A Role of Iron in DNA Strand Breaks in the Reaction System of Alloxan with Reduced Glutathione: Iron(III) Binding to the DNA

Koichi Sakurai, * Kaori Haga, and Taketo Ogiso

Hokkaido Institute of Pharmaceutical Sciences, 7-1, Katsurakusa-cho, Otaru 047-02, Japan.
Received July 8, 1993; accepted August 31, 1993

DNA strand breaks were easily induced in a reaction system involving alloxan with reduced glutathione (GSH) in the presence of FeCl₃ in a HEPES-NaOH buffer, pH 7.4. Increasing concentrations of FeCl₃ in the reaction system caused DNA strand breaks in a concentration-dependent fashion, suggesting that iron is required to induce the DNA strand breaks. Catalase, scavengers of hydroxyl radicals (HO·) and iron-chelators almost completely inhibited the DNA strand breaks, but superoxide dismutase (SOD) did not do so, suggesting that the HO·, formed by a Fenton-type reaction, was the species responsible for the DNA strand breaks. The addition of FeCl₃ to the solution containing DNA caused the formation of a DNA–Fe(III) complex, in which Fe(III) was reduced by an alloxan radical (HA·) but not by a superoxide radical. Only when apotransferrin was added to the reaction mixtures before the addition of FeCl₃ were both the DNA strand breaks and the reduction of Fe(III) strongly inhibited. These results suggest that the Fe(III) bound to DNA catalyzes the DNA strand breaks which may be caused by the generation of site-specific HO· via an HA·-dependent Fenton-type reaction.

Keywords alloxan; reduced glutathione (GSH); DNA strand break; hydroxyl radical; DNA–iron(III) complex; alloxan radical

Alloxan is widely used in studies of experimental diabetes because this agent destroys pancreatic β-cells with a specific selectivity. Since this cytotoxic action is inhibited by superoxide dismutase (SOD), catalase and several scavengers of hydroxyl radicals (HO·), it has been proposed that the cytotoxicity of alloxan is mediated by some active oxygen. Several workers have shown, with in vitro experimental systems using isolated pancreatic islets of rats, that the primary step of alloxan toxicity is the generation of HO· by which the DNA of pancreatic islets is attacked to produce strand breaks. More recently, we showed that DNA strand breaks were easily induced by HO· generated via an alloxan radical (HA·) through a Fenton-type reaction in the reaction system of DNA with reduced glutathione (GSH) in the presence of Fe³⁺–EDTA.

The involvement of iron in the alloxan-induced cytotoxicity has been identified by several lines of studies, in vivo and in vitro. However, under physiological conditions, the concentration of free iron capable of inducing these toxicity is probably low, because iron is tightly controlled and regulated. On the other hand, HO· can attack and destroy biomolecules such as DNA at diffusion-control rates. These facts suggest that iron bound to the biomolecules will lead to the site-specific damage of the biomolecules. The present study shows that iron bound to DNA catalyzes the radical reaction that leads to DNA strand breaks in a reaction system of alloxan with GSH.

MATERIALS AND METHODS

Materials Alloxan GSH, α,α′-dipyridyl, thiourea, FeCl₃, sodium benzoate and disodium-1,2-dihydroxy-benzene-3,5-disulfonate (Tiron) were purchased from Wako Pure Chemical Industries, Ltd., Japan. A solution of FeCl₃ was made up freshly just before use. Apotransferrin, transferrin (the iron saturation was approximately 98%), SOD (from bovine erythrocytes) and catalase (from bovine liver, thymol-free), were obtained from Sigma Co., St. Louis, MO, U.S.A. Apotransferrin was dissolved in 10 mM HEPES-NaOH buffer, pH 7.4, containing 5 mM NaHCO₃. SOD and catalase were used without further purification. DNA (M.W. 31.5 × 10⁶ Da; 48502 base pairs) was from Nippon Gene, Ltd., Japan; bathophenanthroline disulfonate from Dojindo Laboratories Co., Ltd.; dimethylthiourea (DMTU) from Aldrich Chemical Co., Inc.: diethylthraminepentaacetic acid (DETPAC) from Kanto Chemical Co., Inc.; Sephadex G-50 DNA grade column from Pharmacia Co., and desferrioxamine from CIBA Laboratories. Other reagents used in the experiments were of the highest grade.

Detection of DNA Strand Breaks To remove the Tris–HCl buffer and EDTA, the commercial DNA solution was dialyzed against sterilized 10 mM HEPES–NaOH buffer, pH 7.4, containing 0.15 M NaCl at 4 °C for 2 d with three changes of the same buffer. The reaction mixtures consisted of 1 mM alloxan, 2 mM GSH, 20 μg/ml DNA and 10–100 μM FeCl₃ (nucleotide:iron = approximate 6:1–10) in 10 mM HEPES–NaOH buffer, pH 7.4, containing 0.15 M NaCl. The reactions were initiated by the addition of alloxan and then incubated for 10 min at 37 °C. The DNA strand breaks were observed as described previously by using the electrophoresis method, and were photographed in ultraviolet (UV) light.

Binding of Iron to DNA The mixture (100 μl) containing 20 μg/ml DNA and 40 μM FeCl₃ in 10 mM HEPES–NaOH buffer, pH 7.4, was applied to a Sephadex G-50 DNA grade column equilibrated with Tris–HCl buffer, pH 7.4. The column was eluted with Tris–HCl buffer, pH 7.4, and then eluted with the same buffer containing 1 mM Tiron. Each fraction of 400 μl was collected, and the concentrations of iron were measured with an atomic absorption flameless spectrophotometer (Hitachi, 180-80). The concentrations of DNA in the eluted fractions were determined by UV spectrophotometry using...
an $A_{260}$ ($E_{1\%}^{1\text{cm}} = 200$).

Assay of Iron Reduction The reduction of Fe(III) bound to DNA was measured as described previously.\textsuperscript{14}

Detection of Alloxan Radical The generation of HA\textsuperscript{-} was observed as described previously by using an ESR (JEOL model JES-RE1X) at room temperature.\textsuperscript{14} The relative intensity of HA\textsuperscript{-} was determined by measuring the peak height at the center hyperfine line of the spectrum.

RESULTS

Effect of Iron on DNA Strand Breaks As shown in Fig. 1, under aerobic conditions, when DNA was incubated in the reaction system of alloxan with GSH in the presence of various concentrations of FeCl\textsubscript{3}, DNA strand breaks were caused in an iron concentration-dependent fashion. However, the DNA incubated with iron alone or without iron did not show any strand breaks. When alkaline sucrose density gradient centrifugation was used to observe the DNA strand breaks in the reaction system of alloxan with GSH in the presence of 40 $\mu$m of FeCl\textsubscript{3}, DNA strand breaks were observed as shown in the previous results.\textsuperscript{8} These results indicate that the DNA strand breaks are easily induced in the reaction system in the presence of FeCl\textsubscript{3}.

Effect of SOD, Catalase, HO\textsuperscript{-} Scavengers and Iron-Chelators To clarify the participation of oxygen radicals in DNA strand breaks, we examined the effect of SOD, catalase, several HO\textsuperscript{-} scavengers and iron-chelators. As shown in Fig. 2, catalase and HO\textsuperscript{-} scavengers (thiourea, DMTU and benzoate) almost completely inhibited the DNA strand breaks, but SOD had little effect, suggesting the participation of H\textsubscript{2}O\textsubscript{2} and HO\textsuperscript{-} in DNA strand breaks.

On the other hand, desferrioxamine and DETAPAC as a ferric iron-chelator, and bathophenanthroline and $\alpha,\alpha'$-dipyridyl as a ferrous iron-chelator, strikingly inhibited the DNA strand breaks (tracks 8—11). These results suggest a possibility that both ferrous and ferric iron are involved in DNA strand breaks under the experimental conditions. The addition of a Fe(III)-binding protein, apotransferrin (4 mg/ml), which has 100 $\mu$m Fe binding capacity, to the reaction system did not inhibit the DNA strand breaks (track 7).

Iron Binding to DNA In order to confirm the interaction of Fe(III) with $\lambda$DNA, the sample containing Fe(III) and $\lambda$DNA was applied to a Sephadex G-50 DNA grade column. Studies of Fe(III) binding to calf thymus DNA by Sephadex chromatography have been reported previously.\textsuperscript{15} As shown in Fig. 3, Fe(III) was eluted together with $\lambda$DNA at the void volume of the column. On the other hand, in the sample containing only Fe(III), Fe(III) was detected as a broad peak at around fraction number 9 only when the column was eluted with Tris--HCl buffer containing 1 mm Tiron. More than 99% of iron and $\lambda$DNA applied to the column were recovered in each fraction. These results indicate a strong interaction between Fe(III) and $\lambda$DNA to form a DNA--Fe(III) complex at pH 7.4. The insert of Fig. 3 shows that the added iron caused a linear increase in the formation of DNA--Fe(III) complex in a concentration-dependent fashion up to about 150 $\mu$m.

Reduction of Fe(III) Bound to DNA Figure 4 shows the time course of the reduction of Fe(III) bound to $\lambda$DNA in the reaction system of alloxan with GSH. Fe(III) was rapidly reduced and the amount of ferrous iron increased with the time of incubation. Neither alloxan nor GSH alone reduced Fe(III) under the experimental conditions employed. The addition of SOD (50 $\mu$g/ml) to the reaction system had no significant effect, while catalase (50 $\mu$g/ml) brought about a slight increase in the reduction of Fe(III).
Fig. 3. Binding of Iron to DNA
After FeCl₃ (40 µM) was mixed with (○) or without (●) DNA (20 µg/ml) in 10 mM HEPES-NaOH buffer, pH 7.4, each mixture was applied to a Sephadex G-50 DNA grade column. Each point represents the mean of triplicate experiments. The inset shows the binding of Fe(III) to DNA in the reaction of 20 µg/ml of DNA with various concentrations of FeCl₃.

Fig. 4. Reduction of Fe(III) in the Reaction System of Alloxan with GSH in the Presence of DNA
(A) The reaction mixture consisted of 1 mM alloxan, 2 mM GSH, 40 µM FeCl₃, and 1 mM bathophenanthroline sulfonate in 10 mM HEPES-NaOH buffer, pH 7.4, in the presence of 20 µg/ml DNA. ○, control; △, + 50 µg/ml SOD; ●, + 50 µg/ml catalase.

These results suggest that O₂⁻ and H₂O₂ generated in the reaction system are not directly involved in the reduction of Fe(III) bound to DNA but H₂O₂ could reoxidize Fe(II) to Fe(III).

Generation of the Alloxan Radical As previously reported, the alloxan radical (HA⁻) generated in the reaction system of alloxan with GSH could reductively release iron from ferritin. This finding suggests the possible participation of HA⁻ in the reduction of Fe(III) bound to DNA. As shown in Fig. 5a, the ESR spectrum of HA⁻ obtained in the reaction system of alloxan with GSH in the presence of DNA (aₜ = aₜH = 0.45G, g = 2.005) was similar to that reported in the previous paper. When GSH was omitted from the reaction mixtures, no generation of HA⁻ was observed (data not shown). The addition of the DNA-Fe(III) complex to the reaction system resulted in a marked decrease in the signal intensity of HA⁻ depending on the concentrations of Fe(III). These results indicate that HA⁻ directly reacts with Fe(III) bound to DNA, which is easily reduced to Fe(II).

Effect of Apotransferrin The addition of apotransferrin to the reaction mixtures was unable to inhibit the DNA strand breaks (Fig. 2, track 7). This result was unexpected; therefore, further studies were performed on the effect of apotransferrin on the DNA strand breaks and the reduction of Fe(III). As shown in Fig. 6, when apotransferrin (4 mg/ml) was premixed with DNA immediately before the addition of FeCl₃ to the reaction mixtures, the DNA strand breaks were almost completely inhibited at FeCl₃ concentrations up to 60 µM (A). In contrast, when apotransferrin was added after the addition of FeCl₃ to the reaction mixtures, DNA strand breaks were scarcely observed at any concentration of FeCl₃ up to 120 µM (B).

As shown in Fig. 7, the initial rates of Fe(III)-reduction
Fig. 6. Effect of Apotransferrin on DNA Strand Breaks

Apotransferrin (4 mg/ml) was added to the reaction mixture before (A) or after (B) the addition of FeCl₃ at various concentrations. Other conditions were the same as described in Fig. 1. FeCl₃ concentrations were as follows: track 1, 0 µM; 2, 40 µM; 3, 60 µM; 4, 100 µM; 5, 120 µM.

Fig. 7. Effect of Apotransferrin on the Reduction of Fe(III) Bound to DNA

The experimental conditions were the same as given in Fig. 4, except for varying the concentration of FeCl₃ (A). Apotransferrin (4 mg/ml) was added to the reaction mixture before (□) or after (○) the addition of FeCl₃.

in the reaction system increased with increasing concentrations of Fe(III) up to 120 µM. When apotransferrin (4 mg/ml) was premixed with DNA before the addition of Fe(III), the rates of Fe(III) reduction were markedly inhibited at all concentrations of FeCl₃. But when apotransferrin was added after the addition of FeCl₃, there was no effect. Although data were not shown, the reduction of Fe(III) bound to the transferrin (the iron-saturation was approximately 98%) was scarcely observed in the reaction system without DNA. These results suggest a possible requirement that Fe(III) be previously bound to DNA to produce DNA strand breaks, and that Fe(III) bound to apotransferrin, having a high affinity for Fe(III), is inactive in promoting the DNA strand breaks.

DISCUSSION

It is generally believed that a highly reactive species such as HO is involved in cytotoxicity, and that a transition metal such as iron is strictly required in the generation of the active oxygens. We reported previously that HO and oxidizing species of iron generated in the GSH–alloxan system in the presence of Fe³⁺-EDTA attacks DNA to produce strand breaks. However, under the experimental conditions used here, various HO scavengers almost completely inhibited the DNA strand breaks (Fig. 2), suggesting that the strand breaks of λDNA were induced by HO generated in the reaction system of alloxan with GSH in the presence of FeCl₃. In our previous works with the reaction system of alloxan with GSH in the presence of Fe³⁺-EDTA, we suggested that HO was generated by HA through a Fenton-type reaction as follows:

\[
\text{alloxan} + \text{GSH} \rightarrow \text{HA} + \frac{1}{2}\text{GSSG}
\]
\[
\text{HA} + \text{O}_2 \rightarrow \text{alloxan} + \text{O}_2^{-}
\]
\[
\text{O}_2^{-} + \text{O}_2^{-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]
\[
\text{HA} + \text{Fe³⁺-EDTA} \rightarrow \text{alloxan} + \text{Fe²⁺-EDTA}
\]
\[
\text{H}_2\text{O}_2 + \text{Fe²⁺-EDTA} \rightarrow \text{HO}^{-} + \text{HO}^{-} + \text{Fe³⁺-EDTA}
\]

Several studies have been carried out to determine what forms of iron are able to generate HO under physiological conditions, demonstrating that iron ions bound to adenine nucleotide, citrate, adriamycin, bleomycin or deoxyribose could participate in Fenton-type reactions. Iron bound to DNA has also been reported to be attributed to DNA damage mediated by the Fenton reaction. In the present studies, the interaction between λDNA and FeCl₃ to form a DNA–Fe(III) complex at pH 7.4 was demonstrated by column chromatography (Fig. 3), suggesting that the iron bound to DNA could play a role in DNA strand breaks in the reaction system of alloxan with GSH. Since the DNA strand breaks were inhibited by either ferric or ferrous iron-chelators (Fig. 2), the redox-cycling of iron bound to DNA appears to occur.
during the generation of HO·.

The reduction of Fe(III) bound to DNA could not be inhibited by the addition of SOD or catalase (Fig. 4), indicating that O₂- or H₂O₂ are not involved in the reduction of Fe(III) bound to DNA. Moreover, the addition of a DNA–Fe(III) complex to the reaction system resulted in a decrease in the signal intensity of HA· depending on concentrations of Fe(III) bound to DNA (Figs. 4 and 5). In our previous works, HA· generated in the reaction system of alloxan with GSH directly reduces Fe³⁺-EDTA to Fe²⁺-EDTA 23 and reductively releases iron from ferritin. 22 These results suggest that HA· directly reduces Fe(III) to Fe(II). We have observed, although data are not shown, that the addition of ferrous iron (FeSO₄) to the solution of 5DNA in the presence of H₂O₂ causes the DNA strand breaks. Floyd has also shown that DNA binds to ferrous iron in a manner such that HO· formation from H₂O₂ is enhanced, as compared to the amount formed in the absence of DNA. 23 Thus, the DNA strand breaks induced by alloxan may depend on the formation of a DNA–Fe(III) complex that catalyzes the site-specific production of OH· through the Fenton reaction; the target site to which Fe(III) are bound will tend to be preferentially attacked by HO·.

The Fe(III)-binding proteins, apotransferrin or apolactoferrin, have two separate binding sites to which Fe(III) attaches tightly at physiological pH, preventing the reaction of iron-dependent HO· generation. 24 The present study, however, demonstrated that apotransferrin did not inhibit DNA strand breaks when apotransferrin was added after the addition of FeCl₃ to the reaction system (Fig. 6). These results suggest that the apotransferrin was unable to remove iron from the DNA–Fe(III) complex previously formed under the experimental conditions, probably because apotransferrin (pI=5.6) negatively charged at physiological pH 25 made it difficult to attain close proximity to the Fe(III) binding site of the DNA molecule. On the other hand, apotransferrin pre-mixed with DNA before the addition of FeCl₃ inhibited the DNA strand breaks (Fig. 6). The binding constant of an iron–DNA complex and transferrin is reported to be 2.1×10¹⁴ 15 and 4.7×10²⁵ 2⁵ at pH 7.4, respectively. Therefore, most Fe(III) probably forms a complex with apotransferrin and not DNA. The results presented here suggest that Fe(III) bound to DNA could participate in the generation of HO· and lead to DNA strand breaks in the reaction system of alloxan with GSH.

Samuni et al. proposed a “site-specific” Fenton-type reaction in which the binding of a transition metal such as iron or copper to the target is a prerequisite for the production of HO·. 26 On the other hand, alloxan has been known to induce DNA strand breaks to cause experimental diabetes mellitus 27 which is protected by the administration of several iron-chelators. 9,10 These findings suggest that the diabetogenic action of alloxan may be initiate through the involvement of Fe(III) bound to DNA. The source of the iron is unknown; however, the rat pancreas has been reported to contain a significant amount of ferritin. 28 We previously reported that the reactive iron was reductively released from ferritin by alloxan. 22 Further studies are required to elucidate the exact mechanism of the diabetogenic action of alloxan.

REFERENCES