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GENERATION OF ALLOXAN FREE RADICALS IN CHEMICAL AND BIOLOGICAL SYSTEMS: IMPLICATION IN THE DIABETOGENIC ACTION OF ALLOXAN

Mamoru Nukatsuka, Hiromu Sakurai and Jun Kawada

Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi 1, Tokushima 770, Japan

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SUMMARY: Electron spin resonance (ESR) studies showed that on reaction with NADPH, alloxan was reduced forming labile anion radicals giving a 7-line signal with g=2.005. These radicals were also produced on incubation of alloxan with rat liver subcellular fractions and their production was greatly enhanced by NADPH. Alloxan effectively scavenged superoxide anion generated by a xanthine-xanthine oxidase (XOD) system in association with its reduction to these anion radicals. These radicals were also formed during incubation of alloxan with rat pancreatic β-cells. These results suggest that the cytotoxicity of alloxan is related to the formation of alloxan anion radicals . © 1989 Academic Press, Inc.

Alloxan (2,4,5,6(1H,3H)-pyrimidinetetrone; Fig.1) destroys pancreatic β-cells selectively and causes insulin-dependent diabetes in animals [1]. The exact mechanism of induction of diabetes by alloxan is unknown, although various possible mechanisms have been proposed. One of these is that alloxan is reduced to dialuric acid (5-hydroxy barbituric acid; Fig.1) and then autooxidized rapidly by molecular oxygen, with formation of cytotoxic superoxide anion radicals (O_2^-) [2, 3]. It is based on the observation that pretreatment with radical scavengers such as superoxide dismutase (SOD), catalase [2, 3] or N,N-dimethyl urea [4] and ferrous ion chelator as diethylenetetraaminepenta acetic acid (DETAPAC) [5] prevent alloxan-induced diabetes [2, 3]. However, as far as we know, no free radicals formed from alloxan or molecular oxygen have yet been detected directly in biological systems. From results in chemical and biological systems, we here propose an alternative mechanism that the cytotoxicity of alloxan is due to alloxan anion radicals.

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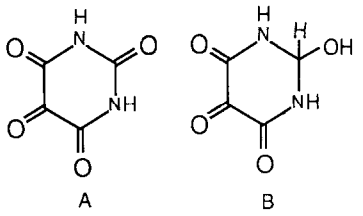


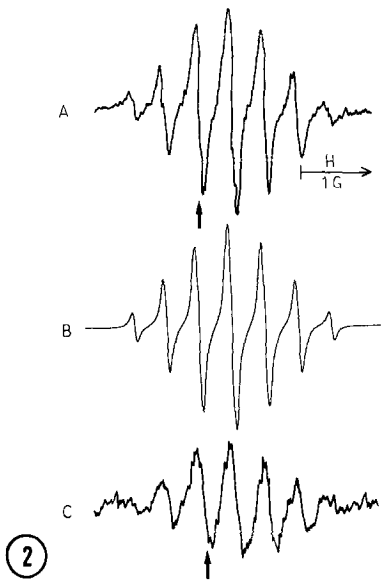
Figure 1.
Chemical structures of (A) alloxan and (B) dialuric acid.

MATERIALS AND METHODS

Alloxan and trypsin were purchased from Wako Pure Chemicals (Osaka). NADPH was from Oriental Yeast Co. (Tokyo). Xanthine oxidase (XOD), collagenase (Type I) and bovine serum albumin (BSA, Fraction V) were from Sigma Co. 5,5'-Dimethyl-1-pyrroline N-oxide (DMPO) was from Daiichi Chem. Co. (Tokyo). Fetal calf serum and medium-199 were obtained from Flow Lab. (Sydney) and Nissui (Tokyo), respectively. ESR spectra were measured at 22 °C in a JEOL-FE1XG ESR spectrometer. As standards, TCNQ-Li salt, Fremy's salt and Mn^{2+} doped in MgO were used. Five groups of experiments were performed:
(1) Alloxan (6.3 mM) in Krebs-Ringer phosphate buffer (KRPB, pH= 7.5) was mixed with NADPH (1-12 mM) and the ESR spectra were measured.
(2) Alloxan (6.3 mM) in 10 mM citrate buffer (pH=4-5.5) or in 10 mM phosphate buffer (pH=6-8.5) was mixed with NADPH (1 mM) and the ESR spectra were recorded.
(3) Rat liver was homogenized in 10 volumes of KRPB and subcellular fractions were obtained by differential centrifugation [6]. Then each rat subcellular fraction (8 mg protein/ml and 0.51 nmole cytochrome P-450/mg protein for microsomal fractions) was mixed with alloxan (6.3 mM) with or without NADPH (1 mM) and the ESR spectrum was measured. The protein concentration of each fraction was measured by the method of Lowry et al. [7] with BSA as a standard. Cytochrome P-450 in microsomal fractions was determined by the method of Omura and Sato [8].
(4) The reaction of alloxan with rat pancreatic B-cells (6 X 10^6 cells/sample), isolated from neonatal rats [9], was monitored by ESR spectra.
(5) Hypoxanthine in KRPB (2 mM, 100 μl), XOD (12 mU, 10 μl), various concentrations of alloxan (0-150 μM, 10 μl) and DMPO (10 μl) were mixed and the ESR spectra were recorded within 30 sec after mixing [10]. The activity of XOD was estimated by the method of Hashimoto [11].

RESULTS

A freshly prepared solution of alloxan in KRPB (pH 7.5), gave no ESR signal (data not shown), but when the solution of alloxan was mixed with NADPH or NADH, an intense ESR signal was seen, consisting of 7 symmetrical peaks with a relative intensity ratio of 1:3:5:7:5:3:1. The g-value was 2.005 and the coupling constant of adjacent peaks was 0.46 G (Fig. 2 A). The optimum conditions



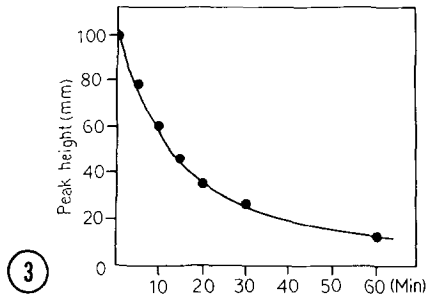


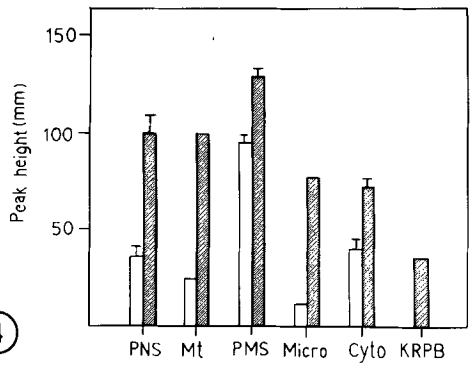
Figure 2.
ESR spectra generated in a mixture of alloxan (6.3 mM) and NADPH (1 mM) at pH 7.4 and 22 °C. (A) Observed spectrum. Magnetic field = 3371-3381 G, modulation amplitude = 0.1 G at field modulation frequency, 100 KHz; power, 8 mW; amplitude, 1600; response 0.1 sec at 22 °C. The arrow shows 3366 G in the magnetic field. (B) Computer-simulated spectrum. (C) ESR spectrum of alloxan anion radicals generated by alloxan (6.3 mM) and rat pancreatic β-cells (6 X 10^6 cells). The arrow shows 3366 G in the magnetic field.

Figure 3.
Time course of disappearance of alloxan anion radicals generated by alloxan (6.3 mM) and NADPH (7.6 mM) at pH 7.0.
The ordinate indicates the signal height in arbitrary units (mm) of the 4th peak from the lower magnetic field.

for the generation of the radicals were an approximate molar ratio of NADPH to alloxan of 1.2 at pH 7.0.

The signal of radicals appeared within 30 sec after mixing and disappeared time-dependently, with a half life of 13 min (Fig. 3).

When alloxan was added to subcellular fractions of rat liver, the same radicals were seen as in the chemical systems. The post-mitochondrial supernatant (PMS, 12000 X g supernatant) showed the strongest activity for formation of the radicals, followed by the post-nuclear supernatant (PNS, 700 X g supernatant) and cytosol (Cyto, 105000 X g supernatant) (Fig. 4). The mitochondrial and microsomal fractions exhibited weaker activities. Addition of NADPH to the fractions greatly enhanced generation of radicals, especially in the microsomal fractions (6.6-fold increase; Fig.4). In the microsomal systems, the half life of these radicals was estimated to be 39 min (data not shown).



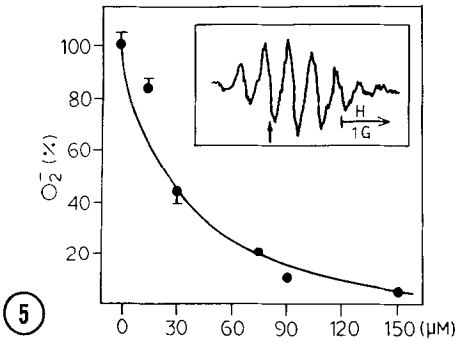


Figure 4.
Formations of alloxan anion radicals in reactions with subcellular fractions of rat liver. Open columns , reactions of alloxan and subcellular fractions containing 8 mg protein/ml. Closed columns, the same reactions in the presence of 1 mM NADPH. The ordinate indicates the signal height in arbitrary units (mm) of the 4th peak from the lower magnetic field of alloxan anion radicals. Values are means ± SEM (n = 4).

Figure 5.
Effect of alloxan in scavenging superoxide anion radicals ($O_2^{\cdot -}$) generated in the hypoxanthine-XOD system and ESR spectrum of alloxan anion radicals produced in the reactions of alloxan with $O_2^{\cdot -}$. The arrow shows 3366 G in the magnetic field (inset). The scavenging effect was monitored by ESR spin-trapping method with DMPO. The ordinate indicates the signal height at the lowest magnetic field of DMPO-OOH (%). Values are means ± SEM (n = 4).

Addition of alloxan to the hypoxanthine-XOD system decreased the signal height due to the DMPO-OOH adduct dose-dependently, but during the reactions, no loss of specific activity of XOD was observed. The results showed that alloxan has strong superoxide-scavenging activity, the ED_{50} value being 0.03 mM (Fig. 5). In these experiments, the ESR signal due to the alloxan radical was detected, in the presence of 6.3 mM alloxan (Fig.5, inset), but not in the presence of less than 1 mM alloxan (data not shown). To elucidate the mechanism of alloxan-induced diabetes, we examined the reaction of alloxan with intact rat pancreatic β-cells . After addition of alloxan (6.3 mM) to β-cells (6 X 10^6 cells/sample), a weak signal of exactly the same alloxan anion radicals were detected (Fig. 2 C).

DISCUSSION

A previous study by ESR spectrometry showed that alloxan is reduced to alloxan anion radicals during reaction with reducing reagents such as glutathione or ascorbic acid [12]. In this work, we examined the reactions of alloxan with NADPH and rat liver subcellular fractions. Both NADPH and rat liver subcellular

fractions showed strong activities to induce alloxan-derived free radicals (Fig. 2 A). These radicals were identified as alloxan anion radicals, on the basis of both ESR parameters ($g = 2.005$ and $\Delta H = 0.46G$) as reported previously [12] and computer-simulation with coupling constants of $a^N = 0.425G$ and $a^H = 0.415G$ (Fig. 2 B). The cytotoxic mechanism of alloxan has been explained by supposing that alloxan is reduced to dialuric acid by two-electron reduction and autooxidized in the presence of O_2 to regenerate alloxan and form O_2^- [2, 3], which is then converted to cytotoxic ·OH by a Haber-Weiss like reaction ($3O_2^- + 2H^+ \rightarrow$ ·OH + OH^- + $2O_2$) [13]. However, we found that during the reaction of alloxan with O_2^-, O_2^- was scavenged effectively by alloxan with reduction of the latter to anion radicals (Fig. 5, inset).

From these results we propose an alternative mechanism for the cytotoxic activity of alloxan; namely that it depends on alloxan anion radicals produced by one-electron reduction of alloxan in chemical and biological systems. There seem to be four pathways for producing alloxan anion radicals: a direct reaction with NADPH (Fig. 2 A), reaction with NADPH and cytochrome P-450 systems in microsomes (Fig. 4), reaction with intact pancreatic β-cells (Fig. 2 C) and reaction with O_2^- (Fig. 5, inset).

Pancreatic β-cells contain a high level of XOD, which is a typical source of O_2^-, but a low level of SOD [14], so alloxan may be reduced to alloxan anion radicals in β-cells.

In summary, we found that the diabetogenic reagent alloxan was reduced to anion radicals on reaction with NADPH, liver sub-cellular fractions, intact β-cells or superoxide anion. These results suggest that alloxan anion radicals are involved in the cytotoxicity of alloxan in β-cells.

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