Enzymatic Generation of Alloxan Radicals in Rat Liver Microsomes: Possible Participation of Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH)–Cytochrome P-450 Reductase

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Electron spin resonance studies showed that addition of rat liver microsomes to the reaction system of alloxan with reduced nicotinamide adenine dinucleotide phosphate (NADPH) resulted in a marked increase in the generation of alloxan radicals (AH·), whereas heat-denatured microsomes were without such effect. Oxidation of NADPH by alloxan was also stimulated by microsomes. The microsomes from rats treated with phenobarbital, an inducer of cytochrome P-450 reductase, greatly stimulated both the AH· generation and the NADPH oxidation. However, the microsomes from rats treated with 3-methylcholanthrene, an inducer of DT-diaphorase, did not have stimulative effect greater than the control microsomes. These results suggest the possibility that NADPH-linked AH· generations in microsomal membranes is catalyzed by NADPH–cytochrome P-450 reductase.

Keywords alloxan; NADPH; alloxan radical; NADPH–cytochrome P-450 reductase; DT-diaphorase; microsome; phenobarbital; ESR

Injection of alloxan to animals causes a selective cytotoxicity on pancreatic β-cells and insulin-dependent diabetes.[1-3] The toxic mechanism of alloxan is not fully understood, however, it has been proposed that alloxan gives rise to active oxygens such as hydrogen peroxide, superoxide and hydroxyl radical (OH·) during the reduction to dialuric acid and its reoxidation.[3-5] These cyclic reduction and autoxidation processes are thought to play a central role in the mechanism of alloxan toxicity.[4-6]

We previously demonstrated the generation of intermediate alloxan radicals (AH·) in the reaction of alloxan with glutathione, suggesting the possible participation of AH· in the generation of oxygen radicals.[5,6] The toxic actions of alloxan have also been shown to be caused by rapid oxidation of cellular reduced nicotinamide adenine dinucleotide phosphate (NADPH)[7] and generation of dialuric acid catalyzed by thioredoxin and NADPH–thioredoxin reductase.[8]

The present study using rat liver microsomes as a well-characterized model system revealed the possibility of the generation of AH· from alloxan by NADPH–cytochrome P-450 reductase (NADPH: ferricytochrome oxidoreductase, EC 1.6.2.4).

Experimental

Materials Alloxan and 2,6-dichlorophenolindophenol (DCPIP) were purchased from Wako Pure Chemical Industries, Ltd., Japan. NADPH was obtained from Oriental Yeast Co., Japan. Cytochrome c (type III) and 3-methylcholanthrene (MC) were from Sigma Co., St Louis, Mo. and phenobarbital (PB) was from Sankyo Ltd., Japan. Other chemicals used in this experiment were of analytical grade from commercial suppliers.

Preparation of Rat Liver Microsomes Male rats of the Wistar strain, weighing about 190 g, were starved overnight and then killed with a blow on the head. The liver was perfused, rapidly removed, finely minced and a homogenized with homogenizer (HG-30, Hitachi) in 5 vol. of 0.25 M sucrose. Hepatic microsomes were prepared by ultracentrifugation as described by Ernster *et al.*[9] and washed once with 0.15 M KCl. The washed pellet was suspended in 0.25 M sucrose and stored at −85 °C until used. Injections of PB and MC were made intraperitoneally each day for 4 d. The daily doses of PB and MC were 80 and 20 mg/kg body weight, respectively. Control rats were injected with a physiological saline. Protein was estimated according to the modified Lowry method of Markwell *et al.*[10] using bovine serum albumin as a standard.

Assay of Enzymes The activity of cytochrome P-450 reductase was measured at 37 °C by monitoring the rate of reduction of cytochrome c at 550 nm (using an extinction coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$). The assay mixture contained 10 mM phosphate buffer pH 7.4, 0.15 M NaCl, 20 μM cytochrome c, 0.1 mM potassium cyanide and 0.1 mM NADPH in a final volume of 3.0 ml. The reaction was started by the addition of microsomes and the enzyme activity was expressed as the rate of cytochrome c reduction (nmol·min^{-1}·mg^{-1} protein). The DT-diaphorase activity was measured at 37 °C by monitoring the rate of reduction of DCPIP at 600 nm (using an extinction coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$). The assay mixture contained 10 mM phosphate buffer pH 7.4, 0.2% Tween-20, 40 μM DCPIP and 0.2 mM NADPH in a final volume of 3.0 ml. The reaction was started by the addition of microsomes and the enzyme activity was expressed as the rate of DCPIP reduction (nmol·min^{-1}·mg^{-1} protein). The cytochrome b_5 reductase activity was measured at 37 °C by monitoring the rate of reduction of ferricyanate at 420 nm using an absorption coefficient of 1.02 $mM^{-1} \cdot cm^{-1}$. The assay mixture contained 10 mM phosphate buffer, pH 7.4, 0.22 mM potassium ferricyanide and 0.1 mM reduced nicotinamide adenine dinucleotide (NADH) in final volume of 3.0 ml. The reaction was started by addition of NADH and the enzyme activity was expressed as the rate of ferricyanide reduction (μmol·min^{-1}·mg^{-1} protein).

Detection of Alloxan Radicals Electron spin resonance (ESR) detection of the alloxan radical (AH·) was performed with a JEOL model JES-RE1X. Samples were incubated outside of the cavity for 1 min at 37 °C, then rapidly aspirated into the aqueous flat cell for ESR measurements. Spectrometer settings for AH· were the same as described previously[5,6] The signal intensity of AH· was evaluated by the peak height of the center hyperfine line of the ESR spectrum.

NADPH Oxidation The rate of NADPH oxidation was measured at 37 °C with a Shimadzu UV-3000 spectrophotometer equipped with a magnetic stirrer. The reaction mixture consisted of 0.3—1.0 mM alloxan, 0.4 mM NADPH and microsomes (0.5—1.0 mg protein/ml) in 3.0 ml of 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl. The reaction was initiated with the addition of alloxan and a decrease in absorbance was recorded at 340 nm. The concentrations of NADPH were determined at 340 nm using an extinction coefficient of 6.3 $mM^{-1} \cdot cm^{-1}$. The rate of reaction during the first 1 min was considered as the "initial velocity". Each value represented in the data was corrected by substracting the value of NADPH oxidation by alloxan and microsomes from the total value. The initial velocity of NADPH oxidation was plotted in a double-reciprocal form. The apparent K_m and V_{max} of the Michaelis–Menten equation were obtained by a method of least squares fit with the Taylor expansion.[11]

Statistical Analysis Data were expressed as a mean±S.E. and statistically analyzed by Student's t-test for paired data. $p<0.05$ was considered statistically significant.

Results

Alloxan Radical Generation AH· generation was observed by ESR spectroscopy. The spectra obtained here exhibited a g-value of 2.005 and a hyperfine structure consisting of seven equally spaced lines with a splitting field of about 0.45 G, and agreed with that of AH· determined previously.[5,6] As shown in Table I, a slight generation of AH· was observed in the reaction of alloxan with NADPH,

however, no AH· were detected in alloxan alone or in the reaction of alloxan with microsomes. Addition of microsomes to the reaction system of alloxan with NADPH resulted in a marked increase in the signal intensity of AH·, whereas the denatured microsomes heated for 5 min at 100 °C were without any effect.

Figure 1 shows the AH· generation in the reaction of various concentrations of alloxan with NADPH in the presence of microsomes. When alloxan at a concentration of from 0.2 to 2.0 mM was incubated with 1.0 mM NADPH in the presence of microsomes, the signal intensity of AH· increased with higher alloxan concentration, in accord with the microsome concentrations up to 3.0 mg protein/ml. These results suggest that the AH· generation in the reaction of alloxan with NADPH may be catalyzed by some enzyme in the microsomes.

TABLE I. Generation of Alloxan Radicals by the Reaction of Alloxan with NADPH in the Presence of Microsomes

Conditions	Alloxan radicals (mm)
Alloxan (1.0 mM)	ND
+microsomes (2.0 mg protein/ml)	ND
+NADPH (1.0 mM)	36.7±4.3 (9)
+NADPH (1.0 mM) and microsomes (2.0 mg protein/ml)	103.6±6.1 (9)
+NADPH (1.0 mM) and denatured microsomes (2.0 mg protein/ml)	40.2±1.8 (3)

The reaction was carried out for 1 min at 37 °C, then rapidly aspirated into an aqueous flat cell for ESR measurement. Denatured microsomes were prepared by heating for 5 min at 100 °C. Other conditions are described in Experimental. Each value represents the mean±S.E. of the number of experiments indicated in parenthesis. ND: not detectable.

NADPH Oxidation Figure 2 shows the NADPH oxidation by alloxan in the presence of microsomes. The addition of alloxan to the reaction system immediately resulted in NADPH oxidation, which continuously increased for at least 5 min. When either the alloxan or microsomes was omitted from the reaction mixture, only a slight oxidation of NADPH was observed. The increasing concentrations of alloxan up to 1.0 mM caused an increase in the rate of NADPH oxidation, depending on the concentration of microsomes. When heat-denatured microsomes were used, such activation was scarcely observed (data not shown). These results indicate that AH· are generated through the NADPH-linked reduction of alloxan catalyzed by microsomal enzymes.

NADPH-Linked Alloxan Radicals Generation by Liver Microsomes from Rats Treated with Phenobarbital or Methylcholanthrene Experiments using liver microsomes of PB- and MC-treated rats were carried out to evaluate the role of the microsomal enzymes in NADPH-linked AH·

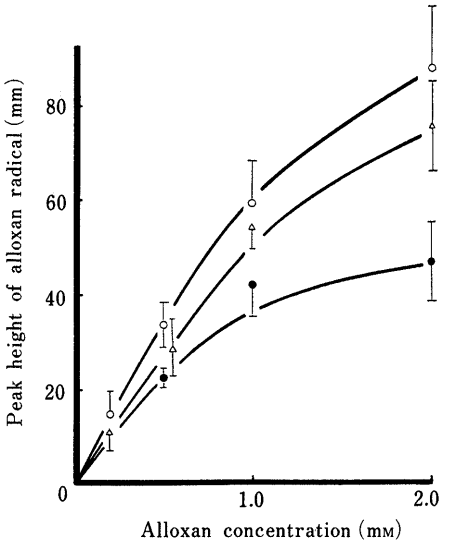

Fig. 1. Generation of Alloxan Radicals in the Presence of Various Concentrations of Microsomes

The reaction mixture contained 1.0 mM NADPH, alloxan at the concentrations indicated in the figure and microsomes in a final volume of 3.0 ml of 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl. Other conditions were as noted in Experimental. Microsome concentrations were —●—, 1.0; —△—, 2.0; —○—, 3.0 mg protein/ml. Peak heights of AH· were corrected by subtracting the values obtained by the reaction of alloxan with NADPH. Each point is the mean±S.E. of 3 to 7 experiments.

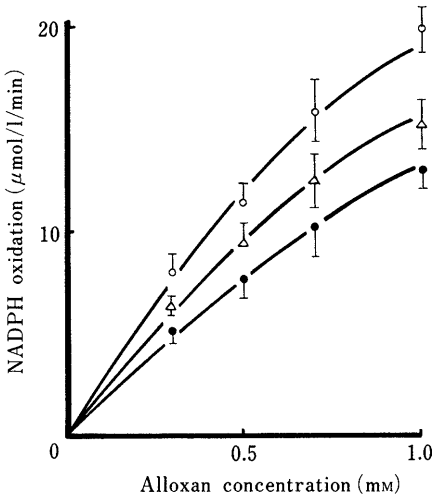

Fig. 2. Effect of Alloxan on NADPH Oxidation in the Presence of Microsomes

The reaction mixture contained 0.4 mM NADPH and alloxan at the concentrations indicated in the presence of microsomes in a final volume of 3.0 ml of 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl. Other conditions were as noted in Experimental. The microsome concentrations were —●—, 0.5; —△—, 0.7; —○—, 1.0 mg protein/ml. Each value was corrected by subtracting the values obtained by the reaction with alloxan or microsomes alone. Each point represents the mean±S.E. of 5 to 11 determinations.

TABLE II. Alloxan Radicals Generation and NADPH Oxidation in Liver Microsomes from Rats Treated with Phenobarbital or Methylcholantrene

Conditions	Alloxan radical (mm)	NADPH oxidation (μM/min/mg protein)	Cyt. P-450 reductase (nmol/min/mg protein)	DT-diaphorase (nmol/min/mg protein)	Cyt. b_5 reductase (μmol/min/mg protein)
Control	73.3±3.5 (7)	10.4±1.1 (11)	114.2±4.2 (8)	35.5±1.8 (5)	4.5±0.4 (3)
PB-treated	136.0±3.3 (5)[a]	14.8±0.2 (4)[a]	210.1±8.7 (3)[a]	46.1±1.5 (5)[b]	3.8±0.1 (3)
MC-treated	69.0±3.8 (3)	8.8±0.4 (3)	131.4±7.3 (4)	75.9±3.4 (5)[a]	4.5±0.0 (3)

The generation of alloxan radical and NADPH oxidation were determined under the same conditions as described in Table I and Fig. 2, respectively. The activities of cytochrome P-450 reductase and DT-diaphorase were determined by the methods described in Experimental. Each value was corrected by subtracting the blank and represents the mean±S.E. of the number of experiments indicated in parenthesis. Statistically significant compared to the respective control: *a*) $p<0.01$; *b*) $p<0.05$.

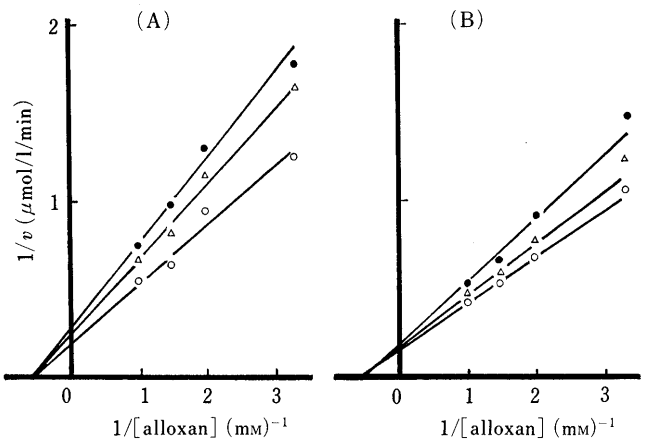

Fig. 3. Double-Reciprocal Plots of the Initial Velocity of NADPH Oxidation *versus* Varying Alloxan Concentrations

The reaction mixture consisted of 0.4 mM NADPH and the indicated concentration of alloxan in the presence of control (A) or PB-treated (B) microsomes. Microsome concentrations were —●—, 0.5; —△—, 0.7; —○—, 1.0 mg protein/ml. Each point is the mean of at least 5 experiments.

generation. As shown in Table II, cytochrome P-450 reductase and DT-diaphorase activity in PB-treated microsomes were higher by about 1.8- and 1.3-fold than those of untreated controls, respectively, accompanied by a significant increase in AH· generation and NADPH oxidation. Cytochrome b_5 reductase activity in PB-treated microsomes was a little lower than that of untreated controls.

DT-diaphorase activity in MC-treated microsomes, in contrast, was about 2.0-fold higher than that of controls, but neither significant changes in cytochrome P-450 reductase nor in cytochrome b_5 reductase activity were observed. Treatment with MC had no significant effect on the AH· generation and NADPH oxidation compared with controls. These results suggest that the NADPH-linked AH· generation may be catalyzed by cytochrome P-450 reductase in liver microsomes.

Determination of Apparent K_m Value We next attempted to determine an apparent K_m value for microsomal AH· generating enzyme at different concentrations of alloxan in control and PB-treated microsomes. Data points represented in Fig. 2 were subjected to a Lineweaver–Burk analysis and typical results are shown in Fig. 3A. In the control microsomes, the apparent K_m for alloxan in the range of 0.3—1.0 mM was 1.78 ± 0.35 mM and V_{max} was 53.2 ± 9.5 μM/min/mg protein. Figure 3B shows the reciprocal plots for PB-treated microsomes, and indicate that the K_m was 1.87 ± 0.28 mM and V_{max} was 69.7 ± 7.3 μM/min/mg protein. The two apparent K_m values for the two cases were similar within experimental error. These results suggest that the NADPH-linked AH· genration is catalyzed by cytochrome P-450 reductase induced in the liver microsomes by the administration of PB.

Discussion

Although the generation of AH· has been reported to be enhanced by incubation of alloxan with rat liver subcellular fractions and NADPH,[12] the exact mechanism of the metabolic activation of alloxan is still unclear. The study presented here demonstrated that the AH· generation in the reaction of alloxan with NADPH was catalyzed by an enzyme in rat liver microsomes (Table I). In addition, the NADPH-linked AH· generation was greatly enhanced by liver microsomes from PB-treated rats. Microsomes from MC-treated rats, however, had no significant effect on the AH· generation compared with that of controls (Table II). Other authors have reported that the treatment of rats with PB gives rise to a large increase in both cytochrome P-450 content and NADPH–cytochrome c (cytochrome P-450) reductase in liver microsomes accompanied by a slight increase in DT-diaphorase activity.[13,14] In contrast, treatment with MC has been reported to result in a marked increase in DT-diaphorase activity of microsomes, but a slight decrease in the NADPH–cytochrome c reductase activity.[14] These results are largely consistent with our data described above. Previous authors have reported that another quinone-reducing activity in microsomes may be attributed to cytochrome b_5 reductase,[15,16] however, this possibility for the generation of AH· is ruled out by results that cytochrome b_5 reductase activity in microsomes was little affected by treatment of rat with PB or MC (Table II). Neither the NADPH oxidation nor the AH· generation in microsomes from control and PB-treated rats was inhibited by CO (data not shown). These results suggest a possibility that NADPH-linked AH· generation in microsomal membranes is catalyzed by cytochrome P-450 reductase.

Bachur *et al.*[17] showed that purified cytochrome P-450 reductase catalyzes the single-electron reduction of several quinone antibiotics such as adriamycin and mitomycin C to semiquinone free radicals, which can transfer their single electron to molecular oxygen to form superoxide. Moreover, Powis and Appel[18] showed that the K_m range for quinone reduction by rat liver microsomes in the presence of NADPH is from 6.2 to 25.0 mM. The present studies found that the apparent K_m value for alloxan was about 1.9 mM (Fig. 3) and that somewhat lower than the magnitude reported for various quinones.[18]

Earlier reports demonstrated the generation of AH· during the incubation of alloxan with pancreatic β-cells.[19] The presence of NADPH–cytochrome P-450 reductase in β-cells is interesting in view of the fact that the toxic action of alloxan on these cells may be caused by rapid oxidation of cellular NADPH and generation of the site-specific free radical intermediate, AH·. The exact significance of microsomal NADPH–cytochrome P-450 reductase in alloxan toxicity requires further study.

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