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Iron Release from Ferritin and Generation of Hydroxyl Radical in the Reaction System of Alloxan with Reduced Glutathione; A Role of Ferritin in Alloxan Toxicity

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Release of iron from ferritin and deoxyribose degradation were caused by alloxan in the presence of reduced glutathione (GSH). Superoxide dismutase, catalase, diethylenetriaminepentaacetic acid and hydroxyl radical scavengers such as mannitol and benzoate inhibited the degradation of deoxyribose in the alloxan–GSH system, suggesting that hydroxyl radical was generated in the alloxan–GSH system in the presence of ferritin. Iron released from ferritin may catalyze hydroxyl radical generation *via* the Haber–Weiss reaction. However, the inhibition of the iron release from ferritin in the alloxan–GSH system required a large amount of superoxide dismutase. These results suggest that the iron release from ferritin in the alloxan–GSH system is not primarily due to superoxide. These findings may help to explain the precise mechanism of alloxan toxicity.

Keywords——alloxan; reduced glutathione; ferritin; hydroxyl radical; superoxide dismutase

Alloxan specifically damages β-cells in pancreatic islets.[1] The deleterious effect of alloxan can be blocked by a number of hydroxyl radical-reducing agents including superoxide dismutase (SOD), catalase, the iron-chelator diethylenetriaminepentaacetic acid (DETAPAC) and some hydroxyl radical scavengers.[2-11] On the basis of these findings, the toxic action of alloxan has been related to generation of hydroxyl radicals ($HO^{\cdot}$) *in vivo* or *in vitro*.

When alloxan is exposed to reducing substances under aerobic conditions, a redox cycle is formed between alloxan and its reduced analogue dialuric acid.[12] Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are produced during the autoxidation of dialuric acid.[12] Iron catalyzes the reaction of $HO^{\cdot}$ generation from O_2^- and H_2O_2 (Haber–Weiss reaction).[13] However, the possibility has not been explored that iron within cells participates in the diabetogenic action of alloxan. Most intracellular iron except for heme iron is stored within ferritin, a large multisubunit protein in which iron is stored as ferric micelles.[14] Release of iron from ferritin requires reduction of the ferric iron.[15]

Evidence for iron release from ferritin by alloxan would cast light on the role of ferritin in the alloxan toxicity. Therefore, we examined the interaction of alloxan with ferritin. The present study indicates that alloxan in the presence of glutathione (GSH) is able to release iron from ferritin, and $HO^{\cdot}$ is generated in the alloxan–GSH system.

Experimental

Materials——SOD (from bovine erythrocytes), catalase (from bovine liver, thymol-free) and ferritin (from horse spleen) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Xanthine oxidase was purchased from Biozyme Co., Ltd. Great Britain. Alloxan and deoxyribose were from Wako Pure Chemical Industry Co., Ltd. while bathophenanthroline sulfonate was from Dojindo Laboratories Co., Ltd. Thiobarbituric acid (TBA) was obtained from Merck Japan Ltd. and recrystallized twice from water prior to use. Sepharose 6B was purchased from Pharmacia Japan Ltd. All other chemicals were of highest purity commercially available.

Enzyme Assay——SOD activity was measured by the method of McCord and Fridovich.[16] Specific activity was 3000 units/mg protein for SOD. The assay of catalase activity was based on the decomposition of H_2O_2, which was followed spectrophotometrically at 240 nm. One unit of catalase was defined as 1.0 μmol of H_2O_2 decomposed per min.[17] Specific activity was 20000 units/mg protein for catalase. Contaminating protease in the commercial xanthine oxidase used in this study was inhibited as follows. A stock enzyme in phosphate buffer containing 3.4 M ammonium sulfate was diluted to a concentration of about 20 units/ml in 10 mM phosphate buffer, pH 7.4. Aprotinin was added to inactivate serine protease. To remove ammonium sulfate, this solution (0.2 ml) was applied to a Sephadex G-25 column (1.0 × 20 cm) and eluted with 10 mM phosphate buffer containing 0.15 M NaCl at 4°C. Fractions were monitored for xanthine oxidase activity by measuring the rate of conversion of hypoxanthine to uric acid. Fractions containing xanthine oxidase activity were collected and pooled at 4°C. One unit of xanthine oxidase was defined as 1.0 μmol of uric acid produced per min at 25°C at pH 7.5. Protein concentration was estimated by the bicinchoninic acid method.[18]

Preparation of Ferritin and Iron Determination——Commercial ferritin was incubated in 10 mM phosphate buffer, pH 7.4, containing 10 mM ethylenediaminetetraacetic acid at 4°C and the solution (2.0 ml) was applied to a Sepharose 6B column (2.6 × 80 cm). Ferritin fractions were collected and concentrated by ultrafiltration. The iron released from ferritin was determined according to Thomas and Aust with minor modifications.[19] The ferrous iron chelator used in this study was bathophenanthroline sulfonate ($\varepsilon = 22.14\ mM^{-1}cm^{-1}$) and the increase in absorption at 530 nm was continuously monitored with a Hitachi model 200-20 spectrophotometer. Iron in ferritin was determined with a Hitachi 180—80 polarized Zeeman atomic absorption spectrophotometer. The iron content of ferritin was 0.22 mg Fe^{3+}/mg protein. For anaerobic experiments, all solutions were purged with N_2 gas for 10 min.

Degradation of Deoxyribose——TBA-reactive oxidation products of deoxyribose were determined according to Gutteridge with minor modifications.[20] Reaction of the deoxyribose was terminated after 5 min at 37°C by mixing 3.0 ml of samples with 0.15 ml of 30% trichloroacetic acid (TCA). After centrifugation of the reaction mixture for 10 min at 1500 × *g*, 0.5 ml of 0.6% TBA was added to the supernatant. The solution was heated for 10 min at 100°C and cooled, and the absorbance at 532 nm was measured.

Results and Discussion

Iron Release from Ferritin by Alloxan in the Presence of GSH

As shown in Fig. 1, when ferritin was incubated with alloxan in the presence of 0.3 mM GSH, absorption at 530 nm, caused by formation of bathophenanthroline-Fe^{2+}, increased with time, indicating that iron in ferritin was reductively released. Iron release from ferritin was dependent on alloxan concentration in the range from 0.01 to 0.10 mM. Little increase in absorption at 530 nm was observed when ferritin was incubated with GSH (0.3 mM) alone. It is likely that GSH reduces alloxan but does not release iron from ferritin in the reaction system.

Formation of HO·

It has been assumed that the diabetogenic action of alloxan is due to HO· generated by the iron-catalyzed Haber–Weiss reaction.[2-11] We therefore tested whether HO· was generated or not in the reaction system of alloxan with GSH in the presence of ferritin. As shown in Fig. 2, absorption at 532 nm, based on the formation of degradation products of deoxyribose, increased with increase of alloxan concentration in the presence of ferritin with GSH. In the absence of ferritin, no significant increase in absorption at 532 nm was observed. These results indicate a requirement of ferritin for degradation of deoxyribose in the reaction system of alloxan with GSH. Furthermore, as summarized in Table I, addition of 10 μg/ml of SOD to the reaction system of alloxan with GSH containing deoxyribose and ferritin inhibited the degradation of deoxyribose by about 20%. Catalase completely inhibited the degradation of deoxyribose. An iron chelator, DETAPAC, and scavengers of HO·, mannitol and benzoate, also inhibited the degradation of deoxyribose. These results indicate that HO· is produced in the alloxan–GSH system in the presence of ferritin. Iron released from ferritin may catalyze to the generation of HO· *via* the Haber–Weiss reaction.

Effect of SOD

The iron release from ferritin was further investigated by addition of SOD to both

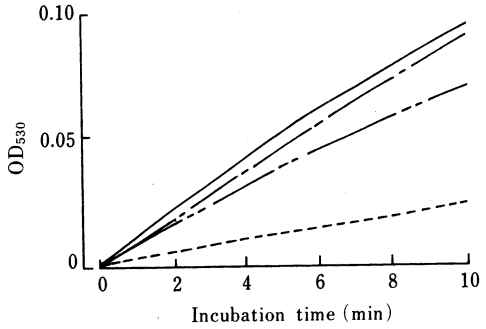

Fig. 1. Release of Iron from Ferritin by Alloxan with GSH

The reaction mixture contained ferritin (0.3 mg/ml), bathophenanthroline sulfonate (1.0 mM), 0.15 M NaCl, GSH (0.3 mM) and various concentration of alloxan in 10 mM phosphate buffer, pH 7.4. Reactions were initiated by the addition of alloxan at 37 °C and continuously monitored at 530 nm. ——, 0.10 mM alloxan; —·—, 0.05 mM alloxan; — — —, 0.03 mM alloxan; ----, 0.01 mM alloxan.

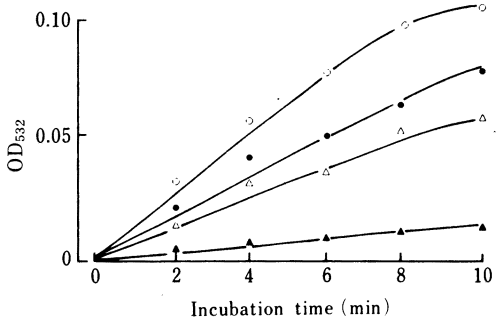

Fig. 2. Deoxyribose Degradation by Alloxan with GSH in the Presence of Ferritin

The reaction mixture contained ferritin (0.3 mg/ml), 2 mM deoxyribose, 0.15 M NaCl, 0.3 mM GSH and various concentrations of alloxan in 10 mM phosphate buffer, pH 7.4. The reaction was initiated by the addition of alloxan. After incubation for various times at 37 °C, the reaction was stopped with TCA and TBA-reactive products were determined. Each point represents the mean of duplicate experiments. —○—, 0.10 mM alloxan; —●—, 0.05 mM alloxan; —△—, 0.03 mM alloxan; —▲—, 0.01 mM alloxan.

TABLE I. Inhibition of Deoxyribose Degradation by Hydroxyl Radical Reducing Agents

Agent	Concentration	OD 532 nm	Inhibition (%)
None		0.067	0
SOD	10.0 (μg/ml)	0.056	16.4
Catalase	10.0 (μg/ml)	0.002	97.0
DETAPAC	1.0 (mM)	0.054	19.4
Benzoate	10.0 (mM)	0.042	37.3
Mannitol	10.0 (mM)	0.027	59.7

The reaction system consisted of 3.0 ml of 10 mM phosphate buffer containing 0.15 M NaCl, 2 mM deoxyribose, ferritin (0.3 mg/ml), 0.1 mM alloxan and 0.3 mM GSH. Hydroxyl radical reducing agents were added to the reaction system before initiation of the reaction. After incubation for 5 min at 37 °C, formation of TBA-reactive products was measured. Other conditions were the same as in Fig. 2. Each value represents the mean of duplicate experiments.

alloxan–GSH and hypoxanthine–xanthine oxidase systems. As summarized in Table II, the iron release from ferritin by the alloxan–GSH system was inhibited by the addition of 50—300 μg/ml of SOD, although addition of 10 μg/ml of SOD was without effect. This is in contrast to the inhibition of deoxyribose degradation by addition of 10 μg /ml of SOD. Although the data are not shown, catalase had no effect, indicating that H_2O_2 is not involved in the reaction of iron release from ferritin. The possibility that SOD was inactivated or irreversibly inhibited was excluded by pretreatment of the enzyme with the alloxan–GSH system. Cytochrome c reduction induced by hypoxanthine–xanthine oxidase is inhibited about 80% by SOD at 1.0 μg/ml. Pretreatment of SOD (100 μg/ml) with the alloxan–GSH system for 10 min, and then dilution about 100 times did not significantly alter this inhibition.

On the other hand, addition of 10 μg/ml of SOD to the hypoxanthine–xanthine oxidase system resulted in about 80% inhibition of the iron release from ferritin. In this reaction system, even addition of 1.0 μg/ml SOD caused about 60% inhibition. Deoxyribose degradation caused by the hypoxanthine-xanthine oxidase system in the presence of ferritin was

TABLE II. Effect of SOD on the Release of Iron from Ferritin in the Alloxan–GSH or Hypoxanthine–Xanthine Oxidase System

System	nmol Fe^{2+}	Inhibition (%)
Alloxan–GSH	7.25	0
+SOD (10.0 μg/ml)	7.23	0.3
+SOD (50.0 μg/ml)	6.44	11.1
+SOD (100.0 μg/ml)	4.83	33.4
+SOD (300.0 μg/ml)	3.22	55.6
Hypoxanthine–xanthine oxidase	4.75	0
+SOD (1.0 μg/ml)	1.90	60.1
+SOD (10.0 μg/ml)	0.85	82.1

The alloxan–GSH system consisted of 1.0 mM bathophenanthroline sulfonate, 0.3 mM GSH, 0.1 mM alloxan and 0.15 M NaCl in 3.0 ml of 10 mM phosphate buffer, pH 7.4. The hypoxanthine–xanthine oxidase system contained 0.1 mM hypoxanthine, 0.025 unit of xanthine oxidase, 1.0 mM bathophenanthroline sulfonate and 0.15 M NaCl in 3.0 ml of 10 mM phosphate buffer, pH 7.4. Ferritin (0.33 mg/ml) was incubated in both systems for 5 min at 37 °C. Various amounts of SOD were added to the reaction systems before initiation of the reaction. Reactions were initiated by the addition of alloxan or xanthine oxidase and continuously monitored.

TABLE III. Iron Release from Ferritin in the Alloxan–GSH System under Aerobic or Anaerobic Conditions

Conditions	Ferritin conc. (mg/ml)	nmol Fe^{2+}		Inhibition (%)
		−SOD	+SOD	
Aerobic	0.16	3.55	3.00	15.5
	0.33	6.95	4.90	29.5
	0.67	12.00	8.73	27.3
	1.00	17.73	12.82	27.7
Anaerobic	0.16	16.59	16.59	0
	0.33	22.91	22.91	0
	1.00	81.36	83.63	

Various concentrations of ferritin were incubated in the alloxan–GSH system for 5 min at 37 °C in air or N_2 gas. SOD (100 μg/ml) was added to the alloxan–GSH system before initiation of the reaction. Other conditions were the same as in Table II.

inhibited by addition of 10 μg/ml SOD (data not shown). Thus, the amount of SOD required for inhibition of iron release from ferritin in the alloxan–GSH system is much more than that in the hypoxanthine–xanthine oxidase system. It was demonstrated that O_2^- generated by xanthine–xanthine oxidase could directly reduce ferritin iron.[15] If iron release from ferritin were due to only O_2^-, the amount of SOD required for inhibition of the reaction would be independent of the source of O_2^-. SOD was almost active in the alloxan–GSH system as described above. Therefore, iron release from ferritin might be not due primarily to O_2^- in the alloxan–GSH system.

As shown in Table III, the rate of iron release from ferritin induced by alloxan–GSH was dependent on ferritin concentration, and SOD inhibited the reaction by about 30% under aerobic conditions. Under anaerobic conditions, iron was more rapidly released from ferritin in the alloxan–GSH system but SOD had no effect. These results suggests the indirect action of O_2^- on ferritin.

Langercrantz and Yhland[21] have demonstrated that alloxan undergoes one-electron reduction by GSH to form the alloxan radical. On the other hand, Winterbourn[22] indicated that a large amount of SOD was required for inhibition of cytochrome c reduction when a

small amount of alloxan (0.2—1.0 mM) was added to a xanthine–xanthine oxidase reaction system and she assumed that the alloxan radical would be produced by interaction of alloxan with O_2^-. If alloxan radical is produced in the alloxan–GSH system, it may directly release the ferritin iron. SOD may indirectly inhibit the iron release from ferritin. More studies are required to examine the involvement of O_2^- and alloxan radical in the reaction of iron release from ferritin.

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