Methemoglobin Formation and Reduction in Canine Erythrocytes Characterized by Inherited High Na^+, K^+-ATPase Activity with Normal and High Glutathione Concentrations

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ABSTRACT. The methemoglobin formation and methemoglobin reduction in canine erythrocytes characterized by inherited high potassium (K^+) and normal reduced glutathione concentrations (HK-low GSH cells) were compared with those in canine erythrocytes with inherited high K^+ and high GSH concentrations (HK-high GSH cells) and normal canine erythrocytes with low K^+ and low (=normal) GSH concentrations (LK-low GSH cells). The rate of methemoglobin formation induced by sodium nitrite ($NaNO_2$) was in the order; LK-low GSH > HK-low GSH > HK-high GSH cells, and the difference among groups was significant at 7 and 15 min. Methemoglobin reduction in a medium containing glucose occurred rapidly in both HK-high GSH and HK-low GSH cells, and the rate of reduction was 1.7-fold higher than in LK-low GSH cells. Accumulation of pyruvate equivalent to the amount of methemoglobin reduced indicated that methemoglobin was predominantly reduced by NADH-methemoglobin reductase coupled to glycolysis. HK-low GSH cells showed an increased glycolytic rate and high pyruvate kinase activity similar to the levels in HK-high GSH cells. It is therefore evident that HK-low GSH cells offer greater protection against oxidation of hemoglobin to methemoglobin than LK-low GSH cells because of the increased glycolytic rate in HK-low GSH cells attributable to high pyruvate kinase activity in these cells.—KEY WORDS: canine HK cell, glutathione, glycolysis, methemoglobin, sodium nitrite.

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Mature erythrocytes of normal dogs contain low potassium (K^+) and high sodium (Na^+) concentrations because they lack Na^+, K^+-ATPase activity. However, some dogs have high K^+ and low Na^+ concentrations and high Na^+, K^+-ATPase activity and the concentration of reduced glutathione (GSH) is markedly elevated (HK-high GSH cells) [10, 16]. In the HK-high GSH cells, Na^+ gradient-dependent L-glutamate and L-aspartate transport are markedly increased by the large gradient of Na^+ and K^+ across the membrane induced by the Na^+, K^+-ATPase, resulting in high accumulation of these amino acids and GSH in the cells [8, 11, 12]. We subsequently found 2 Japanese Shiba dogs which had erythrocytes with high K^+ and low Na^+ concentrations and increased Na^+, K^+-ATPase activity, but with no accumulation of GSH (HK-low GSH cells) [4]. In HK-low GSH cells, Na^+-dependent glutamate influx was about a sixth of the value in HK-high GSH cells, and the cellular glutamate and GSH concentrations were comparable to those in normal canine cells. The low glutamate level may therefore be the reason for the normal GSH concentration in HK-low GSH cells.

Erythrocyte GSH protects hemoglobin and other cellular constituents from oxidation by decomposing hydrogen peroxide and other peroxides produced within the cells [21]. Our previous studies showed that HK-high GSH cells were more securely protected from oxidative damage induced by sodium nitrite ($NaNO_2$) [17] and acetylphenylhydrazine (APH) [18] because excessive GSH and increased glycolysis lead to greater defense against oxidation. HK-low GSH cells might be expected to have less resistance to oxidative stress than HK-high GSH cells because they have a lower GSH concentration than HK-high GSH cells. In this study, the effect of oxidant stress on HK-low GSH cells was compared with that in HK-high GSH and normal (LK-low GSH) cells.

MATERIALS AND METHODS

Of HK-high GSH, HK-low GSH and LK-low GSH cell groups of Japanese Shiba dogs, three from each group were used in the present study. Two mongrel dogs with HK-high GSH cells and three mongrel dogs with LK-low GSH cells were also studied in comparison. Venous blood was collected into heparinized tubes, washed three times with isotonic saline and the plasma and buffy coat were removed. The erythrocytes were finally washed with a medium consisting of 135 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 2 mM Na_2HPO_4, 20 mM Hepes/Tris (pH 7.4) and 10 mM glucose. This medium was also used for incubating the erythrocytes in subsequent experiments.

Methemoglobin formation and reduction: Erythrocytes were suspended in the medium to a hematocrit value of 20% and incubated with 6 mM sodium nitrite at 37°C. Aliquots of the suspension were withdrawn at 7, 15 and 30 min, and the methemoglobin concentration was determined by the method of Nakamura *et al.* [15].

Erythrocytes containing more than 90% methemoglobin were prepared as follows. Heparinized blood was mixed with 6 volumes of 12 mM sodium nitrite solution and left at 25°C for 15 min. The red cells were collected by centrifugation at 1,500 × g for 10 min and washed 3 times with isotonic saline. The washed cells were suspended in the medium to a hematocrit value of 20%, and incubated for 6 hr at 37°C. The concentrations of methemoglobin [15], pyruvate [2] and lactate [2] were determined at 3 and

6 hr.

Glucose consumption and lactate formation: Erythrocytes were resuspended in the medium to a 20% hematocrit, and incubated for 2 hr at 37°C. Before and after incubation, the cell suspensions were deproteinized with one volume of 1 M ice-cold perchloric acid. The supernatant obtained was neutralized with 5 M KOH, and the concentrations of glucose and lactate were determined [2].

Erythrocyte GSH and enzyme assay: Reduced glutathione was determined as described by Beutler *et al.* [1]. Activities of hexokinase, glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), glyceraldehyde phosphate dehydrogenase (GAPD) triose phosphate isomerase (TPI), pyruvate kinase, NADH-methemoglobin reductase, NADPH-methemoglobin reductase, glutathione peroxidase (GPx), glutathione reductase (GR) and catalase were assayed according to the method of Beutler [2]. Superoxide dismutase (SOD) activity was assayed by the method of McCord and Fridovich [14].

RESULTS

As shown in Figs. 1 and 2, and Tables 1 and 2, the values obtained for HK-high GSH cells and LK-low GSH cells of mongrel dogs were within the range for HK-high GSH and LK-low GSH cells of Japanese Shiba dogs, respectively. Thus, data from Shiba and mongrel dogs with each cell type were pooled and the mean ± SD levels were found.

Rate of methemoglobin formation and reduction: Figure 1 shows the change in the methemoglobin concentration when erythrocytes of HK-high GSH, HK-low GSH and LK-low GSH types were incubated with 6 mM $NaNO_2$. Methemoglobin increased rapidly, and almost reached equilibrium at 30 min when LK-low GSH cells had a significantly higher methemoglobin concentration (81%) than HK-high GSH or HK-low GSH cells (71%). The rate of methemoglobin formation was in the order: LK-low GSH > HK-low GSH > HK-high GSH cells, and the difference in the methemoglobin concentration among groups was significant at 7 and 15 min.

Methemoglobin reduction was compared in erythrocytes from three cell groups which contained more than a 90% methemoglobin concentration and were incubated in a medium containing 10 mM glucose (Fig. 2). Methemoglobin reduction occurred rapidly in both HK-high GSH and HK-low GSH cells, and it was about 1.7-fold greater than in LK-low GSH cells (Table 1). The amounts of heme reduced and pyruvate and lactate produced were also similar in HK-high GSH and HK-low GSH cells, and also 1.7 times greater than in LK-low GSH cells. The molar ratio of heme reduced and pyruvate produced was approximately 2:1 in all cell groups, indicating that methemoglobin is predominantly reduced by NADH-methemoglobin reductase coupled to glycolysis. The NADH-methemoglobin reductase activity determined is given in Table 2. The enzyme activity was slightly lower in

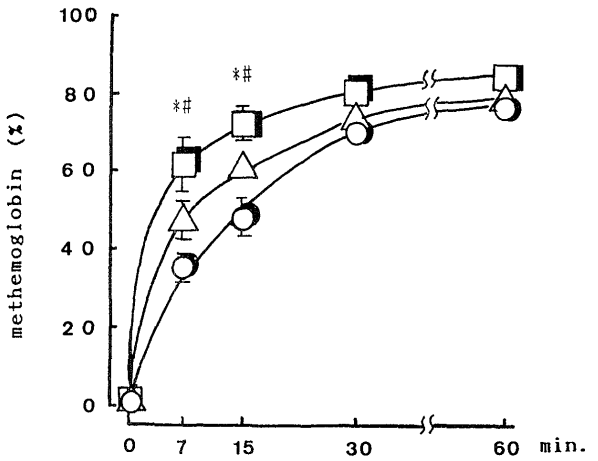

Fig. 1. Methemoglobin formation in HK-high GSH cells of Japanese Shiba dogs (○) and mongrel dogs (●), HK-low GSH cells (△), LK-low GSH cells of Shiba dogs (□) and mongrel dogs (■) incubated with 6 mM sodium nitrite. Data are the mean ± SD for 3 Shiba dogs and mean for 2 mongrel dogs for HK-high GSH cells, 3 Shiba dogs for HK-low GSH cells, and 3 Shiba and 3 mongrel dogs for LK-low GSH cells. Significant ($P<0.01$) difference between groups is indicated as *: HK-low GSH cells vs LK-low GSH cells, and #: HK-low GSH cells vs HK-high GSH cells.

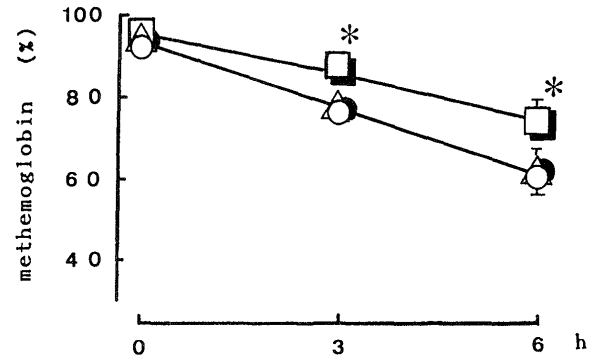

Fig. 2. Methemoglobin reduction in HK-high GSH cells of Japanese Shiba dogs (○) and mongrel dogs (●), HK-low GSH cells (△) and LK-low GSH cells of Shiba dogs (□) and mongrel dogs (■) incubated with 10 mM glucose. *: HK-low GSH cells vs LK-low GSH cells ($P<0.01$). #: HK-low GSH cells vs HK-high GSH cells ($p<0.01$).

HK-low GSH cells than in HK-high GSH and LK-low GSH cells, but the difference was not significant among groups. The activities of NADPH-methemoglobin reductase, catalase and glutathione peroxidase (GPx) were similar in all 3 cell groups. The activities of SOD and glutathione reductase (GR) were significantly higher in HK-high GSH cells than in LK-low GSH cells. HK-low GSH cells showed a large individual difference in the activity of SOD, and GR activity was similar to its level in HK-high GSH cells.

Glycolytic rate and glycolytic enzymes: To determine whether the increased methemoglobin reduction in HK-low GSH cells is attributable to the increased glycolytic

Table 1. Methemoglobin reduction, pyruvate and lactate formation in canine HK-low GSH, HK-high GSH and LK-low GSH cells

	HK-low GSH	HK-high GSH			LK		
	(3)	Shiba (3)	Mongrel (2)	combined (5)	Shiba (3)	Mongrel (3)	combined (6)
% Methemoglobin reduced/hr	5.31±0.57*	5.36±0.54	4.78, 4.63	5.14±0.54	3.11±0.38	3.25±0.25	3.18±0.41
A: heme reduced (μmol/gHb/hr)	3.29±0.35*	3.32±0.33	2.97, 2.87	3.19±0.33	1.93±0.26	2.03±0.16	1.97±0.27
B: pyruvate produced (μmol/gHb/hr)	1.60±0.10*	1.61±0.26	1.50, 1.31	1.56±0.24	0.99±0.12	1.06±0.09	1.03±0.11
Ratio of A and B	2.06±0.16	2.07±0.17	1.98, 2.20	2.07±0.17	1.95±0.15	1.91±0.14	1.92±0.13
Lactate produced (μmol/gHb/hr)	5.11±0.41*	5.99±0.27	5.00, 4.57	5.51±0.64	3.71±0.08	3.48±0.29	3.60±0.24

*: HK-low GSH cells vs LK-low GSH cells ($P<0.01$). #: HK-low GSH cells vs HK-high GSH cells ($P<0.01$).

Table 2. Glycolytic rate and enzyme activities in canine HK-low GSH, HK-high GSH and LK-low GSH cells

	HK-low GSH	HK-high GSH			LK		
	(3)	Shiba (3)	Mongrel (2)	combined (5)	Shiba (3)	Mongrel (3)	combined (6)
Glucose consumption (μmol/gHb/hr)	1.91± 0.43*	1.70± 0.21	1.82, 2.27	1.88± 0.29	0.57± 0.06	0.62 ± 0.03	0.60± 0.05
Lactate production (μmol/gHb/hr)	3.75± 0.95*	3.83± 0.45	3.61, 4.89	4.00± 0.58	1.42± 0.28	1.23 ± 0.21	1.31± 0.26
Hexokinase (IU/gHb)	0.33± 0.04#	0.65± 0.25	0.87, 0.71	0.71± 0.23	0.28± 0.25	0.33 ± 0.04	0.30± 0.05
G-6-PD (IU/gHb)	9.13± 2.05	9.41± 1.30	8.06, 6.82	8.62± 1.45	7.78± 1.13	7.02 ± 1.25	7.40± 1.25
6-PGD (IU/gHb)	5.68± 1.41	4.88± 0.74	4.58, 4.23	4.69± 0.63	5.17± 0.95	4.65 ± 0.49	4.91± 0.76
TPI (IU/gHb)	465.7± 98.8	417.3± 73.6	418.7, 438.5	421.8± 57.6	487.9± 127.4	409.8 ± 49.3	448.8± 104.2
GAPD (IU/gHb)	128.5± 5.3	127.3± 14.8	132.6, 97.4	121.3± 17.9	141.1± 13.6	123.3 ± 19.8	132.2± 19.2
Pyruvate kinase (IU/gHb)	10.50± 0.75*	12.17± 1.23	9.52, 11.70	11.55± 1.40	3.69± 0.46	4.49 ± 0.82	4.09± 0.77
NADH-methemoglobin reductase (IU/gHb)	1.90± 0.34	2.63± 0.70	2.73, 1.50	2.43± 0.72	2.39± 0.38	2.99 ± 1.09	2.69± 0.87
NADPH-methemoglobin reductase (IU/gHb)	0.92± 0.12	0.98± 0.13	1.20, 0.96	1.02± 0.14	0.93± 0.09	1.01 ± 0.12	0.97± 0.12
SOD (IU/gHb×10^3)	2.79± 0.48	3.08± 0.19	3.25, 3.05	3.19± 0.16	2.70± 0.06	2.56 ± 0.20	2.64± 0.15
Catalase (IU/gHb×10^4)	5.24± 1.04	4.10± 0.83	2.88, 4.14	3.87± 0.81	5.78± 1.44	3.94 ± 1.68	4.72± 1.74
GPx (IU/gHb)	184.6± 3.80	218.6± 5.69	252.3, 250.6	231.8± 16.7	213.7± 15.1	221.1 ± 6.40	218.4± 11.1
GR (IU/gHb)	4.47± 0.23*	4.60± 0.59	4.51, 4.52	4.57± 0.48	3.22± 0.20	3.07 ± 0.55	3.13± 0.44
GSH (mg/dl RBC)	67.9± 10.3#	304.1± 65.4	384.5, 362.7	338.9± 58.4	54.2± 8.20	60.5 ± 11.8	57.3± 10.6
GSH (μmol/gHb)	7.04± 1.30#	34.58± 7.10	45.31, 42.74	38.36± 7.23	5.72± 0.86	6.39 ± 1.24	6.06± 1.12

*: HK-low GSH cells vs LK-low GSH cells ($P<0.01$). #: HK-low GSH cells vs HK-high GSH cells ($P<0.01$).

rate, glucose consumption, lactate formation and glycolytic enzyme activities were measured (Table 2). When erythrocytes were incubated with glucose, the glucose consumption and lactate formation occurred to similar degrees in HK-low GSH and HK-high GSH cells, and the levels were approximately 3-fold those in LK-low GSH cells. The activity of pyruvate kinase was similar in HK-high GSH and HK-low GSH cells and much higher than in LK-low GSH cells. The activity of hexokinase was significantly higher in HK-high GSH cells than in the two other cell groups. HK-low GSH cells appear to have slightly increased activities of G6PD, 6PGD and TPI, but the differences among the 3 cell groups were not significant.

DISCUSSION

In this study, HK-low GSH cells showed a greater resistance to the oxidative effect of $NaNO_2$ than LK-low GSH cells, whereas the GSH concentration in HK-low GSH and LK-low GSH cells was almost the same. Sodium nitrite is a well-known oxidizing agent which mainly produces methemoglobin but very few Heinz bodies, and has been used in the study of methemoglobin formation and reduction in the erythrocytes of man and other species [5, 7, 19]. Erythrocytes contain numerous antioxidizing mechanisms which protect hemoglobin from oxidative damage, and the rate of methemoglobin formation and reduction are modified by these mechanisms [6, 9, 17, 20]. It therefore seems reasonable to study methemoglobin formation and reduction with $NaNO_2$ as an oxidizing agent in estimating the cells' resistance to oxidant stress.

Sodium nitrite generates superoxide anion and H_2O_2 in a reaction with oxyhemoglobin, and the accumulation of these activated oxygens causes a further increase in methemoglobin formation [22–24]. To protect hemoglobin from oxidation, the superoxide anion generated is promptly converted to H_2O_2 by SOD, and H_2O_2 is further decomposed by catalase and glutathione peroxidase (GPx) [3, 21]. Glutathione is believed to play a major role in the elimination of H_2O_2 from the erythrocytes through its oxidation by H_2O_2, which is catalyzed by GPx. The

present study showed that the activities of catalase and GPx were almost the same in all three cell groups. The activity of SOD was slightly higher in HK-high GSH cells than in LK-low GSH cells, though HK-low GSH cells showed a large individual difference. The cells' ability to eliminate activated oxygen species may therefore be affected by the GSH concentration, i.e., a higher GSH concentration will provide hemoglobin with a greater protection against the oxidative effect of $NaNO_2$. In fact, the hemoglobin of HK-high GSH cells was oxidized more slowly to methemoglobin than that of LK-low GSH cells when hemolysates were exposed to $NaNO_2$ in our previous study [17], and this was due to excessive GSH in HK-high GSH cells. A similar trend was observed when intact HK-high GSH and LK-low GSH cells were incubated with $NaNO_2$ in the present study. Methemoglobin formation in HK-low GSH cells was also less than that in LK-low GSH cells. However, methemoglobin formation was significantly higher in HK-low GSH cells than in HK-high GSH cells. Since the GSH concentration was similar in HK-low GSH and LK-low GSH cells, and corresponded to 1/5 to 1/6 of the value in HK-high GSH cells, antioxidizing mechanisms other than GSH may account for the difference between methemoglobin formation in HK-low GSH and that in LK-low GSH cells.

The rate of methemoglobin reduction was about 1.7-fold higher in both HK-high GSH and HK-low GSH cells than in LK-low GSH cells. The molar ratio of heme reduced and pyruvate produced was approximately 2 in all three cell types. This ratio indicates that methemoglobin is reduced predominantly by NADH-methemoglobin reductase. The activity of NADH-methemoglobin reductase was similar in all three cell types, which excludes the possibility that the methemoglobin reducing capacity of HK-high GSH and HK-low GSH cells is increased by high activity of the enzyme. NADH is a necessary coenzyme of NADH-methemoglobin reductase and its adequate supply is dependent on glucose metabolism through the Embden-Meyerhof pathway [9]. NADH is generated during the phosphorylation and oxidation of glyceraldyde-3-phosphate, and is used mostly in the reduction of pyruvate to lactate under physiologial conditions. When a considerable amount of methemoglobin is present in the erythrocytes, NADH is used preferentially in methemoglobin reduction, resulting in the accumulation of an equivalent amount of pyruvate. Methemoglobin reduction therefore appears to be greatly affected by glucose metabolism. The glycolytic rate was appreciably higher in both HK-high GSH and HK-low GSH cells than in LK-low GSH cells under the physiological condition and it increased under oxidant stress (see lactate formation in Tables 1 and 2). These results indicate that HK-high GSH and HK-low GSH cells have greater ability to reduce methemoglobin back to oxyhemoglobin because of their higher glycolysis.

Maede and Inaba [13] demonstrated that the glycolytic rate is greater in canine HK cells (HK-high GSH cells) than in LK cells (LK-low GSH cells) under physiological conditions, and that the increased glycolysis in HK cells is mostly due to the increased ATP demand for active cation transport by Na^+, K^+-ATPase to maintain the ionic gradient across the membrane. Hexokinase and pyruvate kinase activities which are responsible for the overall rate of glycolysis were more increased in HK cells than LK cells in their study, and these enzyme activities were similar to the values for HK-high GSH and LK-low GSH cells in the present investigation. In HK-low GSH cells, K^+ and Na^+ concentrations and Na^+, K^+-ATPase activity were almost the same as in HK-high GSH cells, and pyruvate kinase activity was elevated as observed in HK-high GSH cells. HK-low GSH cells showed an increased glycolytic rate. This may be due to high pyruvate kinase activity, because pyruvate kinase is one of the key enzymes that control the glycolytic rate.

The effect of GSH concentration may be relatively small in the reduction of methemoglobin because direct nonenzymatic methemoglobin reduction by GSH is known to be very slow [9, 20]. NADPH-methemoglobin reductase activity which accounts for only 5% of reducing capacity [9], was similar in all three cell groups. Indirect nonenzymatic reduction and NADPH-dependent enzymatic reduction therefore play only a small part in methemoglobin reduction in canine erythrocytes, and these processes cannot be responsible for the increased rate of methemoglobin reduction in HK-high GSH and HK-low GSH cells.

In conclusion, HK-low GSH cells offer greater protection against oxidation of hemoglobin to methemoglobin compared with LK-low GSH cells, and this is due to the high glycolytic rate attributable to high pyruvate kinase activity in HK-low GSH cells.

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