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Formation of Denatured Erythrocytes by Exposure to a Superoxide Radical Generating System of Xanthine Oxidase[1)]

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Exposure of rat erythrocytes to an O_2^--generating system of xanthine oxidase resulted in the formation of cells resistant to hypotonic hemolysis along with the degradation of hemoglobin without evidence of hemolysis. Exposure of cells for 30 min to the xanthine oxidase system caused a shift of the Soret maximum at 415 to 405 nm with the disappearance of three isosbestic points. At this time, a large amount of dark brown pigment was formed in cells and rigid cells resistant to hypotonic hemolysis were formed. Catalase but not superoxide dismutase strongly inhibited the oxidative degradation of oxyhemoglobin and the formation of resistant cells, suggesting possible involvement of H_2O_2 in the xanthine oxidase system. Moreover, cells exposed directly to H_2O_2 were transformed to resistant cells, but this did not occur in the presence of KCN. Presumably, denatured cells are produced in association with the formation of methemoglobin and denatured hemoglobin, which precipitates in the cells throughout the exposure period to H_2O_2 generated in the xanthine oxidase system.

Keywords——erythrocyte; xanthine oxidase; resistance to hypotonic hemolysis; denatured hemoglobin; hydrogen peroxide; catalase

Superoxide radicals (O_2^-), formed by the univalent reduction of molecular oxygen, are known to be generated in a number of biological reactions and to cause cell damage.[2)] Superoxide dismutase, which catalytically scavenges O_2^-, has been proposed to be an essential component of biological defence against oxygen toxicity.[3)] Because of the content of superoxide dismutase in erythrocytes, the damaging effects of O_2^- on cellular components and the protective effects of superoxide dismutase in erythrocytes have been investigated by many workers using many models of O_2^--generating system.[4)] Several workers have shown that the oxidative breakdown of cellular hemoglobin and hemolysis induced by O_2^- were inhibited by superoxide dismutase, suggesting that these protective mechanisms may represent one of the physiological roles of superoxide dismutase in cells.[4a-c,e)] The exact significance of this enzyme in erythrocytes, however, is not clearly understood.

In the previous paper,[5)] we examined the damaging effect of O_2^- on erythrocytes in the O_2^--generating system of photoactivated riboflavin and demonstrated that erythrocytes were denatured to cells resistant to hypotonic hemolysis without evidence of isotonic hemolysis, and that catalase but not superoxide dismutase had a preventive effect on the formation of these resistant cells.

The present paper is concerned with further investigation on the damaging effect of O_2^- on rat erythrocytes, and in particular, with changes in osmotic fragility and hemoglobin degradation in the O_2^--generating xanthine oxidase-hypoxanthine system.

Experimental

Material——Xanthine oxidase (from butter milk) was obtained from P-L Biochemicals, Inc., Milwaukee, Wis., U.S.A. Catalase (from bovine liver, thymol-free) and superoxide dismutase (from bovine blood) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. All other chemicals used were of the highest quality available from commercial sources.

Animals——The rats used in this experiment were males of the Wistar strain, weighing about 200 g. Vitamin E-deficient rats were obtained by feeding infants weighing about 60 g initially with pellets of vitamin E-deficient diet containing 8 mg of vitamin E per kg (Oriental Yeast Co., Ltd.).

Preparation of Erythrocyte Suspension——As described previously,[5] blood was taken from the common carotid artery into heparinized test tubes and centrifuged at $1500 \times g$ for 10 min at 4°. The plasma and buffy coat were removed by aspiration. Erythrocytes were washed three times with 5 volumes of isotonic NaCl solution. Erythrocytes containing carbon monoxide hemoglobin (CO-Hb) were prepared by bubbling CO through the erythrocyte suspension (10% hematocrit) for 15 min. The erythrocytes were collected by centrifugation at $1500 \times g$ for 10 min and washed three times with isotonic NaCl solution. Carbon monoxide was generated by heating a mixture of oxalic acid and sulfuric acid. Carbon dioxide formed concomitantly was removed by passing the gas through 50% NaOH solution and water.

O_2^--Generating System and Reaction with Erythrocytes——Generation of O_2^- in the xanthine oxidase-hypoxanthine system was assayed by the cytochrome c reduction procedure of McCord *et al.*[3] and H_2O_2 by the method of Hildebrandt *et al.*[6] Xanthine oxidase activity was assayed by measuring the absorption of uric acid formed at 293 nm. Unless otherwise specified, all reaction procedures were as follows: packed cells suspended in 3 ml of 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 5 mM hypoxanthine and 100 μg of xanthine oxidase to give a final hematocrit of 0.1% were incubated with shaking at 37°. The anaerobic reaction with cells was carried out in a modified Thunberg tube with a quarz cell under an atmosphere of nitrogen gas.

Determination of Hemolysis and Osmotic Fragility——Extent of hemolysis was followed in terms of the decrease in turbidity of the cell suspension at 740 nm as described in the previous paper.[5] Complete hemolysis was carried out by adding cells to distilled water. Osmotic fragility was tested as follows; cells incubated in the xanthine oxidase-hypoxanthine system or in isotonic saline containing H_2O_2 were collected by centrifugation at $1500 \times g$ for 10 min. After being washed three times with isotonic saline, the packed cells were challenged with 0.2% NaCl. The cell suspension was allowed to stand for 10 min at room temperature, then the turbidity was measured at 740 nm with a spectrophotometer (Hitachi 200-20). The measurements of absorption spectra of hemoglobin in cells were performed between 350 and 700 nm with a Shimadzu MPS-5000 spectrophotometer.

Results and Discussion

Hemolysis of Erythrocytes in Xanthine Oxidase-Hypoxanthine System

As shown in Fig. 1, exposure of normal erythrocytes to the xanthine oxidase system caused a slight decrease in the turbidity at 740 nm and a profound change in cellular color to dark brown. However, the extent of the decrease in turbidity was not significantly different from that of cells incubated in the reaction system without xanthine oxidase or hypoxanthine, in which no changes in the cellular color were observed. These results indicated that the exposure of cells to the xanthine oxidase system did not cause hemolysis but rather caused degradation of hemoglobin in the cells. On the other hand, erythrocytes from the vitamin E-deficient rats were progressively hemolyzed and the time for 50% hemolysis was about 60 min. The inserted figure shows the generation of H_2O_2 to be about 2.8 mM at 120 min after the start of incubation of the reaction system.

Hypotonic Hemolysis of Cells exposed to the Xanthine Oxidase System

After cells from normal rats had been exposed to the xanthine oxidase system for various times, they were tested for hemolysis against 0.2% NaCl. As shown in Fig. 2, the cells that had been exposed for 15 min to the xanthine oxidase system were completely hemolyzed in 0.2% NaCl. However, exposure for 30 min caused only about 30% hemolysis. The cells exposed for over 45 min were no longer sensitive to hypotonic hemolysis. These resistant cells to hypotonic hemolysis were collected by centrifugation. They contained a dark brown pigment and were not soluble in water or Triton X-100 but were readily soluble in 0.1 N NaOH.

Since the xanthine oxidase system is known to produce H_2O_2 and O_2^-,[3] the effects of catalase and superoxide dismutase on the formation of resistant cells were examined. As shown in Fig. 2, addition of catalase to the xanthine oxidase system (10 μg/ml) resulted in complete hemolysis in 0.2% NaCl and no formation of resistant cells or dark brown pigment was detected throughout the incubation, whereas addition of superoxide dismutase (10 μg/ml) was without effect.

Although the data are not shown, microscopic examination revealed that the dark brown pigment in the resistant cells was intensely stained by brilliant cresyl blue and was located

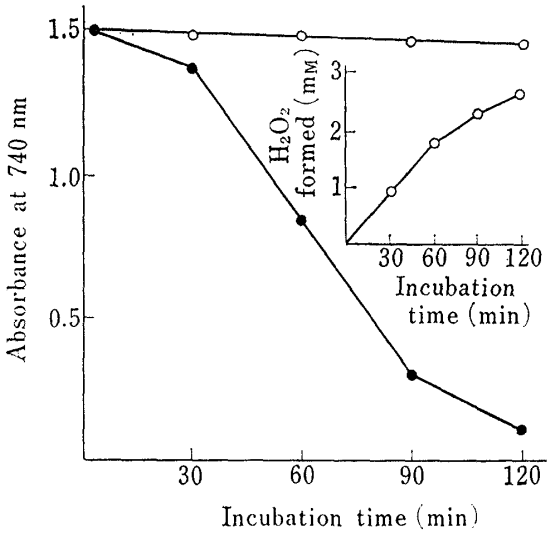

Fig. 1. Time Course of Hemolysis of Rat Erythrocytes in the Xanthine Oxidase–Hypoxanthine System

Reaction mixture containing 0.15 M NaCl, 5 mM hypoxanthine, 100 μg of xanthine oxidase and erythrocytes (0.1% hematocrit) in 3.0 ml of 10 mM phosphate buffer, pH 7.4, was incubated at 37°. Hemolysis was determined by measuring the decrease in turbidity at 740 nm. The insert represents the time course of the generation of H_2O_2 under the same conditions. Each point represents the mean of triplicate experiments. —○—, erythrocytes; —●—, erythrocytes from vitamin E-deficient rat.

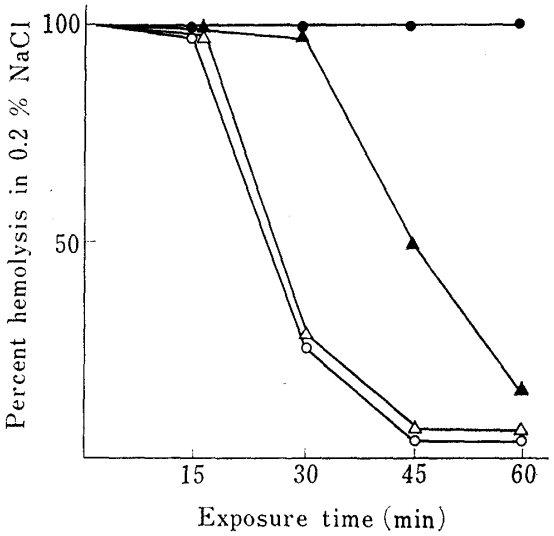

Fig. 2. Hypotonic Hemolysis of Cells Exposed to the Xanthine Oxidase System

Erythrocytes from normal rats were exposed to the xanthine oxidase system for various times. After centrifugation for 10 min at 1500×*g*, the packed cells were challenged with 0.2% NaCl and hemolysis was measured by the method described in "Experimental." Each point represents the mean of duplicate experiments. —○—, complete system; —●—, +catalase (10 μg/ml); —△—, +superoxide dismutase (10 μg/ml); and —▲—, cells containing CO-Hb exposed to the complete system.

uniformly around the interior periphery of cells, as described in the previous paper.[5] With the cells exposed to the xanthine oxidase system for 60 min under anaerobic conditions (under N_2 gas), resistant cells were not formed. This is presumably because active oxygen species, that is O_2^- or H_2O_2, are not generated in the xanthine oxidase system under anaerobic conditions, as reported by McCord *et al.*[3] With the cells containing carbon monoxide-hemoglobin, the formation of resistant cells proceeded slowly after a lag time of about 30 min. Carbon monoxide-hemoglobin is known to be less susceptible than oxyhemoglobin to oxidative attack.[8,9] Therefore, the formation of resistant cells as described above seemed to result from the oxidative degradation of hemoglobin molecules in cells. Moreover, the result suggests a possible involvement of H_2O_2 in the formation of resistant cells in the xanthine oxidase.

Spectral Changes of Hemoglobin in Cells

Fig. 3 shows the spectral changes of hemoglobin during the exposure of cells to the xanthine oxidase system. Exposure of cells for 15 min caused a rapid decrease in the double peaks of absorption at 540 and 576 nm, which are characteristic of oxyhemoglobin, and a slight increase in absorption at around 620 nm. Of greater interest was the finding that the exposure of cells for 30 min resulted in a shift of the Soret maximum at 415 to 405 nm with disappearance of the three isosbestic points at 475, 525 and 590 nm. At this time, a large amount of dark brown pigment was formed in cells and the resistant cells were formed to the extent of about 70% as shown in Fig. 2. After the exposure of cells for 45 min, the double peaks of absorption at 540 and 576 nm disappeared and a decrease in the absorption at 405 nm was observed. Addition of 10 mM KCN to the cell suspension after exposure for 60 min caused a shift of the Soret maximum from 405 to 420 nm. Furthermore, the shoulder around 570 and 620 nm did not completely disappear upon addition of KCN to the cell suspension, demonstrating the presence of oxidatively degraded hemoglobin together with about 30% methemoglobin.

When the hemolysate was exposed to the xanthine oxidase system, the spectroscopic

changes in hemoglobin were quite similar to those of the cell suspension as described above. However, the absorption intensity at 405 nm was about twice that of the cell suspension. These results suggest that the shift of the Soret maximum from 415 to 405 nm and the decrease in the absorbance at 405 nm during prolonged exposure were probably due to the formation of denatured hemoglobin, which precipitated in the cells. Moreover, there was a lag period of about 15 min before the formation of resistant cells as shown in Fig. 2, indicating that the oxidative formation of such denatured hemoglobin may be important in initiating the formation of resistant cells.

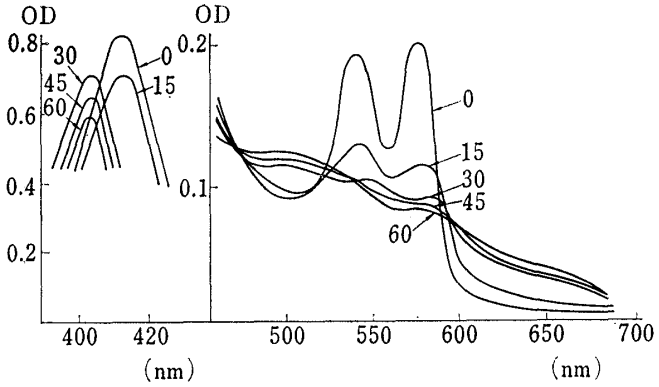

Fig. 3. Spectral Changes of Hemoglobin in Cells exposed to the Xanthine Oxidase System

Erythrocytes were exposed to the xanthine oxidase system. Changes in the absorption spectra of hemoglobin in cells were recorded at 15 min intervals. Numbers on curves refer to exposure times.

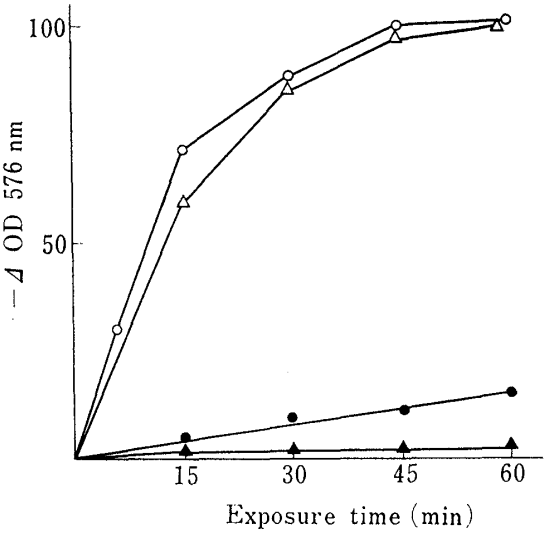

Fig. 4. Effects of Catalase and Superoxide Dismutase on the Oxidative Degradation of Oxyhemoglobin

Catalase (10 μg/ml) and superoxide dismutase (10 μg/ml) were added to the xanthine oxidase system containing erythrocytes (0.1% hematocrit). The decrease in absorption at 576 nm was measured. Each point represents the mean of duplicate experiments. —○—, complete system; —●—, +catalase (10 μg/ml); —△—, +superoxide dismutase (10 μg/ml); and —▲—, +both enzymes.

Since oxyhemoglobin is known to be converted to methemoglobin, hemichrome, choleglobin and other products by H_2O_2 added directly or generated from ascorbate,[7,12] the dark brown pigments produced in the resistant cells were termed here for convenience "denatured hemoglobin." A green breakdown product of hemoglobin such as choleglobin, which has a broad absorption peak at around 700 nm, was not produced under the conditions employed here.

Effects of Catalase and Superoxide Dismutase on the Oxidative Degradation of Hemoglobin

As shown in Fig. 4, the oxidative degradation of oxyhemoglobin in cells was strongly but not completely inhibited by the addition of catalase to the xanthine oxidase system. In this case, only a small amount of methemoglobin was detected in cells. In the presence of both catalase and superoxide dismutase, the changes in the absorption spectrum of oxyhemoglobin were scarcely observed. The observation that superoxide dismutase completely inhibited the oxidative degradation of oxyhemoglobin in the presence of catalase suggests a possible involvement of O_2^- in the formation of methemoglobin, but not further degradation to denatured hemoglobin. On the other hand, the addition of superoxide dismutase alone caused a slight inhibition of the oxidative degradation of oxyhemoglobin, though the time course of spectral changes of oxyhemoglobin was almost the same as those shown in Fig. 3.

These results suggest that denatured hemoglobin is formed in cells by oxidative attack of H_2O_2 generated in the xanthine oxidase system.

Formation of Resistant Cells by H_2O_2

To ascertain whether H_2O_2 takes part in the formation of resistant cells, cells were exposed directly to H_2O_2. As shown in Fig. 5, almost 50% of the cells was transformed to resistant cells by exposure to 1 mM H_2O_2. At high concentrations of 2 mM or above, nearly all of the cells were found to be resistant cells. The concentration of H_2O_2 required for the formation of resistant cells was similar to that of H_2O_2 generated in the xanthine oxidase system as shown in the insert of Fig. 1. Although the data are not shown, the spectroscopic changes in oxyhemoglobin in these resistant cells were almost the same as those shown in Fig. 3, indicating the formation of denatured hemoglobin in cells.

It has been shown that methemoglobin formed by oxidative attack of H_2O_2 is immediately converted to cyanmethemoglobin in the presence of cyanide,[11a] which is expected to inhibit the formation of resistant cells by producing H_2O_2-insensitive cyanmethemoglobin in the cells. As shown in Fig. 5, the formation of resistant cells did not occur in the presence of KCN at a final concentration of 10 mM in isotonic saline containing various concentrations of H_2O_2. Furthermore, the hemolysate obtained from these cells by hypotonic hemolysis in 0.2% NaCl showed a characteristic absorption spectrum of cyanmethemoglobin, indicating the formation of cyanmethemoglobin in the cells during their exposure to H_2O_2 in the presence of KCN. Presumably, the formation of methemoglobin may be an intermediate event in the process of resistant cell formation in the xanthine oxidase system. In addition, the cells containing carbon monoxide hemoglobin were converted more slowly to resistant cells as compared with normal cells. This result is consistent with the result obtained on exposure of cells to the xanthine oxidase system. These results support the view that rigid cells resistant to hypotonic hemolysis are formed in the xanthine oxidase system by oxidative attack of H_2O_2 rather than O_2^-.

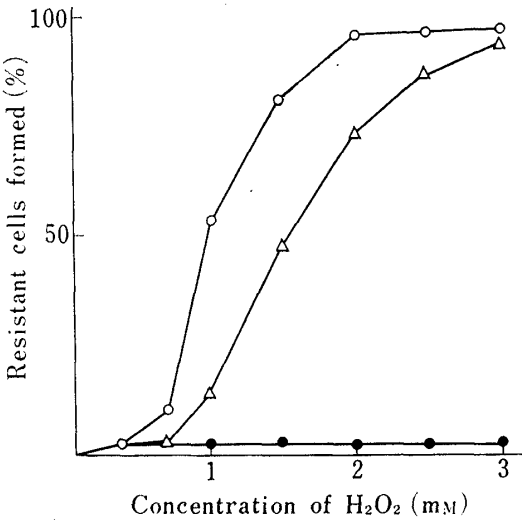

Fig. 5. Formation of Resistant Cells by H_2O_2

Erythrocytes were suspended in isotonic saline containing various concentrations of H_2O_2. After incubation at 37° for 30 min, the packed cells collected by centrifugation for 10 min at 1500×*g* were challenged with 0.2% NaCl solution. The turbidity of the suspensions was measured at 740 nm. —○—, no addition; —●—, + 10 mM KCN; and —△—, cells containing CO-Hb in the absence of KCN.

The data reported here suggest that H_2O_2 generated in the xanthine oxidase system penetrates into the erythrocyte membrane, and thereby causes some sequential degradation of hemoglobin and formation within the cells of water-insoluble, stainable granules resembling Heinz bodies. Earlier workers have shown the importance of catalase and glutathione peroxidase in protecting hemoglobin from oxidative breakdown by H_2O_2 or other chemical oxidants.[12,13] Both enzymes are known to be present in sufficient amounts in erythrocytes to reduce the toxic effects of H_2O_2 produced *in vivo*.[14] The results presented here represent evidence that erythrocytes were denatured to form rigid cells when sufficient H_2O_2 was generated to overwhelm the capacity of the two enzyme activities. On the other hand, the exposure of cells to the xanthine oxidase system brought about oxidative hemolysis in vitamin E-deficient cells, probably as a consequence of lipid peroxidation in the cell membrane.

References and Notes

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