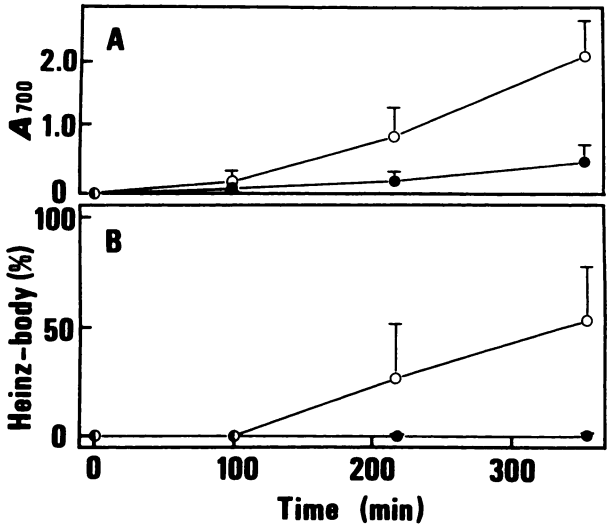


Fig 2. Effects of APH on the formation of Heinz bodies in high- and normal GSH erythrocytes. High- (●) and normal (○) GSH cells were incubated with 30 mmol/L APH in the presence of glucose at 37°C. (A) The turbidity of the erythrocyte lysate was determined by measuring the difference in absorbance of the hemolysate (1 to 14 dilution of blood with 5 mmol/L phosphate buffer) at 700 nm before and after centrifugation at 12,000 g for three minutes, and multiplying the value obtained by 15. (B) The Heinz body count was determined as the percentage of the cell that had five or more Heinz bodies by examination of 500 RBCs. Data are the mean ± SD of three separate determinations.

22% in normal cells as soon as they were incubated without glucose; however, subsequent recovery of their levels was not observed during the incubation in either type of cells (Fig 3).

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; ie, 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:

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

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

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

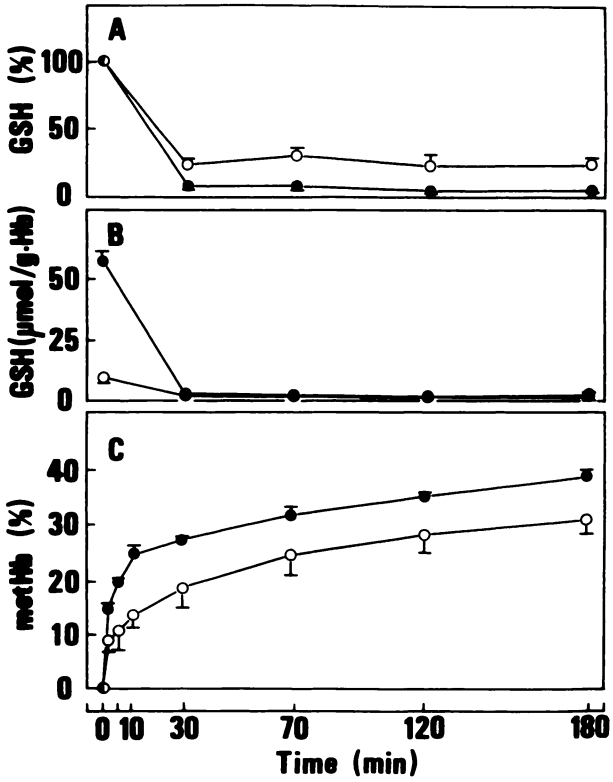


Fig 3. Effects of 4-AD on the concentration of GSH and metHb in high- and normal erythrocytes. High- (●) and normal (○) GSH cells were incubated with 150 μmol/L 4-AD in the absence of glucose at 37°C. Data are the mean ± SD of three separate experiments.

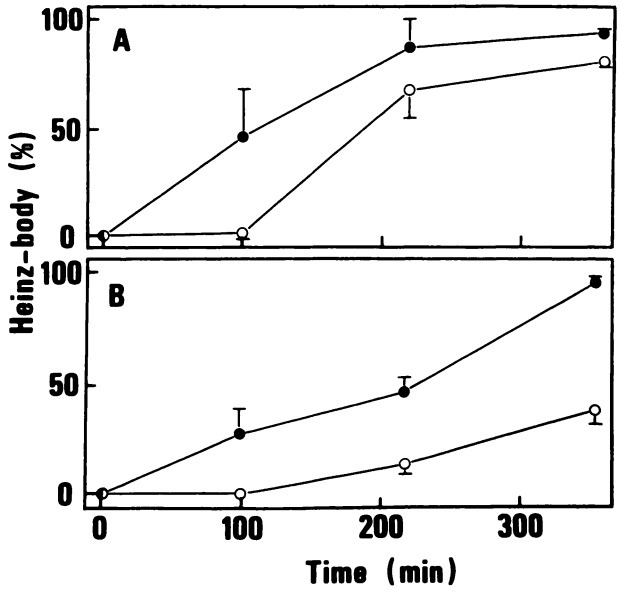


Fig 4. Effect of 4-AD on the formation of Heinz bodies in high- and normal GSH erythrocytes. High- (●) and normal (○) GSH cells were incubated with 150 μmol/L 4-AD in the presence (A) or absence (B) of glucose at 37°C. Heinz body count was measured as described in the legend of Fig. 2.

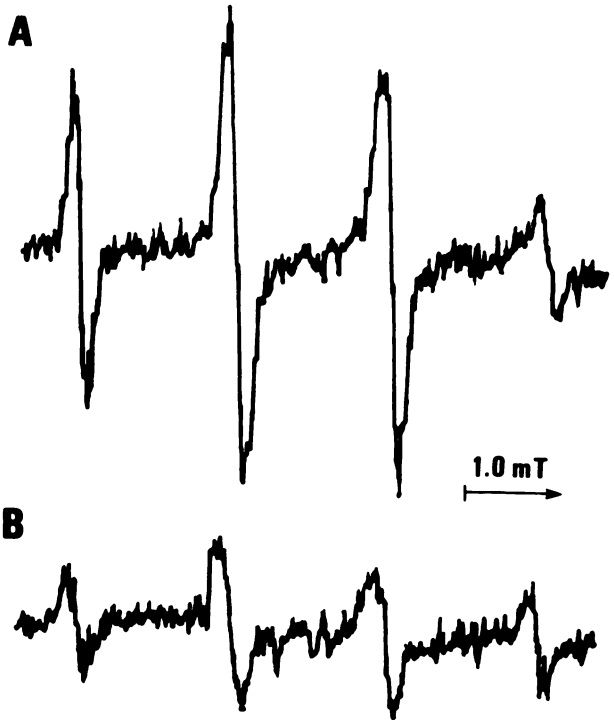
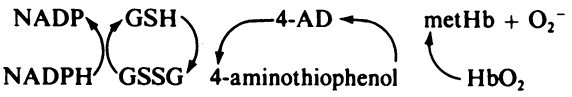


Fig 5. Superoxide formation in high- and normal GSH erythrocytes treated with 4-AD. The scans are sequential (six- to eight-minutes) ESR spectra obtained following incubation of high-GSH (A) and normal (B) cells with 4-AD in the presence of the spin trap DMPO.

reached almost the same level in both erythrocytes at 60 minutes after incubation (Fig 6). In this experiment, H_2O_2 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 H_2O_2 formation within the cells.[16] Thus, the results obtained indicate that H_2O_2 was more readily produced within high-GSH cells than normal cells when both cells were incubated with 4-AD.

DISCUSSION

Recently, Munday[17] 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 metHb 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:



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

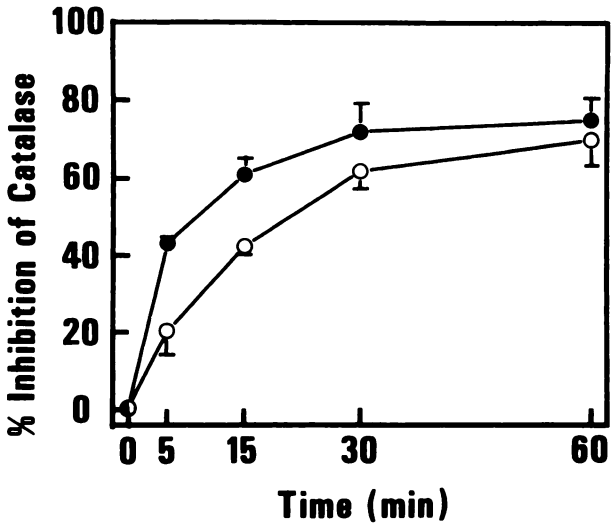


Fig 6. H_2O_2 production in high- and normal GSH erythrocytes incubated with 4-AD. High- (●) and normal (○) GSH cells were incubated with 300 μmol/L 4-AD in the presence of 50 mmol/L aminotriazole at 37°C. The H_2O_2 production was expressed as the rate of inhibition of endogenous catalase. Data are the mean ± SD of three separate determinations.

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.[18] 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_2O_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_2O_2 by a catalytic function of SOD.[19] This was demonstrated by the fact that the generation of H_2O_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_2O_2 induce oxidative damage in the erythrocyte,[20] resulting in the formation of insoluble oxidative products that precipitate within the cell as Heinz bodies.[19] 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-aminothiophenol. 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.[19] 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.[21]

In contrast, APH is a well-known hemolytic agent that also generates O_2^- and H_2O_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.[22,23] The generated O_2^- in erythrocytes is promptly converted to H_2O_2 by SOD, as described. The breakdown of H_2O_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_2O_2 from the erythrocyte through its oxidation by H_2O_2, which is catalyzed by GSH-Px.[19] 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.[19]

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.[24] 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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