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Formation of spiroiminodihydantoin nucleoside from 8-oxo-7,8-dihydro-2′-deoxyguanosine by nitric oxide under aerobic conditions

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ABSTRACT

When 8-oxo-7,8-dihydro-2′-deoxyguanosine in potassium phosphate buffer of pH 7.4 was bubbled by nitric oxide at room temperature under aerobic conditions, two major products were formed. They were identified as the diastereomers of spiroiminodihydantoin deoxyribonucleoside on the basis of their identical ESI-MS and UV spectra and HPLC retention times with those of the major products in reaction of 8-oxo-7,8-dihydro-2′-deoxyguanosine with hypochlorous acid. A 1000-fold excess of 2′-deoxyguanosine did not inhibit the reaction of 8-oxo-7,8-dihydro-2′-deoxyguanosine with nitric oxide. The results suggest that an 8-oxo-7,8-dihydroguanine moiety formed in DNA may react with nitric oxide in the presence of oxygen molecule generating spiroiminodihydantoin in humans.

Nitric oxide (NO) is synthesized in various types of cells by the enzyme nitric oxide synthase and is involved in numerous biological functions, including vasodilation, neurotransmission, and inflammation.[1,2] Although NO is a radical, the reactivity of NO per se is relatively low. However, in the presence of O_2, NO is converted to a reactive nitrosating reagent, dinitrogen trioxide (N_2O_3).[3] N_2O_3 can react with amino groups in various biological molecules by electrophilic reactions resulting in corresponding *N*-nitroso compounds. It is thought that N_2O_3 can act as a carrier of NO^+.[4,5] On the other hand, NO is converted to peroxynitrite ($ONOO^-$) in the presence of superoxide anion radical O_2^- at an almost diffusion-controlled rate.[6] In a neutral solution, peroxynitrite exists as a mixture of $ONOO^-$ and peroxynitrous acid (ONOOH), a protonated form of peroxynitrite, since the pK_a of ONOOH is 6.5.[7] ONOOH is highly reactive and readily causes both oxidation and nitration of various biological molecules.[8–11] 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), a ubiquitous DNA damage, is easily formed by oxidative reactions of 2′-deoxyguanosine (dGuo) with various oxidants.[12] 8-oxodGuo is thought to be present in human cellular DNA at levels in the range of 0.3–4.2 per 10^6 dGuo.[13] 8-OxodGuo is highly susceptible to various oxidizing agents due to its low redox potential.[14] ONOOH easily reacts with 8-oxodGuo generating various products.[15,16] Low flux ONOOH or high flux ONOOH with thiol generates spiroiminodihydantoin deoxyribonucleoside (dSph) from 8-oxodGuo.[16,17] However, little attention has been paid to the reaction of 8-oxodGuo with N_2O_3. In this study, we examined the reaction of 8-oxodGuo with NO under neutral aerobic conditions, and found the consumption of 8-oxodGuo and the formation of products.

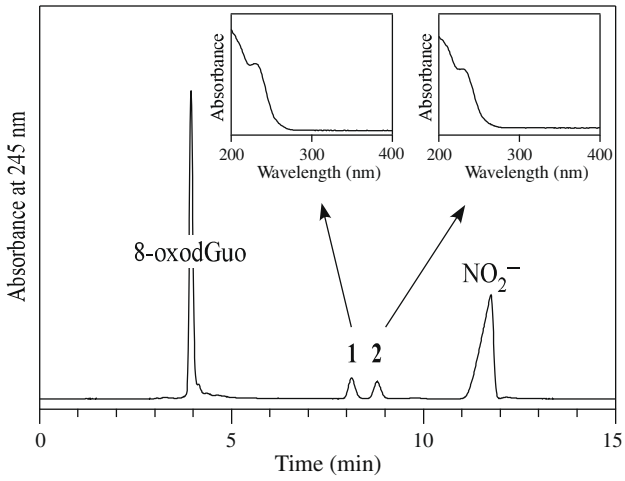

Figure 1. A normal-phase HPLC chromatograph of a reaction solution of 8-oxodGuo with NO gas. The insets are the on-line UV spectra of **1** and **2**. A stirring solution of 1 mM 8-oxodGuo (sigma) with 250 mM potassium phosphate buffer (pH 7.4) in a 6.5 mL open vial was bubbled by 0.5 mL/min NO (99.7%, Sumitomo Seika, Tokyo) in open air at room temperature for 4 min. The flow rate of NO gas was controlled by a Model 3660 mass flow controller (KOFLOC, Kyoto, Japan). The final pH was 6.9. The HPLC system consisted of Shimadzu LC-10ADvp pumps and an SCL-10Avp system controller. On-line UV spectra were obtained with a Shimadzu SPD-M10Avp UV-vis photodiode-array detector. An Inertsil NH_2 column of 4.6 × 250 mm and particle size 5 μm (GL Sciences, Tokyo) was used. The eluent was 20 mM ammonium formate (pH 6.8) and acetonitrile (5/5, v/v). The column temperature was 40 °C and the flow rate was 1.0 mL/min.

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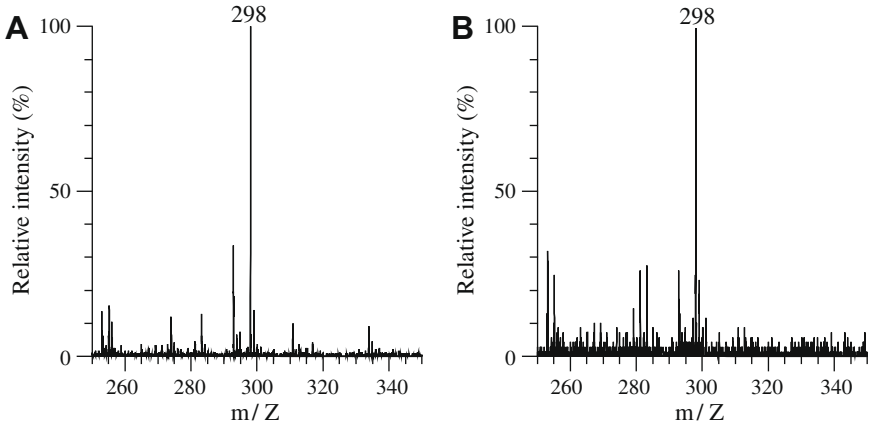

Figure 2. Negative ion electrospray ionization time of flight mass spectrometry (ESI-TOF/MS, MicroTOF, Bruker) spectra of **1** and **2**. The sample isolated by normal-phase HPLC was directly infused into the MS system using a syringe pump.

8-OxodGuo (1 mM) in 250 mM potassium phosphate buffer (pH 7.4) was bubbled by 0.5 mL/min NO in an open vessel in open air at ambient temperature for 4 min. A normal-phase HPLC chromatogram of the reaction mixture is shown in Figure 1. Two product peaks with retention times of 8.1 min and 8.8 min referred to as **1** and **2**, respectively, were detected on the chromatogram in addition to unreacted 8-oxodGuo and formed nitrite (NO_2^-). The products **1** and **2** exhibited similar UV spectra with a shoulder near 230 nm (Fig. 1, insets). The products were isolated by normal-phase HPLC and subjected to mass spectrometry. Negative-ion ESI-TOF/MS for both **1** and **2** showed a signal at *m/z* 298, which was 16 mass units greater than that of 8-oxodGuo, as shown in Figure 2. It has been reported that dSph is formed as the products of 16 mass units greater than that of starting 8-oxodGuo by several oxidation systems including ONOOH, $KHSO_5/CoCl_2$, and HOCl.[17–19] To compare the products in the present system to dSph, an 8-oxodGuo/HOCl system, in which almost exclusive formation of dSph has been reported, was conducted. 1 mM 8-oxodGuo in 250 mM potassium phosphate buffer (pH 7.4) was incubated with 1 mM NaOCl at room temperature for 10 min. Normal-phase HPLC chromatogram of the reaction mixture showed two major product peaks, which had identical HPLC retention times, UV spectra, and ESI-TOF/MS spectra to **1** and **2** (data not shown). From the results, **1** and **2** were identified the diastereomers of dSph (Fig. 3). Figure 4 shows the time course of the concentrations of formed dSph and unreacted 8-oxodGuo after 8-oxodGuo was bubbled by NO gas up to 4 min at room temperature. The consumption of 8-oxodGuo and the yield of dSph increased as the bubbling time increased. At 4 min, the yield of dSph was 0.52 mM while 0.66 mM of 8-oxodGuo was consumed.[20] Thus, the yield of dSph was 78% relative to

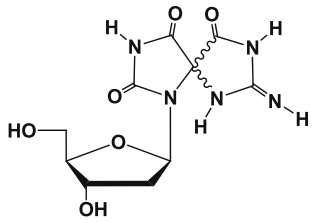

Figure 3. Structure of **1** and **2**, diastereomers of spiroiminodihydantoin deoxyribonucleoside (dSph).

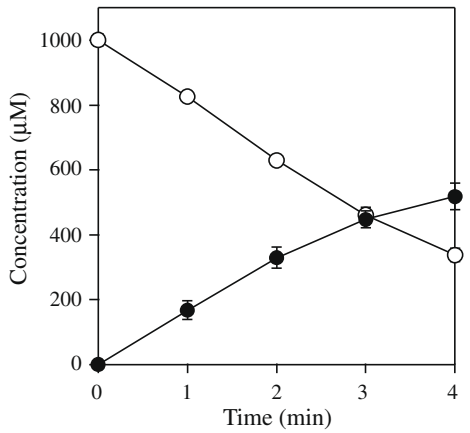

Figure 4. The time course of the concentration changes in 8-oxodGuo (open circle) and dSph (closed circle). A stirring solution mixture (1 mL) of 8-oxodGuo (1 mM) with 250 mM potassium phosphate buffer (pH 7.4) in a 6.5 mL open vial was bubbled by 0.5 mL/min NO at room temperature. The nucleoside concentrations were determined by normal-phase HPLC analysis detected at 245 nm for dSph and 300 nm for 8-oxodGuo. Means ± SD (n = 3) are shown.

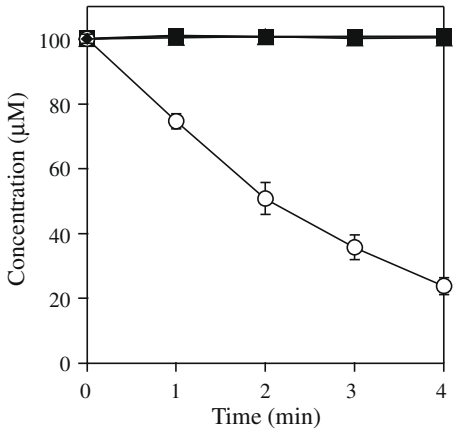

Figure 5. The time course of the concentration changes in 8-oxodGuo (open circle), dCyd (closed square), dGuo (closed circle), dThd (closed triangle), and dAdo (closed rhombus). A stirring solution mixture (1 mL) of 8-oxodGuo, dCyd, dGuo, dThd, and dAdo (100 μM each) with 225 mM potassium phosphate buffer (pH 7.4) in the 6.5 mL open vial was bubbled by 0.5 mL/min NO at room temperature. The nucleoside concentrations were determined by reversed-phase HPLC analysis detected at 260 nm. An Inertsil ODS column of 4.6 × 250 mm and particle size 5 μm (GL Sciences) was used. The eluent was 20 mM triethylammonium acetate (pH 7.0) containing methanol. The methanol concentration was increased from 0% to 50% for 30 min with a linear gradient mode. The column temperature was 40 °C and the flow rate 1 mL/min. Means ± SD (n = 3) are shown.

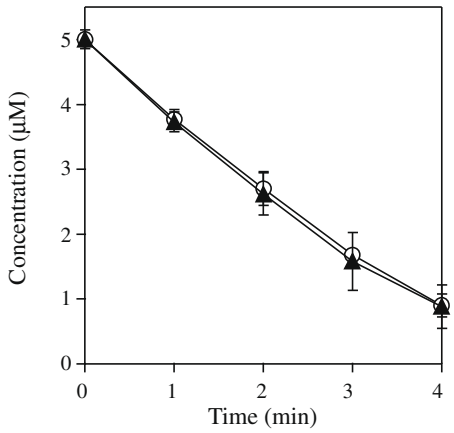

Figure 6. The time course of the concentration changes in 8-oxodGuo in the absence (open circle) and presence (closed triangle) of dGuo. A stirring solution mixture (1 mL) of 8-oxodGuo, (5 μM) with 250 mM potassium phosphate buffer (pH 7.4) in a 6.5 mL open vial was bubbled by 0.5 mL/min NO at room temperature in the absence and presence of 5 mM dGuo. The nucleoside concentrations were determined by reversed-phase HPLC analysis detected at 300 nm. Means ± SD (n = 3) are shown.

the consumed 8-oxodGuo. The pH was 6.9 and the formed NO_2^- concentration was 55 mM.[20] A subsequent incubation of the reaction mixture at 37 °C for 7 days showed no decrease of the dSph concentration, suggesting that dSph is stable under physiological conditions. To compare the reactivity of 8-oxodGuo to other deoxynucleosides, a nucleoside mixture containing 8-oxodGuo, 2′-deoxyguanosine (dGuo), 2′-deoxyadenosine (dAdo) 2′-deoxycytidine (dCyd), and 2′-deoxythymidine (dThd) (100 μM each) in 225 mM potassium phosphate buffer (pH 7.4) was bubbled by NO gas. The nucleoside concentrations were determined by reversed-phase HPLC analysis detected at 260 nm. Figure 5 shows the time course of concentration changes of the nucleosides up to 4 min. At 4 min, the pH was 6.9 and the NO_2^- concentration was 52 mM. Although the concentration of 8-oxodGuo decreased greatly with increasing bubbling time, the concentrations of the other four nucleosides, dGuo, dAdo, dCyd, and dThd, were unchanged. To compare the reactivity of 8-oxodGuo with that of dGuo, we examined a possible inhibitory effect of dGuo (5 mM) on the reaction of 8-oxodGuo (5 μM) with NO bubbling. Figure 6 shows a plot of the concentrations of unreacted 8-oxodGuo as a function of the NO bubbling time in the absence and presence of dGuo. At 4 min, the pH was 6.9 and the NO_2^- concentration was 54 mM. The 1000-fold excess of dGuo did not inhibit the reaction of 8-oxodGuo by NO.

Possible mechanisms of the formation of dSph from 8-oxodGuo by NO under aerobic conditions are shown in Figure 7. N_2O_3 is a nitrosating reagent, and can act as a carrier of NO^+.[4,5] NO^+ may combine to the double bond between C4 and C5 of 8-oxodGuo forming a cation adduct. This cation can then be hydrolyzed to 5-hydroxy-8-oxodGuo, which is proposed as an intermediate for dSph formation by other oxidation systems.[17,18] 5-Hydroxy-8-oxodGuo then rearranges via an acyl shift, giving rise to dSph. N_2O_3 is also a relatively mild oxidant with the oxidation potential of 0.7 V.[24] Since the one-electron oxidation potential of 8-oxodGuo is reported to be 0.74 V versus NHE at pH 7,[14] N_2O_3 may oxidize 8-oxodGuo generating a radical cation, 8-oxodGuo^{+}. This cation then is hydrolyzed to 5-hydroxy-8-oxodGuo and the subsequent rearrangement generates dSph. In addition to N_2O_3, we should pay attention to nitrogen dioxide, NO_2. The autoxidation of NO generates NO_2 which then reacts with NO to form N_2O_3 both in gas-phase and aqueous solution.[25,26]

$$2NO + O_2 \rightarrow 2NO_2$$
$$NO_2 + NO \leftrightarrow N_2O_3$$

It is reported that NO_2 reacts with 8-oxodGuo.[27] The oxidation potential of NO_2 is 1.04 V versus NHE.[28] Thus, NO_2 can oxidize 8-oxodGuo generating 8-oxodGuo^{+}. We cannot exclude the possibility that a small amount of NO_2 formed in the present system in gas-phase or in solution reacts with 8-oxodGuo generating dSph.

It has been reported that when dSph containing templates are replicated by Klenow fragment and Klenow fragment exo$^-$, the replication is strongly blocked at the dSph moiety.[29,30] When the replication occurs over the lesions, the DNA polymerase inserts predominatly adenine and guanine opposite the lesions, which would result in G to T and G to C mutations, respectively. Several repair enzymes can excise the dSph moiety, but their efficiencies are relatively low.[31,32] Recently, dSph moiety was detected in vivo using *Escherichia coli*.[33] Its levels largely increased in Nei (endonuclease VIII) deficient mutant after Cr(VI) treatments. Nei has high affinity to a substituted hydantoin, 5-hydroxy-5-methylhydantoin.[34] These results suggest that dSph is an important genotoxic lesion generated from 8-oxodGuo. Various oxidants such as ONOOH and HOCl generate dSph from 8-oxodGuo. The present study shows that NO can also generate dSph from 8-oxodGuo under aerobic conditions.

In conclusion, we found that 8-oxodGuo reacts with NO under aerobic conditions, generating diastereomers of dSph as major

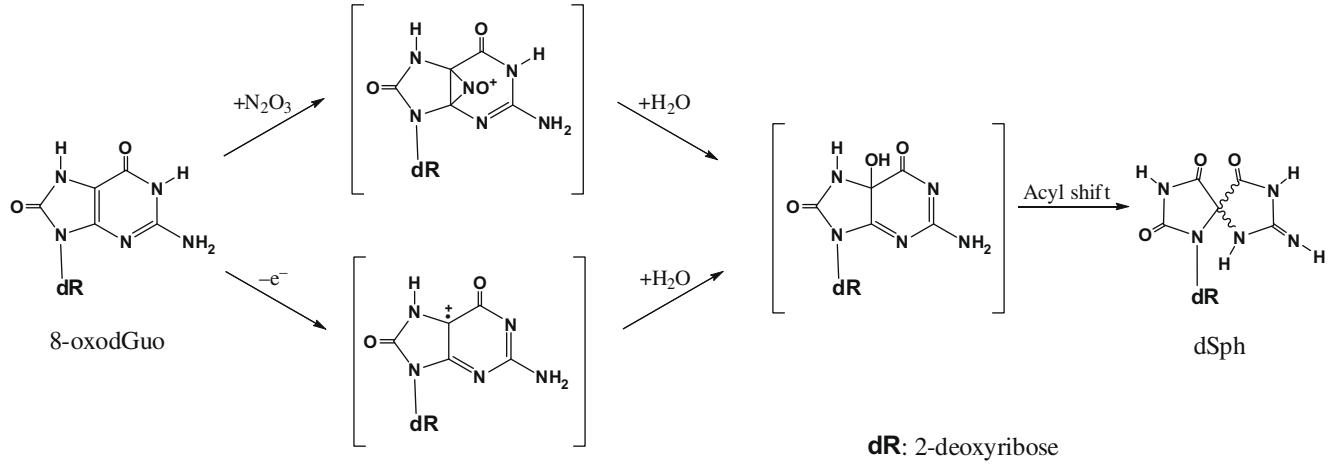

Figure 7. Proposed reaction mechanism for the formation of dSph from 8-oxodGuo with NO under aerobic conditions.

products. It is unclear whether physiological concentrations of NO and O_2 generate dSph from 8-oxodGuo or not. If the reaction mechanisms proposed in this paper, however, involved, the formation of dSph by low concentrations of NO and O_2 would be possible. The present result suggests that dSph may form from 8-oxodGuo in DNA by NO generated by nitric oxide synthases in humans.

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