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A formation mechanism of 8-hydroxy-2'-deoxyguanosine mediated by peroxidized 2'-deoxythymidine

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Running title: 8-OHdG formation by peroxidizing thymidine

Abstract

The oxidative formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in DNA is closely associated with the induction of degenerative diseases including cancer. However, the oxidant species participating in the formation of 8-OHdG has yet to be fully clarified. On the basis that peroxyl radicals are a strong candidate for this species, we employed 2,2'-azobis(2-amidinopropane) (AAPH) as a peroxyl radical generator. Exposure of calf thymus DNA to AAPH formed 8-OHdG, but the exposure of 2'-deoxyguanosine (dG) alone did not. From the exposure of various combinations of nucleotides, 8-OHdG was formed only in the presence of dG and thymidine (dT). A mix of dG with an oxidation product of dT, 5-(hydroperoxymethyl)-2'-deoxyuridine (5-OOHmdU), produced 8-OHdG, but the amount formed was small. On the contrary, 8-OHdG was produced abundantly by the addition of dG to peroxidized dT with AAPH. Thus, the formation of 8-OHdG was found to be mediated by the peroxidized dT. Instead of artificial AAPH, endogenous peroxyl radicals are known to be lipid peroxides, which are probably the oxidant species for 8-OHdG formation mediated by thymidine *in vivo*.

Keywords: **Oxidation of DNA; 8-oxo-2'-deoxyguanosine, Formation mechanisms; Peroxyl radicals; Peroxidized thymidine; 5-OOHmdU**

Introduction

8-Hydroxy-2'-deoxyguanosine (8-OHdG) (Fig. 1) is one of the major products in oxidative DNA damage caused by reactive oxygen species (ROS) [1]. The formation of 8-OHdG in DNA is believed to induce degenerative diseases including cancer [2-4]. 8-OHdG can pair with adenine and lead to G:C→T:A transversion mutations unless repaired before replication [5, 6]. These mutations are widely seen in mutated oncogenes [7] and tumor suppressor genes [8], and consequently 8-OHdG levels are closely associated with the number and size of cancerous tissues [9-13]. In mice lacking repair enzymes for 8-OHdG, cancer incidence rates increased and an accumulation of 8-OHdG was found in the cancerous lesions of these mice [14, 15]. Prior to the formation of tumor lesions, 8-OHdG levels increased in the target tissues in nickel (II)-challenged rodents [16]. A supply of antioxidant vitamin E suppressed both the formation of 8-OHdG and tumors in rats with renal cancer induced by ferric nitrilotriacetate [17]. Thus, the formation of 8-OHdG is one of the direct causes of cancer. However, the oxidant species participating in 8-OHdG formation has yet to be fully clarified.

Candidate ROS for the oxidant species are superoxide anions, OH radical, peroxynitrite, and singlet oxygen, and they have been well recognized to form 8-OHdG. Superoxide anions are generated by several systems such as mitochondrial electron

transfer and xanthine oxidase and produce 8-OHdG [18]. In this reaction, the superoxide does not attack dG directly, and an OH radical generated from H_2O_2 attacks dG to form 8-OHdG after the superoxide is transformed into H_2O_2 [19]. Peroxynitrite is made by the reaction of nitric oxide and a superoxide anion [20]. Singlet oxygen is electromically excited molecular oxygen and causes the 8-OHdG production [21], and is generated by photochemical reaction and also by biological dark reactions as lactoperoxidase, lipoxygenase, and chloroperoxidase. These oxidant species are certain to cause 8-OHdG formation, but it may be unreasonableness that they frequently cause the formation, because the generation of these oxidant species can occur under limited conditions. For example, the generation of OH radicals from H_2O_2 requires transition metal ions for the Fenton reaction near DNA [22], and further the OH radical is very unstable with a very short half-life time of approximately 10^{-9} s [23]. Peroxynitrite is more unstable than superoxide, and both nitric oxide and superoxide anion are limited in the location where if can be generated [20]. The generation of singlet oxygen is also limited in biological systems. Then, we assumed that there was another oxidant to be responsible for the formation of 8-OHdG.

Peroxyl radicals (ROO·) are ubiquitously formed in cells with membranous lipid peroxidation, which is a chain reaction of enzymatic and nonenzymatic peroxidation of

polyunsaturated fatty acids (PUFAs) [24]. The radicals can attack DNA remote to their site of formation because of their longer half-life, approximately 7 s, compared with that of other oxygen-centered radicals including OH radicals [23]. Peroxyl radicals arise in nuclear or mitochondrial membranes. In particularly, peroxyl radicals generated in the inner mitochondrial membrane can easily attack DNA, since the mitochondrial DNA is located close to the membrane with the oxygen radical generating system of the electron-transport chain. Hruszkewycz et al. showed that isolated mitochondria produced 8-OHdG after treatment to induce lipid peroxidation [25]. Park et al. showed that calf thymus DNA formed 8-OHdG in a mixture with peroxidizing lipids or liposomes [26]. These findings assume that peroxyl radicals drived from lipid peroxidation participate in 8-OHdG formation directly or indirectly.

To investigate the participation of peroxyl radicals in 8-OHdG formation, we employed 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator, since AAPH decomposes thermally, reacts with oxygen, and produces alkylperoxyl radicals stoichiometrically at 1.3×10^{-6} s^{-1} at 37°C [27]. In previous studies, we exposed a single nucleoside, dG, to AAPH and detected stoichiometric formation of a peroxyl derivative 8-hydroperoxy-2'-deoxyguanosine (8-OOHdG) but not 8-OHdG [28]. Furthermore, only the formation of 8-OHdG but not 8-OOHdG, was detected when

nuclei isolated from HepG2 cells were exposed to AAPH [29]. Thus, the formation process of 8-OHdG was considered to implicate several intermediates and peroxyl radicals did not attack dG directly. In the present study, an important role of another nucleoside, thymidine (dT), was investigated in the formation mechanisms of 8-OHdG.

Materials and methods

Chemicals

Nucleosides dG and dT, and AAPH were purchased from Wako Pure Chemical Ind. (Osaka, Japan). The dG was more than 99.0% pure by HPLC analysis and contained 8-OHdG at a level of $0.55 \pm 0.17/10^5$ dG. Other nucleosides, 2'-deoxyadenosine (dA) and 2'-deoxycytidine (dC), were obtained from Sigma (St.Louis, MO, USA), and thymine (T) was from Tokyo Chemical Ind. (Tokyo, Japan). Calf thymus DNA (type I, highly polymerized) and standard chemicals 8-OHdG and 5-(hydroxymethyl)-2'-deoxyuridine (5-OHmdU) were from Sigma. For the preparation of 8-OOHdG, dG (2.5 mM) was oxidized with 250 mM AAPH in 10 ml water at 37°C for 6 h, and 8-OOHdG was purified by preparative HPLC after removing AAPH from the reaction mixture by ion exchange chromatography [28]. The resulting 8-OOHdG

was free from dG and 8-OHdG when analyzed by HPLC. Water was distilled twice, and all other reagents used were of the highest grade available from commercial sources.

Exposure to AAPH-derived peroxyl radicals

Calf thymus DNA (125 μg) was incubated with 25 mM AAPH in 1 ml of 10 mM potassium phosphate buffer (pH 7.4) at 37°C for 1 h as described previously [29]. The DNA was precipitated in 50% *n*-propanol containing 0.5 M NaCl, stored at -80°C for 15 min, and centrifuged at 17,000 *g* for 15 min at 4°C. The pellet was washed twice with 70% ethanol, dissolved in 1 mM disodium ethylenediamine tetraacetate (EDTA), and heated to 95°C for 5 min followed by cooling on ice. The denatured DNA solution was mixed with 35 mM sodium acetate and 2.5 units of nuclease P_1 (Wako) at 37°C for 30 min. After stopping the reaction by the addition of 0.1 M Tris-HCl (pH 7.5), the DNA was treated with 1.5 units of alkaline phosphatase (Sigma) at 37°C for 1 h and centrifuged at 17,000 *g* for 15 min at 4°C. The supernatant was filtered through a 0.2 μm membrane filter and a 50 μl aliquot was submitted for HPLC analysis as described below.

Similarly, 2'-deoxyribonucleosides were incubated in AAPH solution in water

singly or combined with other nucleosides at concentrations of 0.25 mM each, and then analyzed to determine the oxidation products by HPLC.

Determination of oxidation products of nucleosides by HPLC

The oxidation products of dG and dT were determined as described previously [29]. Briefly, the hydrolyzed DNA products and incubation mixtures of 2'-deoxyribonucleosides were analyzed by HPLC using the following conditions: column, Capcell pak C18 UG 120 maintained at 35°C; mobile phase, a mixed solvent of 6.5% methanol and 93.5% 20 mM potassium phosphate buffer (pH 4.5) containing 0.1 mM EDTA; flow rate, 1.0 ml/min. The 8-OHdG concentration was determined using an electrochemical detector at +600 mV using a calibration curve made with commercial standard 8-OHdG. Non-oxidized dG and 8-OOHdG were detected using a UV detector (L-7420, Hitachi) and their concentrations were determined with the respective molecular extinction coefficients λ_{253} (ε) =12,800 and λ_{254} (ε) =15,300 [29]. The detection limits of 8-OHdG and 8-OOHdG were 0.25 pmol and 10 pmol (0.044/10^5 dG and 400/10^5 dG), respectively. The commercial calf thymus DNA and dG originally contained 8-OHdG at levels of 5.8$\pm$0.8 and 0.55$\pm$0.17 number per 10^5 dG, respectively.

The present results were expressed after subtracting these original amounts of 8-OHdG. The oxidized products of dT, 5-(hydroperoxymethyl)-2'-deoxyuridine (5-OOHmdU) and 5-OHmdU were determined by the same method with λ_{254} (ε) =9,680 [30], and both had detection limits of 0.31 pmol.

Synthesis of the oxidation product of thymidine

5-OOHmdU was synthesized according to the method of Hahn and Wang [31]. 5-OHmdU (25 mg) dissolved in 2.5 ml of 15% H_2O_2 and was mixed dropwise with 1 ml of 15% H_2O_2 containing 10 μl concentrated HCl and allowed to stand for 24 h. The reaction was stopped by placing in liquid nitrogen and then lyophilized. The dried matter was dissolved in 10 ml water and subjected to preparative HPLC under the following conditions: column, Column Inertsil Prep-ODS (5 μm mesh and ø 20.0×250 nm, GL Science, Tokyo, Japan); mobile phase, 6.5% methanol in water; flow rate, 8.0 ml/min. Two peaks were detected on the HPLC trace and one coincided with the retention time of the original 5-OHmdU. The other peak was collected and dried with a centrifugal concentrator. This compound was further purified by HPLC, dried, and stored at -30°C in the dark until use.

The purified product was identified chemically and spectrometrically. The peroxide value (PV) was determined by the absorbance at 410 nm of iodine liberated by the reduction of the product with KI [32]. Mass spectra were measured with a LC/MS-QP8000 spectrometer (Shimadzu, Kyoto, Japan) using the following conditions: mobile phase, 50% acetonitrile in water containing 0.1% acetic acid; spray voltage, 3.5 kV in negative mode; capillary temperature, 250°C; *m*/*z* values, from 50 to 500. In addition, ^{1}H and ^{13}C NMR spectra were recorded in Fourier transform mode using a JNM-AL300 FT-NMR (JEOL, Japan). Chemical shifts were calibrated in deuterium oxide with reference to 0.1% 3-(trimethylsilyl) propionate-2,2,3,3-d_4 (Euriso-top, France).

Results

Differences in oxidation products after exposing DNA and dG to peroxyl radicals

It has been found previously that 8-OOHdG was formed when nucleoside dG was exposed to AAPH-derived peroxyl radicals [28], while 8-OHdG was detected when the nuclei of HepG2 cells were exposed to peroxyl radicals [29]. To understand the differences in the oxidation products, in the present study, calf thymus DNA and dG

were incubated with AAPH (Table 1). Calf thymus DNA produced 8-OHdG, but dG produced 8-OOHdG. The results indicated two possible pathways for 8-OHdG formation; 8-OHdG was a degradation product of 8-OOHdG during the treatment of DNA before submitting the sample for analysis, or the production of 8-OHdG required the coexistence of nucleosides other than dG.

To examine the former possibility, 8-OOHdG was prepared, purified, and treated in a similar manner to the calf thymus DNA before analysis (Table 2). The incubation of 110 μM 8-OOHdG in sodium acetate with hydrolysis enzymes nuclease and alkaline phosphatase or with Fe^{2+}-EDTA $\pm$ dT partly decreased 8-OOHdG to 91, 100, and 86 μM respectively, but did not produce 8-OHdG. In addition, other reducing agents such as sodium borohydrite, litium aluminum hydrite, potassium iodide, 2-mercaptoethanol, and dithiothreitol were also added at excess concentrations, ranging between 20 and 2 μM, to the incubation mixture of 100 μM 8-OOHdG, but none of these treatments resulted in the conversion 8-OOHdG to 8-OHdG (data not shown). These results clearly showed that 8-OHdG was neither formed during the treatments of oxidized DNA nor by the decomposition of 8-OOHdG.

To assess the other potential route of 8-OHdG formation, dG was exposed to AAPH with various combinations of the other bases (Table 3). Combinations with dA

and dC did not give 8-OHdG, but the systems containing dT or single base T produced 8-OHdG. These results strongly indicated that thymine bases were essential for the formation of 8-OHdG in the exposure systems with AAPH.

Oxidation products of thymidine in the AAPH exposure system

The requirement for dT or T assumes two possible roles of thymine bases in the formation of 8-OHdG. One is that thymine base reducibly converts 8-OOHdG to 8-OHdG, and the other is that the thymine base is oxidized first and its oxidized product oxidizes dG to 8-OHdG. To assess the former possibility, 110 μM 8-OOHdG was incubated with 250 μM dT in the presence of Fe^{2+}-EDTA (Table 2). This incubation decreased the amount of 8-OOHdG to 77 μM but did not form 8-OHdG. For the latter possibility, dT was first exposed to AAPH and was added to dG after removing AAPH (Table 4). The addition of oxidized dT formed increasing amounts of 8-OHdG in an incubation time-dependent manner and produced $23/10^5$ dG of 8-OHdG in 4 h, though the exposure of dG or dT alone to AAPH did not produce 8-OHdG. This indicated that the oxidation products of dT participated in 8-OHdG formation. Both 5-OHmdU and 5-OOHmdU have been reported as the oxidation products of dT [33]. 5-OHmdU is

available commercially but 5-OOHmdU is not, so 5-OOHmdU was synthesized according to the method of Hahn and Wang [31]. Table 5 shows the chemical characteristics of the synthesized product after purification by HPLC. The product exhibited a spectrum at *m/z* 273 for the parent ion $[M-H]^-$, while commercial 5-OHmdU and dT showed spectra at *m/z* 257 and 241 for $[M-H]^-$, respectively. The synthesized product was 32 mass units larger than dT indicating the addition of an oxygen molecule to dT. The UV spectrum and molecular extinction coefficient of the synthesized product were similar to those of dT and 5-OHmdU, indicating that the product remained unchanged in the pyrimidine structure. The synthesized product responded to PV reagent and showed 0.79 eq/mol of PV. ^{1}H and ^{13}C NMR spectra of the synthesized product were consistent with the chemical shifts of 5-OOHmdU assigned by Wagner *et al*. [34]. Thus, the present synthesized product was 5-OOHmdU.

Participation of peroxidized dT in the formation of 8-OHdG

dG was incubated with the oxidation products of dT, 5-OHmdU, and 5-OOHmdU at 37°C for 1 h (Table 6). The incubation with 5-OOHmdU clearly produced 8-OHdG but dT and 5-OHmdU did not. The mode of production of 8-OHdG

was determined by the incubation of dG with 5-OOHmdU and plotted to show the second-order kinetics (Fig. 2). The production of 8-OHdG followed a linear curve *versus* reaction time, indicating that 8-OHdG production was a bimolecular reaction of 5-OOHmdU with dG [35]. Table 7 shows the amounts of 8-OHdG produced when dG was incubated with various concentrations of 5-OOHmdU. The amounts of 8-OHdG produced increased with the concentration of 5-OOHmdU, but the ratio of 8-OHdG produced to the amount of 5-OOHmdU consumed was small. The ratio was 1/4600 when 1000 nM 5-OOHmdU was added to dG and 460 nM of it was consumed, and the ratios were 1/3000, 1/1000, and 1/800 in the consumption of 1200, 300, and 800 nM of 5-OOHmdU, respectively. The formation of 5-OHmdU was low or under the detection limit. Alternatively, 5-OOHmdU barely decomposed to form 5-OHmdU and/or 5-formyldU when it was incubated alone for 2 h (Table 7). These results indicated that a part of 5-OOHmdU participated in the formation of 8-OHdG and most 5-OOHmdU decomposed to undetectable chemicals in the incubation with dG.

In the presence of Fe^{2+}, 5-OOHmdU may produce a large amount of 8-OHdG by decomposition by the Fenton reaction. dG was incubated with 5-OOHmdU in the presence of the Fenton reagents (Table 8). 8-OHdG was produced in 7-fold higher amount compared to the production level in the absence of Fenton reagents. In the

reaction, 236 μM of 5-OOHmdU was consumed and 0.06 μM 8-OHdG and 20.8 μM 5-OHmdU were produced. The production ratios to the amount of 5-OOHmdU consumed were 1/4000 for 8-OOHdG and 1/12 for 5-OHmdU. These indicated that 5-OOHmdU decomposed partly to 5-OHmdU and mostly to undetectable chemicals by the Fenton reaction and that OH radicals generated by the decomposition of 5-OOHmdU did not participate significantly in the production of 8-OHdG.

On the other hand, dT was exposed to AAPH for time-lengths similar to those taken to produce 1000-10,000 nM 5-OOHmdU in Table 7 and then dG was added (Table 9). In this reaction system, the amounts of 8-OHdG formed did not changed linearly and were between 19±9.0 and 2.6±0.42 μM when the dT amount was increased from 1000 μM to 10,000 μM, indicating that dT was a catalyst and not a substrate. The results in Table 9 show the production ratios of 8-OHdG of 1/30, 1/90, 1/1000, and 1/2600 in the reactions that include 1000-10,000 nM of 5-OOHmdU would be produced theoretically and 580, 1300, 3600, and 6900 nM would be consumed, respectively. Comparing the reaction with smaller amounts of 1000 and 2500 nM 5-OOHmdU, the ratios of 8-OHdG formation were dozens of times higher than in Table 7, and the amounts of 5-OHmdU formed were substantially larger than in Table 7. In addition, dG was increasingly consumed with extension of exposure time to peroxidizing condition

in Table 9 where peroxyl radicals of dT were formed, although dG did almost not decreased in exposure to 5-OOHmdU in Table 7. Ravanat et al. [35] found that 8-OHdG was degraded to ring-opening products as soon as it was generated under the condition where peroxyl radical acts as one electron oxidant. These information shows that dG was easily oxidized by the peroxyl radicals of dT and mostly decomposed and partly formed 8-OHdG, compared to the system in which 5-OOHmdU participated. Thus, the exposure to AAPH formed peroxyl radicals of dT, and the peroxyl radicals attacked dG and produced 8-OHdG while mostly decomposing to 5-OHmdU.

Discussion

The formation of 8-OHdG in DNA induces mutations and is closely associated with the etiology of degenerative diseases including cancer [1-4]. An important issue for disease prevention is to understand the oxidant species that converts dG to 8-OHdG. The present study demonstrates that peroxidized dT is the oxidant species and suggested that the peroxyl radicals are derived from lipid peroxidation.

In the present study, AAPH was employed as a peroxyl radical generator instead of lipid peroxides. AAPH thermally produces tertiary carbon centered

tert-amidinopropyl radicals, which react rapidly with oxygen molecules to give peroxyl radicals [27]. The peroxyl radicals from AAPH are positively-charged. On the other hand, lipid peroxyl radicals that are generated endogenously during lipid peroxidation are negatively-charged or neutral and are considered to react more weakly with negatively-charged DNA than AAPH-derived radicals [37]. However, when DNA is exposed to either peroxidized PUFAs or peroxyl radical generators including AAPH, both showed nearly identical patterns of oxidative base damage [38-40]. These findings strongly indicate that there is a mediator when lipid peroxyl radicals act on DNA endogenously.

Thymine is known to be frequently modified by lipid peroxides as well as guanine [41, 42]. Djuric et al. showed that an intake of peroxidizable PUFAs significantly increased the level of 5-OHmdU in the DNA of women with breast cancer [43], and a supplementation of dietary antioxidants suppressed this outcome [44]. These findings suggested that lipid peroxides first oxidized thymidine and the peroxidized thymidine plays a mediator role in 8-OHdG formation.

In the present study, the exposure of calf thymus DNA to AAPH formed 8-OHdG, but the exposure of dG alone did not (Table 1). 8-OHdG was formed only by the combination of dG and dT (Table 3), indicating that dT was indispensable for the

generation of 8-OHdG. To identify the participating product of dT, 5-OOHmdU and 5-OHmdU were employed, and 5-OOHmdU was found to form 8-OHdG in a bimolecular reaction with dG (Table 6 and Fig. 2). However, the production ratio of 8-OHdG to the amount of 5-OOHmdU consumed was small (Table 7). The production ratio was also small when 5-OOHmdU was placed with dG in the Fenton reaction (Table 8). Then, dG was added to the exposure system of dT to AAPH. This degraded dG largely and produced a larger amount of 8-OHdG than the system of 5-OOHmdU and dG (Table 9). Thus, the production of 8-OHdG was small when hydrogen terminated the peroxidation of dT and formed the end product 5-OOHmdU, and the production was large when dG instead of hydrogen terminated the peroxidation. In the former case, 5-OOHmdU must interact with dG for the production of 8-OHdG. Since this reaction was additional in the former system, the production ratio of 8-OHdG was relatively small compared to the latter system. These results clearly indicate that an interaction of dG with radicals generated from 5-OOHmdU produced 8-OHdG. In the termination products of peroxidized dT by dG, Douki et al. reported that C-5 or C-6 peroxyl radicals of dT formed a complex with dG and its decomposition produced 8-OHdG [45]. In the present system, one potential alternative product is a dimer of dG and the C-5 methyl peroxyl radical of dT. However, more study using of a model of

synthetic DNA containing poly dG-dT will be needed to make the complex formation of dT and dG more clear.

In biological system, a peroxide donor to dT must be lipid peroxides. The lipid peroxidation frequently occurs in several systems, mitochondrial electron transfer [46], myeloperoxidase in neutrophile and macrophage [47], monooxygenation by cytochrome P450 enzymes [48], and others. The lipid peroxides are considered to peroxidize dT and then form 8-OHdG. This event probably causes the direct inductions of degenerative diseases, since the association of lipid peroxidation with degenerative diseases has been generally recognized.

Conclusion

The proposed mechanism of endogenous 8-OHdG formation by peroxyl radicals is summarized as shown in Fig. 3. The formation of 5-OOHmdU by peroxyl radicals has been reported by Martini and Termini et al. [33]. Peroxyl radicals (LOO·) derived from lipid peroxides abstract hydrogen at the C-5 methyl position of dT and provide a C-5 methyl-2'-deoxyuridylyl radical. This reacts with an environmental oxygen to yield a C-5 methyl peroxyl radical of dT. If there is dG near the radicals, the peroxyl radical is

trapped by dG and forms an intermediate dimer of dG and 5-peroxyl dT [33]. If there is no dG present the radical is reduced by hydrogen and forms the end product 5-OOHmdU. The intermediate dimer will easily decompose to 8-OHdG and 5-OHmdU. On the other hand, 5-OOHmdU will spontaneously decompose to methyl alkoxyl radical and may partly react with dG in the vicinity and produce 8-OHdG.

The formation of 8-OHdG is probably frequent in mitochondria, because DNA is regularly exposed to membranous lipid peroxidation [25] and lesions at dG adjacent to dT will form 8-OHdG. The 8-OHdG formed is well recognized to induce gene mutations, which is in turn associated with degenerative diseases. *In vivo* the donors of peroxyl radicals are lipid peroxides. Therefore, endogenous lipid peroxidation is concluded to be linked with degenerative diseases including cancer.

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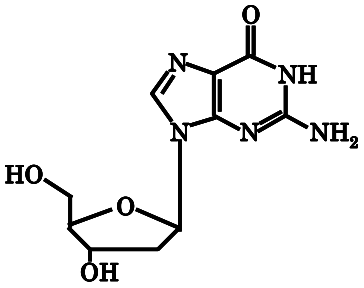
Legends to figures

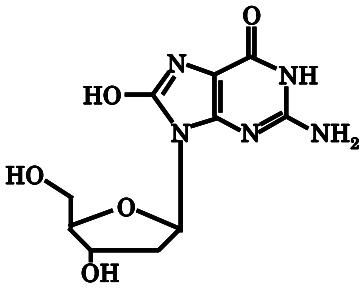
Fig. 1. Chemical structures of oxidized products of dG and dT

Fig. 2. A kinetic curve of 8-OHdG formation by the reaction of dG with 5-OOHmdU. dG (0.25 mM) was incubated with 5-OOHmdU (0.034 mM) in water at 37°C. The production of 8-OHdG was determined by HPLC and plotted to show the second-order kinetics, where $[dG]_0$ is the initial concentration of dG (0.25 mM), $[5\text{-OOHmdU}]_0$ is the initial concentration of 5-OOHmdU (0.034 mM), and $[8\text{-OHdG}]_t$ is the concentration of 8-OHdG at the times indicated. Values are the means of three independent experiments.

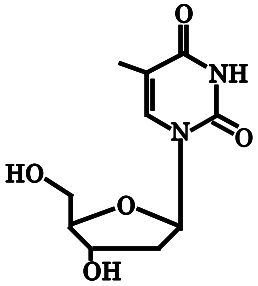
Fig. 3. Proposed mechanism of 8-OHdG formation by peroxyl radicals. Lipid peroxyl radicals (LOO·) abstract a C-5 methyl hydrogen of dT and produce C-5 methyl peroxyl radicals after the incorporation of oxygen. The peroxyl radicals are trapped by dG forming an intermediate dimer of dG and 5-peroxyl dT when dG occurs near the 5-peroxyl dT radical. The intermediate dimer decomposes to 8-OHdG and 5-OHmdU. If there is no dG in the vicinity, the radical is reduced by hydrogen and form 5-OOHmdU, which can spontaneously decomposes to a methyl alkoxyl radical and partly reacts with dG to produce 8-OHdG.



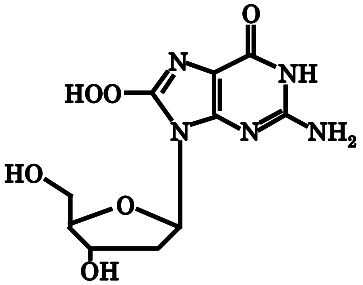
2’-deoxyguanosine (dG)



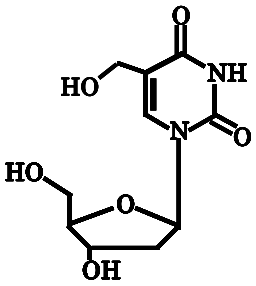
8-hydroxy-2’-deoxyguanosine (8-OHdG)



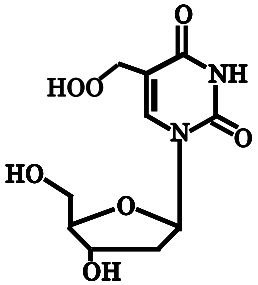
2’-deoxythymidine (dT)



8-hydroperoxy-2’-deoxyguanosine (8-OOHdG)



5-hydroxymethyl-2’-deoxyuridine (5-OHmdU)



5-hydroperoxymethyl-2’-deoxyuridine (5-OOHmdU)

Fig. 1

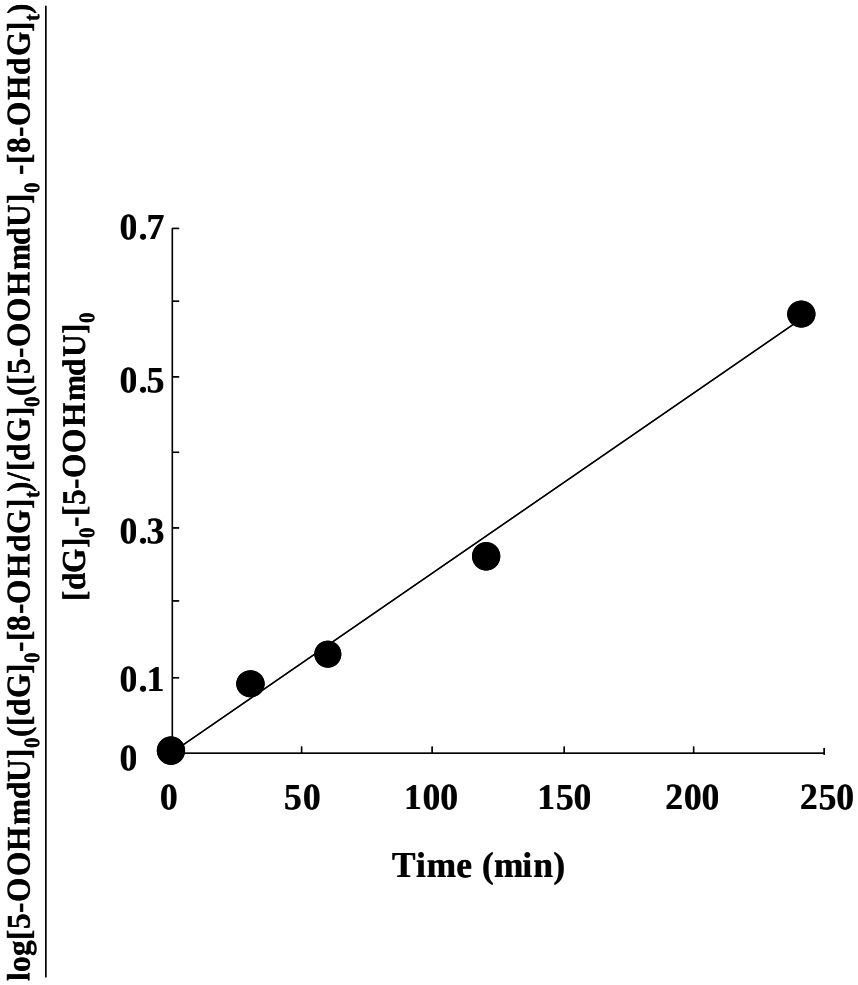

log[5-OOHmdU]0([dG]0-[8-OHdG]t)/[dG]0([5-OOHmdU]0 -[8-OHdG]t)
[dG]0-[5-OOHmdU]0
0.7
0.5
0.3
0.1
0
0
50
100
150
200
250
Time (min)

Fig. 2.

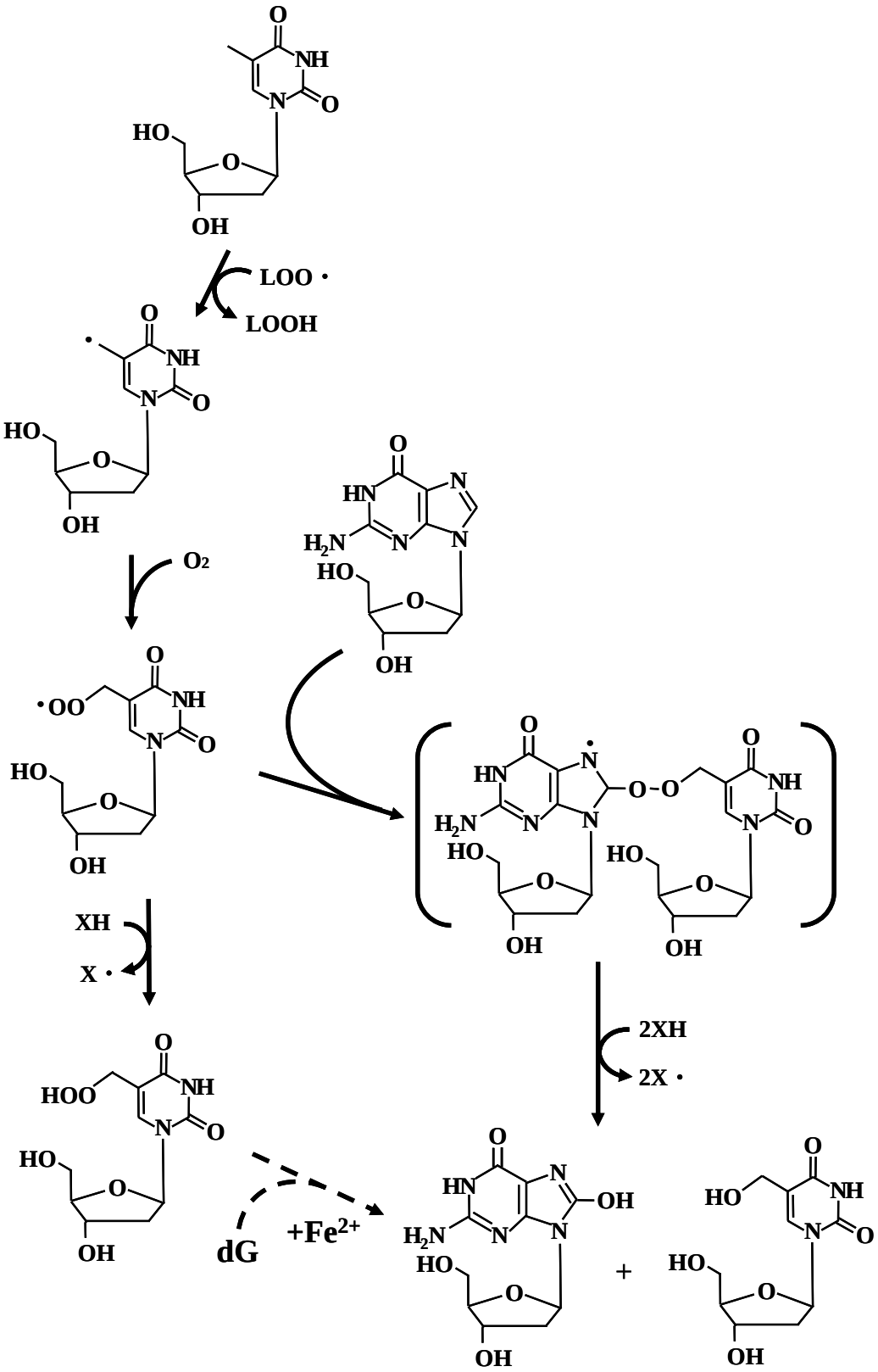


Fig. 3.

Table 1

Oxidation products of calf thymus DNA and dG when exposed to AAPH-derived peroxyl radicals

Substrate	Product (number per 10^5 dG)[a]	
	8-OOHdG	8-OHdG
Calf thymus DNA	ud	154±26
dG	5400±220	ud

[a]Calf thymus DNA or dG was incubated with 25 mM AAPH at 37°C for 1 h, and the oxidation products were examined by HPLC after the DNA was hydrolyzed as described in the "Materials and methods". Values are mean±SD (*n*=6). "ud" is under the detection limits.

Table 2

Stability of 8-OOHdG after various treatments

Treatment[a]	Products (μM)	
	8-OOHdG	8-OHdG
Notreatment	110	ud
Incubation alone	91	ud
with hydrolysis enzymes	100	ud
with Fe^{2+}-EDTA	86	ud
with Fe^{2+}-EDTA and dT	77	ud

[a]The treatments of 8-OOHdG (110 μM) were as follows: incubation alone was in 1 ml 35 mM sodium acetate and 1 mM EDTA at 37°C for 1 h; incubation with hydrolysis enzymes was with 2.5 units of nuclease P_1 for 30 min and with 1.5 units of alkaline phosphatase for another 1 h; and incubation with Fe^{2+}-EDTA was with 0.13 mM $FeSO_4$ and 0.65 mM EDTA in 1 ml 10 mM sodium phosphate buffer at pH 6.8 for 1 h in the absence or presence of 250 μM dT. An aliquot (10 μl) of each incubation mixture was analyzed for the concentrations of 8-OOHdG and 8-OHdG by HPLC. "ud" is under the detection limits.

Table 3

Formation of 8-OHdG when exposed to AAPH-derived peroxyl radicals in combination systems of dG with various bases

Combination	Product (number per 10^5 dG)	
	8-OOHdG	8-OHdG
dG	5000±93	ud
dG + dA	4800±110	ud
dG + dC	4800±45	ud
dG + dT	4800±51	1.6±0.2
dG + dA + dC	4800±58	ud
dG + dA + dT	4900±88	0.45±0.05
dG + dT + dC	4600±73	3.2±1.1
dG + dA + dT + dC	4400±73	0.42±0.04
dG + T	4900±150	1.25±0.02

[a]dG was combined with other deoxyribonucleosides or T at a concentration of 0.25 mM each and exposed to 25 mM AAPH at 37°C for 1 h. Values are mean±SD (*n*=3).

Table 4

Production of 8-OHdG from dG exposed to oxidizing dT with AAPH

Exposure system	Produced 8-OHdG (number/10^5 dG)
dG + AAPH for 1 h[a]	ud
dT + AAPH for 1 h[a]	ud
dT exposed to AAPH + dG for 0 min[b]	ud
dT exposed to AAPH + dG for 15 min[b]	ud
dT exposed to AAPH + dG for 30 min[b]	0.67±0.11
dT exposed to AAPH + dG for 1 h[b]	0.73±0.19
dT exposed to AAPH + dG for 4 h[b]	2.3±0.6

[a]dT (2.5 mM) was incubated with 25 mM AAPH in 1 ml of water at 37°C for 1 h and 8-OHdG production was analyzed by HPLC.

[b]dT (2.5 mM) was incubated with 25 mM AAPH in 1 ml of water at 37°C for 1 h. After removing AAPH by HPLC the dT products were mixed with 1.0 mM dG for the indicated times and 8-OHdG production was analyzed by HPLC. Values are mean±SD (n=3). “ud” is under the detection limits.

Table 5

Chemical characteristics of synthesized 5-OOHmdU

	dT	5-OHmdU	5-OOHmdU
LC/MS (*m/z*)	$[M-H]^-$, 241	$[M-H]^-$, 257	$[M-H]^-$, 273
UVλ_{max} (ε)	267 (9650)	264 (8100)	266 (8400)
PV (eq/mol)	ud	ud	0.79
^{1}H NMR (5-CH_3)	1.9	4.40	4.74
^{13}C NMR (5-CH_3)	14.4	59.5	73.9

"ud" is under the detection limits.

Table 6

Participation of thymine in the formation of 8-OHdG

Incubation[a]	8-OHdG (number per 10^5 dG)
dG + dT	ud
dG + 5-OHmdU	ud
dG + 5-OOHmdU	2.6±0.5

[a]dT, 5-OHmdU, or 5-OOHmdU at concentrations of 0.25 mM was incubated with 0.25 mM dG in water at 37°C for 1 h, and the amount of 8-OHdG produced was determined by HPLC. Values are mean±SD (*n*=3).

Table 7

Production of 8-OHdG in a reaction of dG with 5-OOHmdU

Incubation of 5-OOHmdU (nM)	5-OOHmdU alone [a]			5-OOHmdU with 250 μM of dG [b]			
	Remaining 5-OOHmdU (nM)	Product (nM)		Remaining 5-OOHmdU (nM)	Product (nM)		Remaining dG (μM)
		5-OHmdU	5-formyldU		8-OHdG	5-OHmdU	
1000	950±6.5	ud	19±3.0	540±13	0.10±0.032	ud	250±5.0
2500	2400±32	18±2.0	27±6.6	1300±30	0.40±0.050	ud	250±1.9
5000	4600±70	69±2.0	71±28	4700±54	0.29±0.031	57±7.5	250±3.4
10,000	8600±600	250±74	260±170	9200±140	1.0±0.018	273±68	220±1.7

[a]5-OOHmdU was incubated alone at 37℃ for 2 h, and determined remaining amounts of 5-OOHmdU and produced amounts of 5-OHmdU and 5-formyldU by HPLC (mean±SD, n=4).

[b]5-OOHmdU was incubated with 250 μM dG at 37℃ for 2 h, and the reaction products were determined (mean±SD, n=4).

Table 8

Production of 8-OHdG by the Fenton reaction with 5-OOHmdU and Fe^{2+}-EDTA

Reaction system[a]	Remaining chemicals (μM)		Products	
	dG	5-OOHmdU	8-OHdG (nM)	5-OHmdU (μM)
dG + Fe^{2+}	250±1.8	-	ud	-
dG + Fe^{2+}-EDTA	250±2.1	-	0.69±0.01	-
dG + 5-OOHmdU	246±1.2	217±1.3	8.3±0.07	8.54±0.67
dG+5-OOHmdU+Fe^{2+}	243±5.8	214±5.9	6.7±0.3	8.44±0.78
dG + 5-OOHmdU+ Fe^{2+}-EDTA	250±0.5	14.1±1.6	60±9.0	20.8±1.6

[a]dG 250 μM was incubated with 250 μM 5-OOHmdU in the presence of 250 μM $FeSO_4$ or 1250 μM EDTA plus 250 μM $FeSO_4$ at 37℃ for 1 h.

Table 9

Production of 8-OHdG in a reaction of dG with peroxidized dT

Exposure time (h)[a]	dT alone		dT with 250 μM dG[b]			
	Products (nM)		Product (nM)			Remaining dG (μM)
	5-OOHmdU	5-OHmdU	5-OOHmdU	5-OHmdU	8-OHdG	
1.57	1000	3200	420±19	7200±170	19±9.0	230±7.5
3.83	2500	3300	1200±190	6700±46	15±2.9	180±4.9
7.50	5000	3500	1400±41	130±386	3.5±0.48	140±5.0
14.0	10,000	3800	3100±250	2100±1100	2.6±0.42	40±26

[a]dT (5 mM) was incubated with 25 mM AAPH at 37℃ for the times indicated to produce 5-OOHmdU at levels close to the value shown in Table 7.

[b]In the same system, 250 μM dG were added and incubated, and the amount of reaction products and remaining dG were determined by HPLC (mean±SD, n=4).