Characterization of Acatalasemic Erythrocytes Treated with Low and High Dose Hydrogen Peroxide

HEMOLYSIS AND AGGREGATION* 

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The effects of hydrogen peroxide on normal and acatalasemic erythrocytes were examined. Severe hemolysis of acatalasemic erythrocytes and a small tyrosine radical signal (g = 2.005) associated with the formation of ferryl hemoglobin were observed upon the addition of less than 0.25 mM hydrogen peroxide. However, when the concentration of hydrogen peroxide was increased to 0.5 mM, acatalasemic erythrocytes became insoluble in water and increased the tyrosine radical signal. Polymerization of hemoglobin and aggregation of the erythrocytes were observed. On the other hand, normal erythrocytes exhibited only mild hemolysis by the addition of hydrogen peroxide under similar conditions. From these results, the scavenging of hydrogen peroxide by hemoglobin generates the ferryl hemoglobin species (H-Hb-Fe(IV)=O) plus protein-based radicals (‘Hb-Fe(IV)=O). These species induce hemolysis of erythrocytes, polymerization of hemoglobin, and aggregation of the acatalasemic erythrocytes. A mechanism for the onset of Takarara disease is proposed.

In 1952, Takahara reported that Japanese acatalasemia (cat-
alase deficiency) patients suffered from progressive oral gan-
grene (Takahara disease) as a result of being infected with hydrogen peroxide-generating bacteria (1). Subsequently, Swiss and Hungarian acatalasemia-afflicted people were reported, but they did not evidently suffer from the symptomatic features of the disease (2, 3). The residual catalase (EC 1.11.1.6) activity in Swiss and Hungarian acatalasemic erythrocytes was found to be higher than in the erythrocytes from the Japanese. Ogata et al. (4) suggested the catalase deficiency in the blood to be the etiological cause of the disease. We became interested in the scavenging of hydrogen peroxide in erythrocytes to understand the metabolism of hydrogen peroxide in acatalasemic erythrocytes. The hydrogen peroxide scavenging rates in normal and acatalasemic mouse and human erythrocytes were examined (5–8). It was found that hydrogen peroxide was dominantly scavenged by hemoglobin in hemolysates of acatalasemic erythrocytes. The scavenging rate was enhanced in the presence of reduced pyridine nucleotides (NAD(P)H) or ascorbic acid (AsA).2 A cyclic process has been proposed for the scavenging mechanism; hydrogen peroxide oxidizes hemoglobin, which is then reduced by NAD(P)H or AsA (8).

We suspected that during the process, hemoglobin was oxidized to ferryl hemoglobin (H-Hb-Fe(IV)=O) with hydrogen peroxide, and then a comproportionation reaction of Hb-Fe(IV)=O and ferrous hemoglobin generated methemoglobin (H-Hb-Fe(III)=O) (9). Methemoglobin was then further oxidized to radicals of ferryl hemoglobin (‘Hb-Fe(IV)=O) with hydrogen peroxide (10). In this study, we report that the oxidized hemoglobin species in the scavenging process were identified and that a novel property of acatalasemic erythrocytes was subjected to hydrogen peroxide treatment; erythrocytes aggregated to be insoluble in water at a high concentration (≥0.5 mM) of hydrogen peroxide, whereas the severe hemolysis of acatalasemic erythrocytes resulted at low concentrations of hydrogen peroxide (<0.25 mM).

EXPERIMENTAL PROCEDURES

**Materials—**Male mice (8 weeks old) of the C3H/AnLcs+sCs (normal) and C3H/AnLcs+sCs (acatalasemia) strains established by Feinstein et al. (11) were maintained on a laboratory diet (MF diet, Oriental Yeast Co. Ltd., Tokyo, Japan) and water ad libitum until experiments. Chemicals of analytical grade were purchased from Sigma and Wako Pure Chemical Ind. (Osaka, Japan).

**General Procedures—**Hemoglobin contents were determined by the method of Drabkin and Austin (12), and the concentrations of hemoglobin indicated were calculated as tet-

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2 The abbreviations used are: AsA, ascorbic acid; PBS, phosphate-buffered saline; ESR, electron spin resonance; AAPH, 2,2’-azo-bis-(2-aminopropane) dihydrochloride.
Catalase activity was measured according to a previous method (5). SDS-PAGE was performed according to the method of Laemmli (13). The concentrations of the materials indicated were those of the final ones used in samples.

Preparation of Erythrocyte Suspension and Hemolysates—Mouse blood was collected in heparinized tubes. Erythrocytes were separated by centrifugation and washed three times with 10 mM sodium phosphate buffer containing 152 mM sodium chloride (PBS, pH 7.4). After the last washing, packed cells (100% of erythrocytes) were diluted with PBS, and the suspension was used for experiments. Hemolysates were prepared by dilution of the packed erythrocytes with 9 volumes of water.

Treatment of Normal and Acatalasemic Hemolysates with Hydrogen Peroxide—Hemolysates (0.1%) in PBS were treated with 0.1 mM hydrogen peroxide, and the spectra (380–680 nm) were recorded.

Treatment of Normal and Acatalasemic Erythrocytes with Hydrogen Peroxide—A 2% erythrocyte suspension was treated with 0.0–5.0 mM hydrogen peroxide at 37 °C. For the measurement of the protein leakage from the erythrocytes treated with hydrogen peroxide, 0.06 ml of the mixture was immediately diluted with 0.36 ml of PBS at 5 and 30 min after the addition of hydrogen peroxide. Each mixture was centrifuged at 1,100 × g for 5 min, and the absorbance of the supernatant was measured at 280 nm. For the determination of the water-soluble protein contents in the erythrocytes treated with hydrogen peroxide, 0.06 ml of the incubation mixture was put into a tube containing 0.36 ml of water. The absorbance of the supernatant was measured at 280 nm. For measurement of ESR spectra, a 2% erythrocyte suspension was charged in an ESR tube (inner diameter 5 mm) and incubated with hydrogen peroxide at 37 °C for 5 min. The mixture was immediately frozen in liquid nitrogen. ESR spectra were measured at 120 K using a JES-FE1XG (JOEL, FIGURE 1. Spectral changes of acatalasemic hemolysate (the hemoglobin concentration was 3.50 μM) with 0.1 mM hydrogen peroxide. Spectrum A, spectrum of acatalasemic hemolysate. Spectrum B, 10 min after the addition of 0.1 mM hydrogen peroxide to the hemolysate. Spectrum C, 10 min after the addition of 0.1 mM hydrogen peroxide and 1 mM sodium sulfide to the hemolysate. The inset indicates the spectra between 450 and 650 nm.

FIGURE 2. Properties of the erythrocytes treated with hydrogen peroxide. A 2% erythrocyte suspension was treated with hydrogen peroxide for 5 and 30 min. A, protein leakage from erythrocytes. Reaction mixtures were diluted with PBS, and the absorbance of the supernatant at 280 nm was recorded. ●, acatalasemic erythrocytes treated for 5 min; ○, for 30 min; ▼, normal erythrocytes for 5 min; ▲, for 30 min. B, water-soluble protein contents in the erythrocytes treated with hydrogen peroxide. Reaction mixtures were diluted with water, and absorbance of the supernatant at 280 nm was recorded. Asterisks indicate a significant difference from normal erythrocytes (*, p < 0.001). ●, acatalasemic erythrocytes for 5 min; ○, for 30 min; ▼, normal erythrocytes for 5 min; ▲, for 30 min. The vertical line indicates the standard deviation from more than three experiments.
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Tokyo, Japan). Manganese dioxide was used as the internal standard.

Hemolysis of Erythrocytes Treated with a Low Concentration (0.1 mM) of Hydrogen Peroxide—A 2% erythrocyte suspension containing 5 mM glucose or 5 mM glucose and 0.2 mM AsA was preincubated at 37 °C for 30 min. The hemolysis of erythrocytes by hydrogen peroxide was determined as described above. Hemolysis of erythrocytes with 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) was examined by replacing hydrogen peroxide with 50 mM AAPH.

RESULTS

Catalase Activity in Mouse Erythrocytes—The catalase activities of acatalasemic and normal mouse erythrocytes in the presence of 70 μM hydrogen peroxide at 25 °C were 0.99 ± 0.19 and 7.96 ± 0.69 μmol/s/g of hemoglobin, respectively.

Spectral Changes of Hemolysates by the Addition of Hydrogen Peroxide—The spectral changes of the acatalasemic hemolysates with the addition of 0.1 mM hydrogen peroxide, at which the concentration of hemoglobin was 3.50 μM, are shown in Fig. 1. The spectrum (Fig. 1, spectrum B, λmax 412, 538, 574, and 630...
nm) suggests oxidation of ferrous hemoglobin to a mixture of methemoglobin and ferryl hemoglobin species. The formation of methemoglobin is indicated by the shoulder band at 630 nm and the increase of absorbance at 500 nm (15, 16). The formation of ferryl hemoglobin species is suggested by the weak bands at 538 and 574 nm and confirmed by the spectra of the sulfo-derivative (Fig. 1, spectrum C, $\lambda_{\text{max}}$ 618 nm) with the addition of 1 mM sodium sulfide (9, 16). However, the spectra of normal hemolysates did not change with the addition of hydrogen peroxide (data not shown).

Treatment of Erythrocytes with Various Concentrations of Hydrogen Peroxide—Two percent of the erythrocyte suspension contained 75.3 ± 10.8 $\mu$M hemoglobin. The protein leakage from erythrocytes treated with hydrogen peroxide is shown in Fig. 2A. The protein leakage was not observed after 5 min but was observed 30 min after the addition of 0.1 mM hydrogen peroxide. The water-soluble protein contents in the erythrocytes treated with hydrogen peroxide are indicated in Fig. 2B. The figure indicates that the acatalasemic erythrocytes treated with more than 0.5 mM hydrogen peroxide became insoluble in water 5 min after the addition. Precipitated cells subsequently never became soluble in water again.

Radical ESR signals ($g = 2.005$ and 2.03) were detected when the acatalasemic erythrocyte suspension was treated with 1 mM hydrogen peroxide (concentration of hemoglobin was 69.8 $\mu$M, Fig. 3A). These signals were assigned as tyrosine ($g = 2.005$) and tryptophan peroxyl ($g = 2.03$) radicals, respectively (10, 17). A small signal of $g = 6$ was also observable and was assigned as methemoglobin (high spin). The relation between the intensity of the tyrosine radical signal and the concentration of hydrogen peroxide is indicated in Fig. 3B. The small signal was observed in the acatalasemic erythrocytes, but the signal did not rapidly increase at levels up to 0.25 mM hydrogen peroxide. The signal intensity increased and reached a peak at 2.0 mM hydrogen peroxide.
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Time Course of Hemolysis of Erythrocytes Treated with 0.1 mM Hydrogen Peroxide—The hemolysis of erythrocytes treated with 0.1 mM hydrogen peroxide is indicated in Fig. 4A. After 30 min had elapsed from the addition of hydrogen peroxide, most (≥60%) of the acatalasemic erythrocytes had become hemolyzed, but normal erythrocytes had not. The hemolysis of acatalasemic erythrocytes by hydrogen peroxide was prevented by the addition of glucose and more effectively by the addition of glucose-AsA (Fig. 4C). When compared with the hemolysis caused by the addition of hydrogen peroxide, normal and acatalasemic erythrocytes were treated with AAPH (Fig. 4B). There was no observable difference in hemolysis between them.

Color Change of the Erythrocyte Suspension by Addition of Hydrogen Peroxide—When erythrocytes were treated with 1 mM hydrogen peroxide, the color of the acatalasemic erythrocytes dramatically changed from red to dark brown (1). The color change was attributable to the oxidation of hemoglobin and precipitation of the erythrocytes by hydrogen peroxide.

SDS-PAGE Analysis of Erythrocytes Treated with 1–10 mM Hydrogen Peroxide—The bands for hemoglobin (16 kDa) in the acatalasemic erythrocytes decreased with the treatment of hydrogen peroxide, and the protein bands of greater than 207 kDa increased (Fig. 5). Western blot indicated the latter bands to be polymerized adducts of hemoglobin. On the other hand, the SDS-PAGE pattern of normal erythrocytes was not significantly affected by 1–10 mM hydrogen peroxide treatment.

Microscopic Studies of Erythrocytes—Light microscopy revealed the acatalasemic erythrocytes to be deformed and significantly aggregated (Fig. 6B, arrows), whereas normal erythrocytes exhibited no discernable aggregation after treatment with 1 mM hydrogen peroxide (Fig. 6A).

DISCUSSION

First, the visible spectral changes in the hemolysates were examined subsequent to the addition of hydrogen peroxide. Although the spectra of the normal hemolysates did not change after the addition of hydrogen peroxide, the spectra of the acatalasemic hemolysates did (Fig. 1). The spectral changes indicated the formation of methemoglobin (H-Hb-Fe(III)) and the ferryl hemoglobin species (H-Hb-Fe(IV) = O and Hb-Fe(IV) = O) (9).

Subsequently, we examined the effects of hydrogen peroxide on erythrocytes. Although the protein leakage from normal erythrocytes was hardly observed 30 min after the addition of 0.1 mM...
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hydrogen peroxide, the protein leakage from the aca-
tasalamic erythrocytes was severe. Surprisingly, with the addition of a higher concentration of hydrogen peroxide, protein leakage from the aca-
tasalamic erythrocytes was reduced (Fig. 2A), and the erythro-
cytes immediately became insoluble in water in the presence of more than 0.5 mM hydrogen peroxide (Fig. 2B). On the other hand, normal erythrocytes treated with hydrogen peroxide exhibited only mild hemolysis. These results indicate that aca-
tasalamic erythrocytes are easily distinguishable from normal erythrocytes after the addition of hydrogen peroxide.

A close examination of the time course of hemolysis at a dose level of 0.1 mM hydrogen peroxide would provide a close characterization of hemolysis. It was expected there would be a big difference between normal and aca-
tasalamic erythrocytes (Fig. 4A). However, hemolysis by AAPH, which decomposes with heat to form a carbon-centered radical, indicated that there was in fact no difference in hemolysis between them (Fig. 4B). These results suggested that hydrogen peroxide penetrated through the membrane and generated ferryl hemoglobin species in the aca-
tasalamic but not normal erythrocytes because hydrogen peroxide was decom-
polymerized to become adducts, and the erythrocytes thus would become insoluble in water. The reaction of the ferryl hemoglobin with ferrous hemoglobin pro-
duced methemoglobin (9), which concomitantly reacts with hydro-
gen peroxide to generate the radicals of ferryl hemoglobin (10). This account explains the previous observation that the concentra-
tion of methemoglobin in mouse aca-
tasalamic erythrocytes was higher than that found in normal erythrocytes (15). Furthermore, the formation of ferryl hemoglo-in and the radicals in aca-
tasalamic erythrocytes induces hemolysis. This is because ferryl hemoglobin species are strong oxidants and can promote lipid peroxidation of the membrane (9, 16). Such hemolysis may explain the clinical finding that heme deg-

radation products in the urine of aca-
tasalamic patients are five times higher than in normal patients (18).

The prevention of aca-
tasalamic erythrocyte hemolysis by the addition of glucose-AsA and/or glucose (Fig. 4C) was observed. As the scavenging reaction of hydrogen peroxide in the presence of hemoglobin was enhanced by NAD(P)H (8), the preventive effect by glucose was explained by the reduction with NAD(P)H since NADPH generation through the pentose phos-
phate pathway was enhanced by the generation of hydrogen peroxide and low catalase activity in erythrocytes (19). As the prevention effect of the hemolysis with glucose-AsA is consist-
ent with that of the myocardi-
cal cell damage caused by the ferryl myoglobin species (20, 21), the effect is attributable to the direct reduction of oxidized hemoglobin by AsA (8).

To characterize the water-insoluble erythrocytes that resulted from the treatment with a high concentration of hydrogen peroxide (more than 1 mM), insoluble erythrocytes were examined by SDS-PAGE and Western blot analyses. The results indicate that the amounts of hemoglobin decreased and that hemoglobin polymerized to form adducts in the aca-
tasalamic mouse erythrocytes. The ESR spectra revealed the tyrosine radical of ferryl hemoglobin to be generated and increased at more than 0.5 mM hydrogen peroxide. As it was expected that the amounts of the radical would be increased with more than 0.3 mM hydrogen peroxide, a high concentration of the radical would make hemoglobin polymerize to become adducts, and the erythrocytes thus would become insoluble in water. The abnormal reaction with hydrogen peroxide caused the shape change of the erythrocytes, which resembled that of cardiac myocytes treated with lipid hydroperoxide (22). Furthermore, aggregation was observed in the system (Fig. 6). This aggregation may be due to a change in the charge of the erythrocyte membrane induced by the high concentration of the reactive tyrosine radical. The aggregated erythrocytes might impede the
normal flow of the blood stream and thus contribute to the oral gangrene observed in Japanese acatalasia patients (Takahara disease) (1). Thus, we propose the catabolism of hydrogen peroxide in acatalasemic erythrocytes as depicted in Fig. 7.

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REFERENCES
1. Takahara, S. (1952) Lancet 263, 1101–1104
2. Eaton, J. W., and Ma, M. (1995) The Metabolic and Molecular Bases of Inherited Disease (Scriber, C. R, Beaudet, A. L., Sly, W. S., and Valle, D., eds), Vol. 2, pp. 2371–2383, McGraw-Hill, New York, NY
3. Goth, L. (2001) Blood Cells Mol. Dis. 27, 512–517
4. Ogata, M., Sadamoto, M., and Takahara, S. (1986) Proc. Jpn. Acad. 42, 828–832
5. Masuoka, N., Wakimoto, M., Ubuka, T., and Nakano, T. (1996) Clin. Chim. Acta 254, 101–112
6. Masuoka, N., Wakimoto, M., Ohta, J., Ishii, K., and Nakano, T. (1997) Biochim. Biophys. Acta 1361, 131–137
7. Wakimoto, M., Masuoka, N., Nakano, T., and Ubuka, T. (1998) Acta Med. Okayama 52, 233–237
8. Masuoka, N., Kodama, H., Abe, T., Wong, D. H., and Nakano, T. (2003) Biochim. Biophys. Acta 1637, 46–54
9. Giulivi, C., and Davies, K. J. A. (1990) J. Biol. Chem. 265, 19453–19460
10. Svitunenko, D. A., Patel, R. P., Voloshchenko, S. V., and Wilson, M. P. (1997) J. Biol. Chem. 272, 7114–7121
11. Feinstein, R. N., Braun, J. T., and Howard, J. B. (1967) Arch. Biochem. Biophys. 120, 165–169
12. Drabkin, D. L., and Austin, J. H. (1935) J. Biol. Chem. 112, 51–65
13. Laemmli, U. K. (1970) Nature 227, 680–685
14. Miki, M., Tamai, H., Mino, N., Yamamoto, Y., and Niki, E. (1987) Arch. Biochem. Biophys. 258, 373–380
15. Ogata, M., Kobayashi, H., Ioku, N., and Ishii, K. (1986) Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 62, 367–371
16. Gorbunov, N. V., Osipov, A. N., Day, B. W., Zayas-Rivera, B., Kagan, V. E., and Elsayed, N. M. (1995) Biochemistry 34, 6689–6699
17. Svitunenko, D. A. (2001) Biochim. Biophys. Acta 1546, 365–378
18. Takahara, S., and Ogata, M. (1977) Biochemical and Medical Aspects of Active Oxygen, (Hayaishi, O., and Asada, K., eds) pp. 275–292, University Park Press, Baltimore, MD
19. Gaetani, G. F., Kirkman, H. N., Mangerini, R., and Ferraris, A. M. (1994) Blood 84, 325–330
20. Galaris, D., Eddy, L., Arduini, A., Cadenas, E., and Hochstein, P. (1989) Biochem. Biophys. Res. Commun. 160, 1162–1168
21. Wu, F., Altura, B. T., Gao, J., Barbour, R. L., and Altura, B. M. (1994) Biochim. Biophys. Acta 1225, 158–164
22. Walters, F. P., Kennedy, F. G., and Jones, D. P. (1983) FEBS Lett. 163, 292–296