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Induction of 8,5′-Cyclo-2′-deoxyadenosine and 8,5′-Cyclo-2′-deoxyguanosine in Isolated DNA by Fenton-type Reagents

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Abstract

Exposure of aqueous solutions of DNA to X- or γ-rays, which induces hydroxyl radical as one of the major reactive oxygen species (ROS), can result in the generation of a battery of single-nucleobase and bulky DNA lesions. These include the (5′*R*) and (5′*S*) diastereomers of 8,5′-cyclo-2′-deoxyadenosine (cdA) and 8,5′-cyclo-2′-deoxyguanosine (cdG), which were also found to be present at appreciable levels in DNA isolated from mammalian cells and tissues. However, it remains unexplored how efficiently the cdA and cdG can be induced by Fenton-type reagents. By employing HPLC coupled with tandem mass spectrometry (LC-MS/MS/MS) with the use of the isotope-dilution technique, here we demonstrated that treatment of calf thymus DNA with Cu(II) or Fe(II), together with H_2O_2 and ascorbate, could lead to dose-responsive formation of both the (5′*R*) and (5′*S*) diastereomers of cdA and cdG, though the yields of cdG were 2–4 orders of magnitude lower than that of 8-oxo-7,8-dihydro-2′-deoxyguanosine. This result suggests that Fenton reaction may constitute an important endogenous source for the formation of the cdA and cdG. Additionally, the (5′*R*) diastereomers of cdA and cdG were induced at markedly higher levels than the (5′*S*) counterparts. This latter finding, in conjunction with the previous observations of similar or greater levels of the (5′*S*) than (5′*R*) diastereomers of the two lesions in mammalian tissues, furnishes an additional line of evidence to support the more efficient repair of the (5′*R*) diastereomers of the purine cyclonucleosides in mammalian cells.

Keywords

8, 5′-cyclo-2′-deoxyguanosine; 8,5′-cyclo-2′-deoxyadenosine; Fenton reagent; DNA damage; reactive oxygen species; mass spectrometry

Introduction

Reactive oxygen species (ROS) are constantly induced by endogenous and exogenous sources and they can result in damage to DNA.[1, 2] For instance, exposure of DNA to γ- or X-rays can lead to the formation of a variety of single-nucleobase and bulky DNA lesions.[3–11] During normal aerobic metabolism, electrons leaking from the electron transport chain in mitochondria may couple with molecular O_2 to yield superoxide anion radical ($O_2^{-\bullet}$), which can be subsequently converted to H_2O_2 by superoxide dismutase. Being freely diffusible in the cellular environment, H_2O_2 may reach the nucleus and react with DNA-bound transition metal ions [e.g. Fe(II) or Cu(II)] to yield the highly reactive hydroxyl radical ($^{\bullet}OH$) via the Fenton-type reaction.[12] Along this line, earlier studies by Linn and

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Supporting Information Available: HPLC enrichment trace, LC-MS/MS/MS data, calibration curves, and the numerical values about the levels of DNA lesions. This material is available free of charge via Internet at http://pubs.acs.org.

coworkers [13–15] demonstrated the capability of Fenton-type reagents in inducing oxidatively generated lesions of DNA.

The importance of Fenton-type reaction in human diseases is manifested by genetic disorders associated with defects in handling transition metal ions, including Wilson's disease [16] and iron overload disease.[17] In this vein, we found previously that deficiency in the ortholog of human Wilson's disease gene (i.e., *Atp7b*) and the ensuing aberrant accumulation of copper ions in hepatocytes led to elevated levels of oxidatively induced 8,5′-cyclo-2′-deoxyadenosine (cdA) and 8,5′-cyclo-2′-deoxyguanosine (cdG) lesions in liver tissues of Long-Evans Cinnamon rats.[18]

The cdA and cdG are unique oxidatively induced DNA lesions owing to the presence of an additional C–C bond between the C8 of the purine base and the C5′ of the 2-deoxyribose in the same nucleoside (Scheme 1). This additional covalent linkage introduces helical distortion to DNA and renders the *N*-glycosidic bond resistant toward acid-induced hydrolysis.[19–22] Thus, cdA and cdG are attractive substrates for nucleotide excision repair (NER), but they are poor substrates for DNA glycosylase-mediated base excision repair (BER).[23–25] Both the (5′*R*) and (5′*S*) diastereomers of cdA inhibit primer extension by T7 DNA polymerase as well as human DNA polymerase δ.[23, 26, 27] Nevertheless, results from steady-state kinetic measurements showed that yeast and human polymerase η-mediated nucleotide incorporation opposite (5′*S*)-cdA and (5′*S*)-cdG was largely efficient and accurate.[28] In addition, cdA and cdG strongly impede DNA transcription in mammalian cells and induce transcription mutagenesis.[23, 24, 26]

Recent studies have shown that both the (5′*R*) and (5′*S*) diastereomers of cdA and cdG have been detected *in vitro* and *in vivo,*[18, 29–37] Nevertheless, it remains to be established how efficiently these lesions can be induced in DNA by Fenton-type reagents. Here we treated calf thymus DNA with Cu(II)/H_2O_2/ascorbate or Fe(II)/H_2O_2/ascorbate and analyzed the enzymatic digestion products of DNA by LC-MS/MS or LC-MS/MS/MS. We found that Fenton-type reagents could induce dose-dependent formation of cdA and cdG, with the (5′*R*) diastereomer being produced much more efficiently than its (5′*S*) counterpart.

Experimental Procedures

Materials

$CuCl_2$, $(NH_4)_2Fe(SO_4)_2$•$6H_2O$, L-methionine, L-ascorbic acid, calf thymus DNA, nuclease P1, alkaline phosphatase, and phosphodiesterases 1 and 2 were purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide (30%) and *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) hydrochloride were obtained from Fisher Scientific (Fair Lawn, NJ) and Tocris Bioscience (Ellisville, MO), respectively. The uniformly [^{15}N]-labeled 8-oxodG, cdA and cdG were synthesized previously (Structures shown in Scheme 1).[18, 38]

Treatment of Calf Thymus DNA

Commercially available calf thymus DNA was desalted by ethanol precipitation. The DNA pellet was redissolved in a solution containing 25 mM NaCl and 50 mM phosphate (pH 7.0), and the DNA was annealed by heating the solution to 90°C for 5 min followed by cooling slowly to room temperature.

Aliquots of DNA (75 μg) were incubated with $CuCl_2$ or $(NH_4)_2Fe(SO_4)_2$ (12.5–200 μM), H_2O_2 (0.1–1.6 mM), and ascorbate (1–16 mM) in a 250-μL solution containing 25 mM NaCl and 50 mM phosphate (pH 7.0) at room temperature under aerobic conditions for 60 min. In this vein, ascorbate was added to maintain copper and iron in the reduced state (i.e., Cu^+ and Fe^{2+}) so that they could participate in the Fenton reaction. Chemicals used in the

Fenton-type reagent treatment of DNA were freshly prepared in doubly distilled water. Detailed concentrations of individual Fenton reagents used for the reactions are shown in Table 1. After 60 min, the reactions were terminated by adding excess L-methionine, and the DNA samples were again desalted by ethanol precipitation.

Enzymatic Digestion of DNA

To the above desalted DNA samples were added 8 units of nuclease P1, 0.01 unit of phosphodiesterase 2, 20 nmol of EHNA and a 20-μL solution containing 300 mM sodium acetate (pH 5.6) and 10 mM zinc chloride. EHNA was added to the enzymatic digestion mixture to prevent the possible deamination induced by residual contamination of adenine deaminase present in some commercial preparations of enzymes used in DNA digestion.[39] After a 48-hr incubation at 37°C, 8 units of alkaline phosphatase, 0.02 unit of phosphodiesterase 1, and 40 μL of 0.5 M Tris-HCl (pH 8.9) were added to the digestion mixture. The solution was further incubated at 37°C for 2 h, after which the enzymes were removed by chloroform extraction and the solution dried by Speed-vac. DNA samples were then reconstituted in doubly distilled water and their concentration measured using UV-absorption spectrophotometry. To the mixture were then added uniformly [^{15}N]-labeled 8-oxodG (500 fmol), *R*-cdG (200 fmol), *S*-cdG (100 fmol), *R*-cdA (100 fmol), and *S*-cdA (40 fmol, Scheme 1). The resulting aliquots were subjected directly to LC-MS/MS analysis (for 8-oxodG), or HPLC enrichment prior to LC-MS/MS/MS analysis (for cdA and cdG).

HPLC Enrichment

A 4.6×250 mm Alltima HP C18 column (5 μm in particle size, Grace Davison, Deerfield, IL) was used for the enrichment of the oxidatively induced cdA and cdG lesions from the enzymatic digestion products of DNA. The flow rate was 1 mL/min, and the mobile phases were 10 mM ammonium formate (solution A) and methanol (solution B). A gradient of 25 min 0–2% B, 1 min at 2% B, 14 min 2–5% B, 1 min 5–15% B, and 20 min at 15% B was employed. The HPLC fractions eluting at 12–18, 31.5–41.5, 42.5–50.5, 63–71.5 min were pooled for *R*-cdG, *R*-cdA, *S*-cdG, and *S*-cdA, respectively (Figure S1). The collected fractions were dried in the Speed-vac, redissolved in H_2O, and subjected to LC-MS/MS/MS analysis.

LC-MS/MS Analysis of 8-oxodG

A 3×100 mm Hypersil Gold column (5 μm in particle size, Thermo, San Jose, CA) and an Accela 600 HPLC pump (Thermo) were used, and the flow rate was 50 μL/min. A solution of 0.1% (v/v) formic acid in doubly distilled water (solution A) and a solution of 0.1% (v/v) formic acid in methanol (solution B) were employed as mobile phases. The gradient included 0–90% B in 30 min and 90% B in 5 min. The effluent from the LC column was directed to a TSQ Vantage triple quadrupole mass spectrometer (Thermo). The instrument was operated in multiple-reaction monitoring (MRM) mode, and the MRM transitions for 8-oxodG and its uniformly ^{15}N-labeled standard were *m/z* 284→168 and *m/z* 289→173, respectively.

LC-MS/MS/MS Analysis of cdA and cdG

An Agilent 1100 capillary HPLC pump (Agilent Technologies) and a 0.5×250 mm Zorbax SB-C18 column (particle size, 5 μm, Agilent) were used for the separation. A solution of 0.1% (v/v) formic acid in water (solution A) and a solution of 0.1% (v/v) formic acid in methanol (solution B) were used as mobile phases, and the gradient included 20 min 0–15% B, 10 min 15–35% B, and 10 min 35–60% B. The flow rate was 10 μL/min.

The effluent from the LC column was directed to an LTQ linear ion-trap mass spectrometer (Thermo), which monitored the fragmentation of the $[M+H]^+$ ions of labeled and unlabeled *R*-cdG, *S*-cdG, *R*-cdA, *S*-cdA, and the further fragmentation of the corresponding $[M+H-86]^+$ fragments found in MS/MS.

Results

LC-MS/MS/MS Identification and Quantification of cdA and cdG in Calf Thymus DNA Exposed with Cu(II)/H_2O_2/Ascorbate

We set out to investigate how efficiently the cdA and cdG lesions could be induced by Fenton-type reagents in isolated DNA. To this end, we treated calf thymus DNA with various concentrations of Cu(II)/H_2O_2/ascorbate (Table 1), digested the DNA with a cocktail of four enzymes, enriched cdA and cdG from the resulting nucleoside mixture and subjected them to LC-MS/MS or LC-MS^3 analysis by using the corresponding uniformly [^{15}N]-labeled nucleosides as internal standards (see Experimental Procedures).

In the LC-MS/MS/MS experiment, we monitored the *m/z* 250→164→136 and *m/z* 255→169→141 transitions for (5′*S*)-cdA and its uniformly [^{15}N]-labeled counterpart, respectively. The selected-ion chromatograms (SICs) and MS^3 spectra for the unlabeled and [^{15}N]-labeled (5′*S*)-cdA are displayed in Figure 1. The identity of the component eluting at 22.6 min in the SIC (Figure 1A&B) was found to be (5′*S*)-cdA by the co-elution, and similar fragment ions found in MS^3, of the analyte and its uniformly [^{15}N]-labeled standard. Ions of *m/z* 164 and *m/z* 169 arise from the neutral loss of a 86-Da fragment via cleavages of both the *N*-glycosidic linkage and the bond between the C5′ and C4′ of the 2-deoxyribose moiety of (5′*S*)-cdA and uniformly [^{15}N]-labeled (5′*S*)-cdA, respectively. Further cleavage of the ion of *m/z* 164 led to the elimination of a CO molecule to give the major fragment ion at *m/z* 136. Corresponding fragment ions were observed in the MS^3 of the uniformly [^{15}N]-labeled (5′*S*)-cdA. (5′*R*)-cdA was found to yield the same fragment ions in both MS/MS and MS^3 as (5′*S*)-cdA, though the two diastereomers exhibited different elution time on a reversed phase C18 column (Figure S2). Moreover, the amounts of (5′*R*)- and (5′*S*)-cdA are significantly lower in the control samples without hydrogen peroxide treatment (Figure 2 and Table S1), supporting that Cu(II)/H_2O_2/ascorbate can induce the formation of these two lesions.

We also observed the presence of (5′*R*)- and (5′*S*)-cdG based on the peaks at 11.6 and 21.4 min found in the SIC for monitoring the *m/z* 266→180→163 transition (Figures S3&S4). These components exhibit identical retention times as their respective isotope-labeled internal standards. Moreover, the major fragment ion at *m/z* 180 arises again from the cleavages of the C5′-C4′ bond of 2-deoxyribose and the *N*-glycosidic bond in cdG.[40] Further fragmentation of the ion of *m/z* 180 yielded three major fragment ions of *m/z* 163, 152, and 135 in MS/MS/MS, which emanate from the neutral losses of NH_3, CO, and [NH_3 + CO], respectively (Figures S3&S4). The corresponding fragment ions were found in the MS^3 for both diastereomers of the internal standards (Figures S3&S4). LC-MS^3 quantification results showed that the treatment of calf-thymus DNA with Cu(II)/H_2O_2/ascorbate induced the dose-dependent formation of both diastereomers of cdG (Figure 2 and Table S1).

LC-MS/MS/MS Identification and Quantification of cdA and cdG Lesions Formed in Calf Thymus DNA Treated with Fe(II)/H_2O_2/Ascorbate

Iron is another biologically important transition metal that can participate in Fenton-type reactions.[13, 14, 41] Iron's ability to generate highly mutagenic lesions in genomic DNA has been linked with iron-induced carcinogenesis in iron-overload diseases.[17] Thus, we also assessed the formation of cdA and cdG lesions in calf thymus DNA treated with Fe(II)/

H_2O_2/ascorbate (Table 1). It turned out that there is again a dose-dependent increase in the formation of the (5′*R*) and (5′*S*) diastereomers of cdA and cdG (Figure 3 and Table S2).

Comparison of the levels of the cdA and cdG lesions induced by the two Fenton systems revealed that the yields of cdA and cdG were lower when Cu(II) was replaced with Fe(II) under reaction conditions A through C (Figures 2&3 and Tables S1&S2). However, we observed an opposite trend when the transition metal ion concentrations exceeded 100 μM (i.e., conditions D and E, Figures 2&3 and Tables S1&S2).

LC-MS/MS Quantification of 8-oxodG Formed in Calf Thymus DNA Treated with Fe(II)/H_2O_2/Ascorbate

Ionizing radiation-induced formation of cdA is thought to proceed through an •OH-mediated hydrogen abstraction from the C5′ of the 2-deoxyribose. The resultant C5′ radical then couples with the C8 of the purine base to form the additional covalent bond between the nucleobase and 2-deoxyribose in the same nucleoside. Viewing that •OH can also result in the formation of single-nucleobase lesions, it is important to compare the yields for the formation of cdA and cdG lesions with respect to single-nucleobase lesions in both transition metal systems. Previous studies showed that Fenton reagents, Cu(II)/H_2O_2/ascorbate, can induce single-nucleobase lesion, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in isolated DNA.[38, 42] A comparison of the quantification results for Cu(II)/H_2O_2/ascorbate-induced cdG with these previously published quantification data of 8-oxodG under the same experimental conditions[38] showed that cdG was induced at a level that is 2–4 orders of magnitude lower than 8-oxodG. Here we further assessed the generation of 8-oxodG via the Fe(II)/H_2O_2/ascorbate system, and our results revealed that the yield of 8-oxodG was ~50–2500 fold greater than the combined yield of the two diastereomers of cdG (Figures 3&4 and Tables S2&S3).

It is worth noting that Cu^{2+}/H_2O_2 was found to induce singlet oxygen ($^1O_2$) generation[43, 44] and the incubation of Cu^+ or Cu^{2+} complex with H_2O_2 was observed to induce one-electron oxidation of DNA [45]. Both singlet oxygen and one-electron oxidation could result in the formation of 8-oxodG, but not cdA or cdG. Ascorbate, however, is a known scavenger for singlet oxygen [46] and the inclusion of excess amount of ascorbate in the reaction (Table 1) should also minimize one-electron oxidation of DNA by maintaining copper ion in the +1 oxidation state. Thus, we reason that the markedly lower yield observed for cdA and cdG than 8-oxodG is unlikely due to the involvement of singlet oxygen or one-electron oxidation.

Discussion

Here we demonstrated, by using LC-MS/MS/MS with the standard isotope dilution technique, that the treatment of isolated DNA with Cu(II)/H_2O_2/ascorbate or Fe(II)/H_2O_2/ascorbate can lead to the formation of both the (5′*R*) and (5′*S*) diastereomers of cdA and cdG. Additionally, the yields of the cdA and cdG lesions exhibit a dose-dependent increase at low concentration ranges followed by a marked increase at 800 μM Cu(II) or Fe(II) (Figures 2&3 and Table S1&S2). These results are reminiscent of previous findings about the formation of intrastrand cross-link lesions in calf thymus DNA exposed with Fenton reagents under similar conditions,[38, 42] suggesting that binding to Cu(II) and Fe(II) may result in an alteration in DNA conformation.[41] Although cdA and cdG can be detected in calf thymus DNA without treatment with Fenton reagents, which is consistent with the previous finding,[34] it is apparent that Fe(II)/H_2O_2/ascorbate and Cu(II)/H_2O_2/ascorbate could induce the dose-responsive formation of these lesions. Our results also revealed that Fe(II)/H_2O_2/ascorbate at the two highest concentrations (conditions D and E) were at least ~ 2–8 fold as effective as Cu(II)/H_2O_2/ascorbate system in inducing the cdA and cdG lesions,

though the latter is more efficient in inducing these lesions at lower concentrations. Our observations with calf thymus DNA suggested that Fenton reaction may constitute an important endogenous source for the formation of cdA and cdG in mammalian tissues.

In keeping with previous results from treatment with γ rays,[29] our data demonstrated that treatment with Fenton reagents also led to the preferential formation of (5′*R*)- over (5′*S*)-diastereomers of both cdA and cdG. However, these results were inconsistent with another study showing the preferential formation of (5′*S*)- over (5′*R*)-cdG in calf thymus DNA upon exposure to γ rays.[40] The exact reason for this discrepancy is unclear, though it is possible that less specific LC-MS or GC-MS technique used in the previous study[40] may lead to inaccurate measurements of the cdA and cdG lesions. Recent LC-MS/MS/MS quantification studies revealed that the (5′*S*) diastereomers of cdA and cdG are present at similar or higher levels than the corresponding (5′*R*) diastereomers in DNA isolated from mammalian tissues.[18, 36, 37] This result is in line with the more efficient repair of the (5′*R*) diastereomer than its (5′*S*) counterpart; indeed it was observed that a substrate housing a (5′*R*)-cdA was cleaved more efficiently than the corresponding (5′*S*)-cdA substrate by NER activities in mammalian nuclear extract.[23]

cdA and cdG are known substrates for NER, but not BER.[23, 24, 47] Our results showed that these lesions could be induced by Fenton-type reagent as efficiencies that are 2–4 orders of magnitude lower than that of 8-oxodG under the same experimental conditions (Figures 2–4). The detection of appreciable levels of the cdA and cdG lesions in DNA isolated from mammalian tissues[18, 36, 37, 48] suggests that these lesions might be more resistant to repair than the oxidatively generated single-nucleobase lesions (e.g., 8-oxodG). In addition, cdA was a strong block to DNA polymerase δ,[23] and both cdA and cdG strongly inhibit transcription by RNA polymerase II *in vitro* and in mammalian cells.[23, 24, 26] Thus, these lesions could accumulate in patients with deficiency in NER and become cytotoxic. Neurons consume a vast amount of oxygen, rendering the central nervous system susceptible to ROS-induced DNA damage. In this vein, xeroderma pigmentosum (XP) patients suffer from a progressive, yet massive, neuron loss, which is accompanied with mental deterioration over several decades, and these patients also manifest an elevated frequency of internal cancers.[49] The induction of cdA and cdG lesions by Fenton-type reagents reported here provide important new knowledge toward understanding the role of these lesions in the pathological symptoms of XP patients or with deficiencies in handling transition metal ions, such as Cu(II) or Fe(II).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ROS	reactive oxygen species
cdA	8,5′-cyclo-2′-deoxyadenosine
cdG	8,5′-cyclo-2′-deoxyguanosine
8-oxodG	8-oxo-7,8-dihydro-2′-deoxyguanosine

EHNA	*erythro*-9-(2-hydroxy-3-nonyl)adenine
SIC	selected-ion chromatogram
NER	nucleotide excision repair
BER	base excision repair
XP	xeroderma pigmentosum

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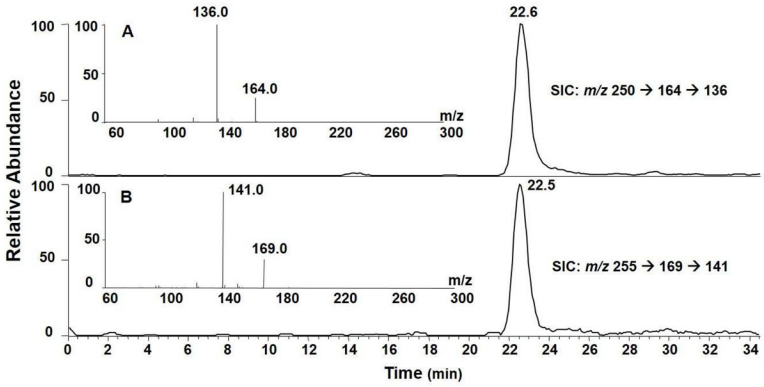


Figure 1.

Selected-ion chromatograms (SICs) for monitoring the *m/z* 250→164→136 [A, for unlabeled (5′*S*)-cdA] and *m/z* 255→169→141 [B, for uniformly ^{15}N-labeled (5′*S*)-cdA] transitions in the Cu(II)/H_2O_2/ascorbate-treated calf thymus DNA after enzymatic digestion. Shown in the insets are the positive-ion MS^3 spectra for the unlabeled and labeled (5′*S*)-cdA. The sample was treated under Conditions B listed in Table 1.

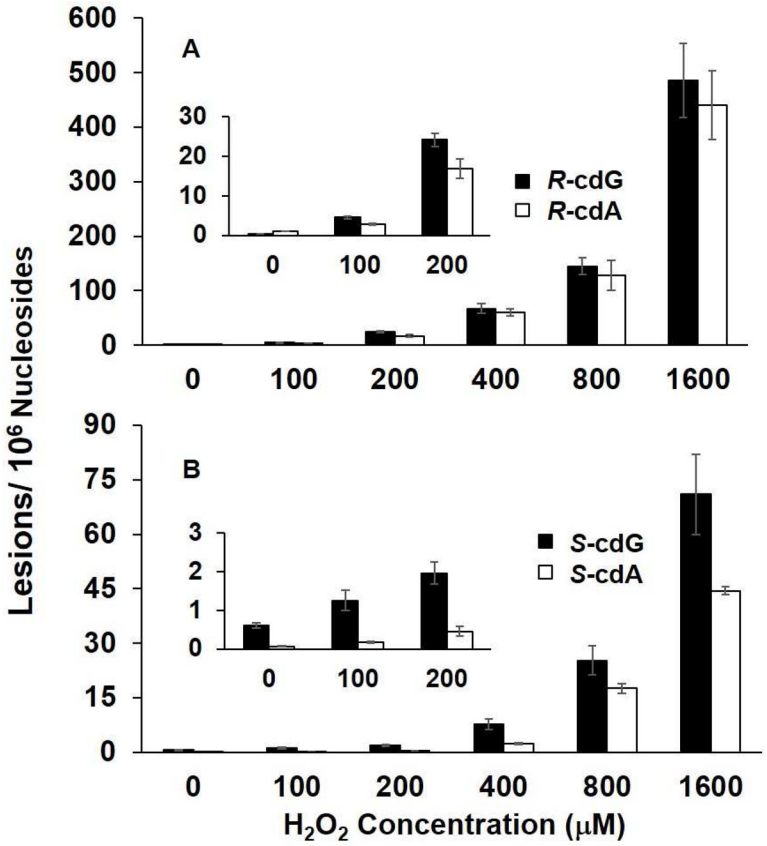


Figure 2.

Cu(II)/H_2O_2/ascorbate-induced formation of cPu lesions in calf thymus DNA: A, (5′*R*)-cdG and (5′*R*)-cdA; B, (5′*S*)-cdG and (5′*S*)-cdA. The values represent the means ± S.D. of results from three independent experiments. The corresponding concentrations of Cu(II) and ascorbate are shown in Table 1.

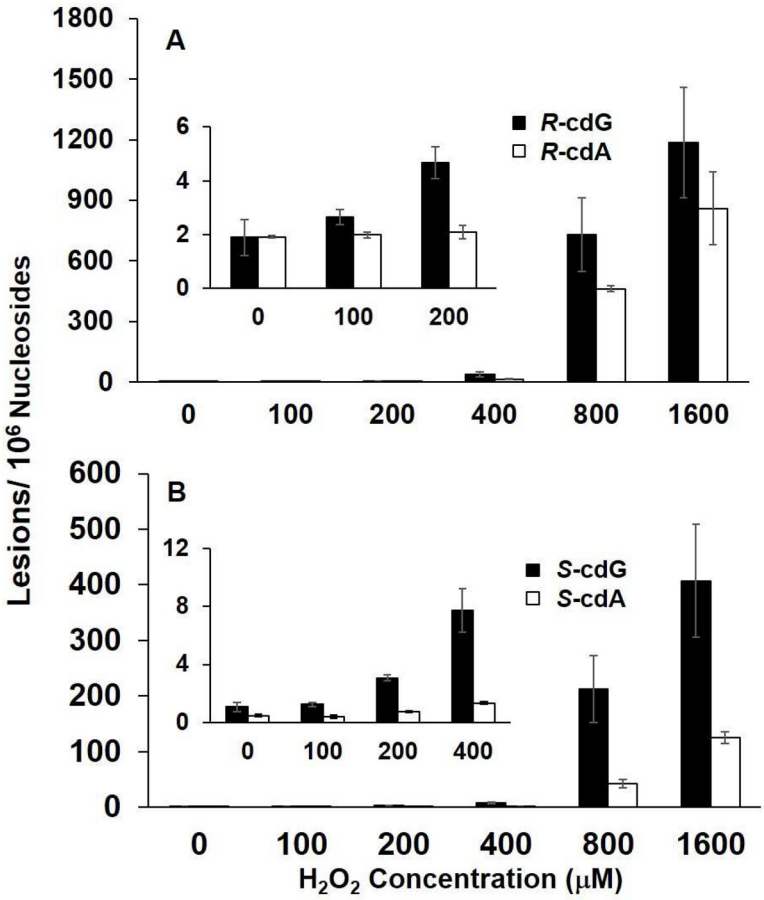


Figure 3.

Fe(II)/H_2O_2/ascorbate-induced formation of cPu lesions in calf thymus DNA: A, (5′*R*)- cdG and (5′*R*)-cdA; B, (5′*S*)-cdG and (5′*S*)-cdA. The values represent the means ± S.D. of results from three independent experiments. The corresponding concentrations of Fe(II) and ascorbate are listed in Table 1.

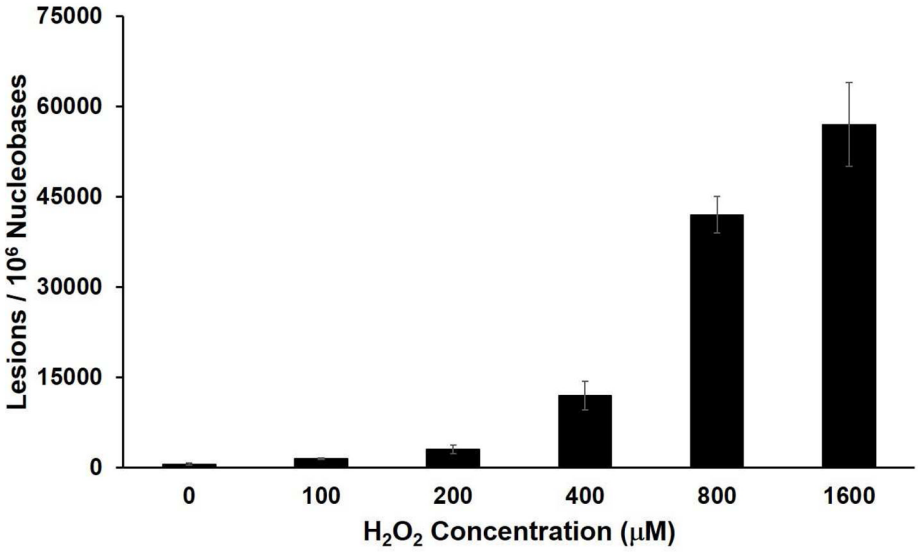
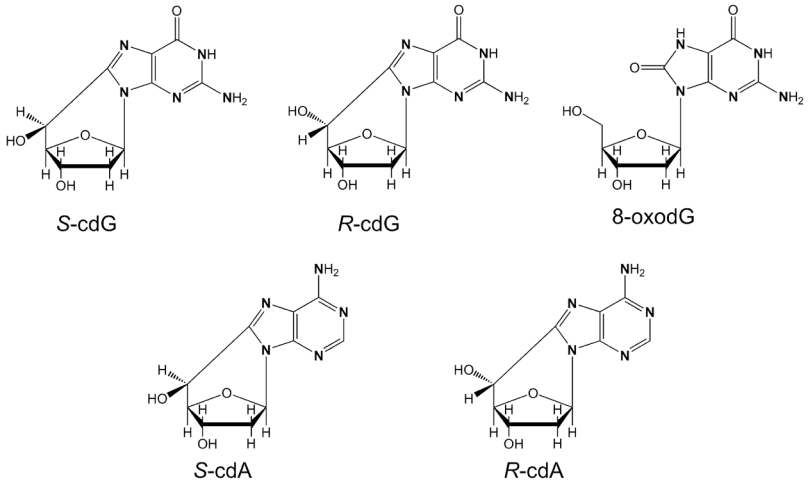


Figure 4.

Fe(II)/H_2O_2/ascorbate-induced formation of 8-oxodG in calf thymus DNA. The values represent the means ± S.D. of results from three independent oxidation and quantification experiments.



Scheme 1.
Chemical structures of synthesized ^{15}N-labeled nucleosides. The “N” in bold represents [^{15}N].

Table 1

Concentrations of Fenton Type Reagents Employed for the Treatment of Calf Thymus DNA.[a]

	Control	A	B	C	D	E
Cu(II)/Fe(II) (μM)	200	12.5	25	50	100	200
H_2O_2 (μM)	0	100	200	400	800	1600
Ascorbate (mM)	0	1.0	2.0	4.0	8.0	16.0

[a]All reactions were carried out in a 250-μL solution containing 75 μg of calf thymus DNA.

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