Role of Nitrite on Nitration of 2′-Deoxyguanosine by Nitryl Chloride

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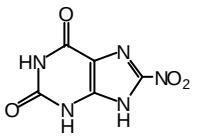
Nitryl chloride and peroxynitrite are reactive nitrogen species generated by activated phagocytes against invading pathogens during infections and inflammation. In our previous report, formation of 8-nitroxanthine and 8-nitroguanine was observed in reaction of 2′-deoxyguanosine or calf thymus DNA with nitryl chloride generated by mixing hypochlorous acid (HOCl) with nitrite (NO_2^-). The present study investigates factors controlling the yields of 8-nitroxanthine and 8-nitroguanine formation in nitration of 2′-deoxyguanosine by nitryl chloride. We found that the yields of 8-nitroxanthine and 8-nitroguanine in reaction of 2′-deoxyguanosine with nitryl chloride were highly dependent on the ratio of NO_2^- versus HOCl concentration. The yields of 8-nitroxanthine and 8-nitroguanine reached a plateau when the ratio of NO_2^- versus HOCl concentration was higher than 2. A possible mechanism was postulated to explain this observation. While 8-nitroguanine is not stable in the presence of peroxynitrite, 8-nitroxanthine is sensitive to HOCl. The stability of these two nitrated adducts might be a factor on their final yields in this reaction. Since HOCl is produced by neutrophils at sites of inflammation where the level of NO_2^- is elevated, it is conceivable that nitryl chloride contributes to DNA base nitration *in vivo*, forming 8-nitroxanthine and 8-nitroguanine.

INTRODUCTION

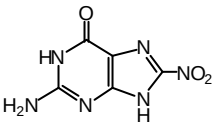
DNA damage caused by peroxynitrite (ONO_2^-), formed by the rapid reaction of superoxide and nitric oxide produced in inflamed tissues,[1-3] is believed to play an important role in the multistage carcinogenesis associated with chronic infections and inflammation.[4,5] Peroxynitrite reacts with DNA mainly by modifying the guanine base, forming xanthine, 8-nitroguanine (8NG) and 8-oxoguanine.[6-8] Formation of apurinic sites induced GC to TA transversion mutations,[9] the same mutations induced by peroxynitrite.[4] It is well documented that peroxynitrite can be formed under oxidative stress in several disease states[10-14] as well as from cigarette smoking.[15,16] Another endogenous nitrating species is nitryl chloride (NO_2Cl) or nitrogen dioxide radical ($NO_2^•$) produced in activated phagocytes promoted by chronic infections and inflammation.[17,18] It is believed that myeloperoxidase uses hydrogen peroxide (H_2O_2) generated by neutrophils to oxidize the chloride ion and produce hypochlorous acid (HOCl), a bacteridal toxin. This toxin reacts with nitrite (NO_2^-), the primary metabolite of NO•, forming NO_2Cl that is capable of nitrating guanine[19] and tyrosine.[20] Nitryl chloride also leads to formation of chlorinated tyrosine and dityrosine,[21] oxidation and nitration of human low-density lipoprotein (LDL), and induced lipid peroxidation.[21,22] A $NO_2^•$ radical may be formed via the one-electron oxidation of NO_2^- by compound I or compound II of myeloperoxidase.[17,18] In reaction of the myeloperoxidase/H_2O_2/Cl^-/NO_2^- system with 2′-deoxyguanosine (dG), 8NG and 8-nitro-2′-deoxyguanosine were formed.[19]

In our previous report, formation of 8-nitroxanthine (8NX) and 8NG (Scheme I) was observed in reaction of dG or calf thymus DNA with nitryl chloride generated by mixing NO_2^- with HOCl.[23] This study examines factors important to 8NX and 8NG formation, such as reaction time, ratio of NO_2^- versus HOCl, and concentrations of nitryl chloride and 2′-

Scheme I Structures of 8-nitroxanthine and 8-nitroguanine



8-nitroxanthine (8NX)



8-nitroguanine (8NG)

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Abbreviations: N_2O_4, dinitrogen tetroxide; dG, 2′-deoxyguanosine; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; NO_2^-, nitrite; $NO_2^•$, nitrogen dioxide radical; 8NG, 8-nitroguanine; NO_2^+, nitronium ion; 8NX, 8-nitroxanthine; NO_2Cl, nitryl chloride; ONO_2^-, peroxynitrite; $NaNO_2$, sodium nitrite.

deoxyguanosine. Since both 8NX and 8NG are rapidly removed from DNA once they are formed, these two nitrated DNA bases might be important sources of apurinic lesions in DNA derived from reactive nitrogen species produced *in vivo* or from exogenous sources. Therefore, 8NX and 8NG might be used as biomarkers for DNA nitration to investigate their role in cancer development.

RESULTS AND DISCUSSION

Nitration of xanthine with peroxynitrite or NO_2^-/HOCl mixture

Reactive nitrogen species, including peroxynitrite and nitryl chloride, are capable of nitrating protein tyrosine, leading to the formation of 3-nitrotyrosine in human plateletes. Peroxynitrite has been shown to be a better nitrating agent than nitryl chloride in this system.[24,25] In reaction of xanthine with various amounts of peroxynitrite, reversed phase HPLC analysis revealed that formation of 8NX reached a maximum when the concentration of peroxynitrite was 10 times in excess of xanthine and it declined with a large excess (50 times) of peroxynitrite. When an equal concentration of HOCl and $NaNO_2$ was mixed in the presence of xanthine, the yield of 8NX was optimum at the concentration of nitryl chloride being 5 times of xanthine and it decreased with increasing concentration of nitryl chloride. No 8NX was detected in the presence of 50 times of nitryl chloride. Interestingly, excess NO_2^- appeared to increase the yield of 8NX. When the concentration of NO_2^- was twice that of HOCl, the yields of 8NX increase with increasing concentrations of HOCl. The nitrating ability for the NO_2^-/HOCl mixture could be higher than peroxynitrite in the presence of excess nitrite. The yield of 8NX (51%) in the reaction with the concentrations of NO_2^- and HOCl being 100 and 50 times in excess of xanthine, respectively, exceeded that (17%) in reaction with a 50 molar equivalent of peroxynitrite (Fig. 1). The reaction yields of 8NX appear to correlate with the stability of 8NX in the presence of excess reactive species. Details will be discussed in a later section.

Effect of incubation time, NO_2^-, nitryl chloride, and dG concentrations on 8NX and 8NG formation in reaction of dG with NO_2^-/HOCl mixture

Our earlier work showed formation of 8NX and 8NG in reaction of dG or DNA with nitryl chloride generated from mixing NO_2^- with HOCl.[23] In this study, a time-dependent formation of 8NX and 8NG from reaction of dG with nitryl chloride was analyzed after quenching with methionine at different time intervals up to 2 hours. Levels of 8NX and 8NG were not a function of the incubation time. They reached the maximum at around 10 min and declined during prolonged incubation and stabilized (Fig. 2). These results indicated that nitrated adducts formed can be decomposed by the excess reagents remaining in the reaction mixture, which was evidenced by examining their stability as described in a later section. No significant change in yields of 8NX and 8NG was observed after 1 h, suggesting that the excess HOCl and NO_2^-

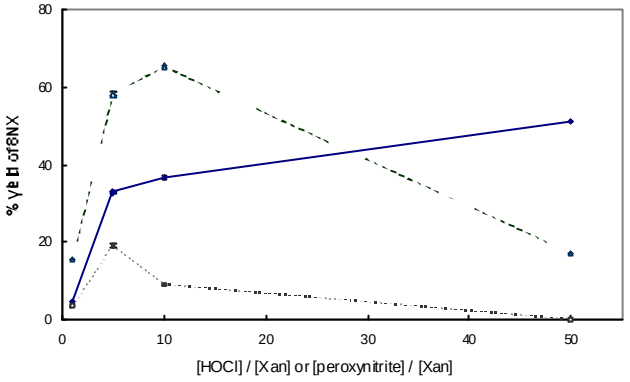


Fig. 1. Effect of NO_2^-/HOCl ratio on formation of 8NX in xanthine reaction with various amounts of nitryl chloride or peroxynitrite. $[NO_2^-]/[HOCl] = 2$ (♦); $[NO_2^-]/[HOCl] = 1$ (□); peroxynitrite (Δ). Results are expressed as means of at least duplicate experiments.

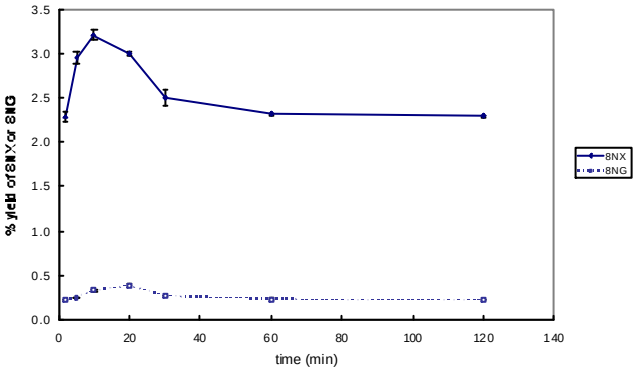
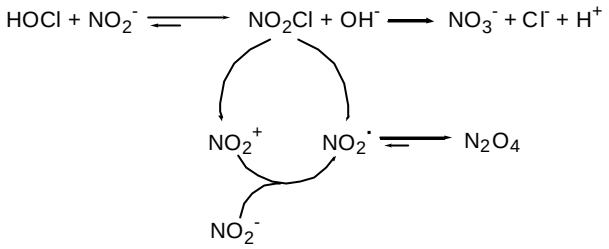


Fig. 2. Effect of incubation time on formation of 8NX (♦) and 8NG (□) in reaction of dG with nitryl chloride. To a solution containing dG (2.0 mM) in 0.1 M potassium phosphate buffer (pH 7.0) and NO_2^- (200 mM) was added NaOCl (100 mM) at room temperature, quenched with methionine (100 mM) and analyzed by HPLC as described in Materials and Methods. Data are expressed as means of at least duplicate experiments.

were converted to NO_3^- and Cl^-, the stable end products (Scheme II).

Scheme II Formation of nitryl chloride from HOCl and NO_2^-



The effect of the relative concentration of NO_2^- versus HOCl on the yields of 8NX and 8NG was investigated. Since the reactivity of nucleoside (dG) with the NO_2^-/HOCl mixture was much lower than the nucleobases, xanthine or guanine,[23] HOCl in the concentration of 50 times that of dG was used, while concentrations of nitrite were varied. In reaction of the HOCl/NO_2^- mixture with dG, the yield of 8NX or 8NG reached a plateau when it was plotted as a function of the ratio of [NO_2^-]/[HOCl]. A ratio of [NO_2^-]/[HOCl] higher than 2 did not significantly affect the yields of the nitrated adducts 8NX and 8NG (Fig. 3). In reaction of dG with the concentration of HOCl being twice that of NO_2^- ([NO_2^-]/[HOCl] = 0.5), no 8NX or 8NG was detected. It therefore appears to be important that the concentration of NO_2^- exceeds HOCl to form nitrated bases. Hypochlorous acid reacts with NO_2^- at physiological pH with a second-order rate constant of 7.4×10^3 M^{-1} s^{-1}.[20] It is conceivable that formation of nitryl chloride by mixing equal amounts of $NaNO_2$ and HOCl is not very efficient. Accordingly, the unreacted HOCl is responsible for low yield of 8NX when the ratio of [NO_2^-] versus [HOCl] is less than 2. However, prior to the proof of this postulation, the concentration of nitryl chloride stated in the text denotes the concentration of NO_2^- or HOCl, whichever is smaller, assuming their complete conversion to nitryl chloride.

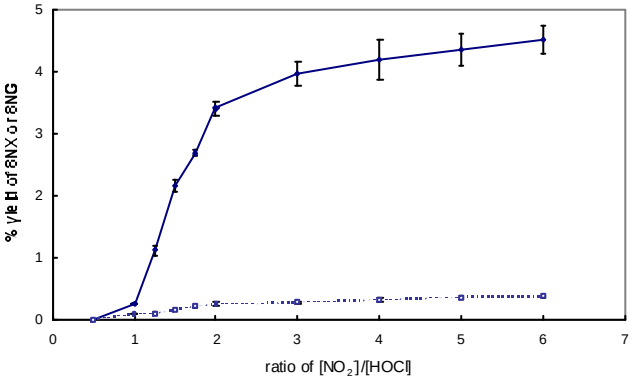


Fig. 3. Effect of [NO_2^-]/[HOCl] ratio on formation of 8NX (♦) and 8NG (□) in reaction of dG (2.0 mM) with nitryl chloride. To a solution containing dG (2.0 mM) with varying concentrations of NO_2^- in 0.1 M potassium phosphate buffer (pH 7.0) was added NaOCl (0.1 M) with stirring at room temperature for 5 min. Results are expressed as means of triplicate experiments.

Another possibility for NO_2^- affecting yield of nitration is the involvement of NO_2^+ as the nitrating agent. Nitryl chloride can be converted to the more reactive nitrating species, such as nitrogen dioxide radical ($NO_2^{\bullet}$) or nitronium ion (NO_2^+) (Scheme II). Since HOCl is a poor one-electron oxidizing agent, but a strong two-electron oxidant with the reduction potential being + 0.2 V and + 1.1 V, respectively,[26] oxidation of NO_2^- to NO_2^+ may be favorable over its conversion to $NO_2^{\bullet}$.[20] In the presence of excess NO_2^-, reduction of NO_2^+ from NO_2^- stoichiometrically results in two $NO_2^{\bullet}$ ($NO_2^+ + NO_2^- \rightarrow 2\ NO_2^{\bullet}$). It is also feasible that the highly unstable NO_2^+ reacts with excess NO_2^- forming the relatively stable dinitrogen tetroxide (N_2O_4) with a second-order rate constant of 4.5×10^8 M^{-1} s^{-1}, the nitrating agent that can slowly decompose into two $NO_2^{\bullet}$ ($k = 6.9\times 10^3$ s^{-1})[27] (Scheme II). Reaction of $NO_2^{\bullet}$ with tyrosyl radicals in proteins forming 3-nitrotyrosine was in a near diffusion controlled rate ($k = 3\times 10^9$ M^{-1} s^{-1}).[28] Combination of $NO_2^{\bullet}$ with the dG(-H)$^{\bullet}$ or the diazonium ion radical intermediate **I**[23] results in formation of 8NG and 8NX, respectively, and thus accounts for the increase in the yield of nitration due to increase of the NO_2^- concentration. So far, the rate constants in these bimolecular pathways have not been investigated.

The yields of 8NX and 8NG also depend on the concentration of the nitrating species relative to that of dG. Using the system giving the optimum yield of nitration (i.e. [NO_2^-]/[HOCl] = 2) with a fixed concentration of dG (0.96 mM), the yields of 8NX increased with increasing nitryl chloride concentrations, i.e. the concentration of HOCl, in a dose-dependent manner with a rapid rise at high concentrations. Conversely, the yields of 8NG increased at lower concentrations of the nitryl chloride to a certain extent (up to 0.29% with [NO_2^-]/[HOCl]/[dG] = 25:12.5:1) and remained constant at higher concentrations. At HOCl concentrations ≤ 12 mM, the yields of 8NG exceeded those of 8NX. The situation was reversed with HOCl concentrations ≥ 24 mM, in which the formation of 8NX was much more favored over 8NG (Fig. 4). Thus, the preference in formation of 8NX or 8NG is a function of the concentration of the nitrating species.

We also examined the effect of dG concentration in the

yields of 8NX and 8NG since the concentrations of DNA *in vivo* vary within cellular compartments such as the nucleus or mitochondria. With a fixed concentration of nitryl chloride generated from $[NO_2^-]/[HOCl] = 2$, the yields of 8NX and 8NG increased linearly with increasing dG concentration ranging from 0.36 to 2.0 mM (Fig. 5). The slope of this graph showed that the yields of 8NX increased 1.66% per millimolar dG while the yields of 8NG remained constant, but the sum of the two nitrated adducts increased 0.04% per millimolar HOCl according to Fig. 4. It is probably due to the fact that nitryl chloride is a reactive species, presumably leading to formation of the non-reactive nitrite and chloride ions (Scheme II). Accordingly, the steady-state level of nitryl chloride is much lower than that from the presumed stoichiometric formation from NO_2^- and HOCl. Collectively, these results indicate that the concentration of dG is the dominating factor in the yield of nitration over the concentration of nitryl chloride and the latter determines which nitrated adduct, 8NX or 8NG, is formed preferentially over the other. It is therefore highly conceivable that both 8NX and 8NG are formed in the vicinity of inflamed tissues *in vivo*.

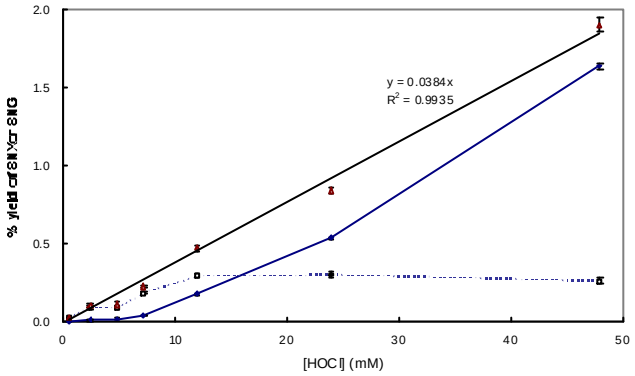


Fig. 4. Formation of 8NX (♦) and 8NG (□) in reaction of dG with various amounts of nitryl chloride. To a solution containing 2′-dG (0.96 mM) in 0.1 M potassium phosphate buffer (pH 7.0) was added with varying concentrations of NO_2^- and HOCl ($[NO_2^-]/[HOCl] = 2$) with stirring at room temperature for 5 min, followed by HPLC analysis as described in Materials and Methods. The sum of 8NX and 8NG (Δ) are expressed by linear regression. Data are presented as means of at least duplicate experiments.

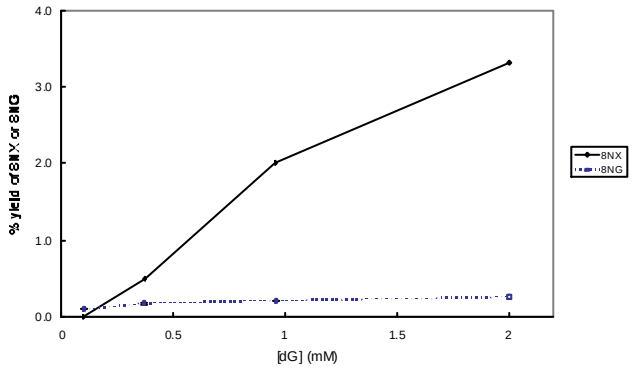


Fig. 5. The relative formation of 8NX (♦) vs. 8NG (□) was a function of dG concentration in its reaction with nitryl chloride. The ratio of $[NO_2^-]/[HOCl]/[dG]$ was 100/50/1. Results are expressed as means of at least duplicate experiments.

The *in vivo* concentrations of NO_2^- have been reported to be at submicromolar levels in plasma,[29] 100 μM in respiratory tract epithelial cells,[30] and up to 210 μM in saliva[31] in healthy individuals. Dietary intake of nitrate (NO_3^-)-rich foods can cause a dramatic increase in the level of NO_2^- in the mouth which is then ingested to the stomach.[32] These levels are elevated during the inflammatory processes. On the other hand, the concentration of HOCl can reach 100 μM in the vicinity of activated neutrophils.[33] Therefore, nitryl chloride generated by mixing HOCl and NO_2^- is likely to play an important role in nonenzymatic DNA nitrative damage. Nitrite inhibits the killing of *E. coli* by HOCl by removing these toxic nitrating species and thus protects the cells,[34,35] which suggests that nitration of biomolecules in *E. coli* does not endanger the immediate survival of the cells. Whether accumulated nitrated biomolecules are related to cancer formation in the long term in higher organisms remains unanswered.

Stability of 8NX and 8NG in the presence of reactive species

In reactions with xanthine or dG, levels of 8NX and/or 8NG decreased substantially when a large excess of nitryl chloride was generated by mixing equal concentrations of HOCl with NO_2^- (see Figs. 1 and 3). Therefore, the stability of 8NX and 8NG in the presence of excess reactive species was examined. When pure 8NX was mixed with a 25 times molar excess of nitryl chloride generated by mixing equal amounts of HOCl with NO_2^-, 8NX was completely decomposed as supported by the reversed phase HPLC analysis. On the other hand, 63% of 8NX survived in a mixture of NO_2^- and HOCl 50 and 25 times in excess, respectively (Table 1). HOCl also effectively destroyed 8NX, as evidenced by the complete decomposition of 8NX when HOCl of 25 times in excess was added to a solution of isolated 8NX at neutral pH. It is conceivable that mixing equal concentrations of NO_2^- and HOCl does not form nitryl chloride efficiently and that

Table 1. Percentage Survival Rate of 8NX or 8NG Reaction with Excess Reactive Species

Reaction	yield of 8NX & 8NG[a]
8NX + $NaNO_2$ + HOCl (1:25:25)	0%
8NX + $NaNO_2$ + HOCl (1:50:25)	63%
8NX + HOCl (1:25)	0%
8NX + ONO_2^- (1:25)	69%
8NX + H_2O_2 (1:25)	95%
8NG + $NaNO_2$ + HOCl (1:25:25)	1.8% 94%
8NG + $NaNO_2$ + HOCl (1:50:25)	2.4% 95%
8NG + HOCl (1:25)	N.D.[b] 98%
8NG + ONO_2^- (1:25)	N.D. 0.3%
8NG + H_2O_2 (1:25)	N.D. 97%

[a] Values are means of at least duplicated experiments.
[b] Not detectable.

the unreacted HOCl is responsible for the decreased yield of 8NX. When the ratio of concentration for NO_2^- versus HOCl was greater than 2 in reactions of dG with mixtures of NO_2^- and HOCl (Fig. 3), excess NO_2^- led the equilibrium toward nitryl chloride formation (Scheme II) and there was a negligible amount of free HOCl. These observations also rationalize the fact that the yield of 8NX in xanthine reaction with the NO_2^-/HOCl mixture was much higher when the amount of NO_2^- exceeded HOCl (Fig. 1). Collectively, these results suggest that the presence of excess NO_2^- increases the flux of the nitrating species and protects 8NX from decomposition, although the decomposition products are not identified at this stage.

In the presence of other reactive species, 8NX was somewhat stable with peroxynitrite (69% survived) but it merely decomposed (95% remained) with H_2O_2, a biologically important oxidant. On the other hand, 8NG was stable in the presence of H_2O_2 or HOCl and it was not affected by nitryl chloride generated from HOCl with equal or excess NO_2^-. However, 8NG almost completely decomposed with the addition of peroxynitrite (Table 1). This result was consistent with previous reports.[36,37]

Since 8NG is not repaired by the enzyme formamidopyrimidine glycosylase[36,38] and that formation of 8NX and 8NG can result in the mutagenic apurinic sites in DNA, they might be important DNA lesions. Because of the labile glycosidic linkages of 8NX and 8NG in DNA, the existence of these two adducts in biological fluids is highly possible. If they are present in high enough levels for detection, measurement of 8NX and 8NG in tissue DNA and in biological fluids should give insights into the roles of these adducts in carcinogenesis. These nitrated DNA adducts should be useful biomarkers for endogenous nitration of DNA. They might also be used to examine the protective effect of antioxidants against reactive nitrogen species-induced nitration of DNA[39] and thus contribute to understanding the correlation between these nitrated DNA adducts and inflammation-induced cancer and other diseases.

EXPERIMENTAL SECTION

Materials

2′-Deoxyguanosine was from Sigma Chemical Co. (St. Louis, MO). Guanine, sodium nitrite, and sodium hypochloride (NaOCl) were obtained from Aldrich Chemical Co. (Milwaukee, WI). All reagents are reagent grade or above. Hydrogen peroxide was purchased from Acros Organic Chemical Co. (Geel, Belgium) and quantified based on the absorbance at 240 nm ($\varepsilon = 43.6\ M^{-1}cm^{-1}$).[40] Peroxynitrite was synthesized according to the previously described procedures using iso-amylnitrite and hydrogen peroxide[41] and was stored at -80 °C. The concentration of peroxynitrite was determined by the absorbance at 302 nm in 1 N NaOH ($\varepsilon = 1670\ M^{-1}cm^{-1}$).[42] The concentration of NaOCl was determined by the absorbance at 292 nm (pH 12, $\varepsilon = 350\ M^{-1}cm^{-1}$).[43] Standard 8NG and 8NX were synthesized as described previously.[23]

HPLC Chromatography

HPLC chromatography was performed by a Hitachi L-7000 pump system with D-7000 interface, a Rheodyne injector, and a L-7450A photodiode array (PDA) detector. **(1) System 1.** A Prodigy ODS (3) 250 mm × 4.6 mm, 5 μm column (Phenomenex, Torrance, CA) was used with the following isocratic conditions: 50 mM ammonium formate buffer (pH = 4.0) at a flow rate of 1.0 mL/min. **(2) System 2.** A Prodigy ODS (3) 250 mm × 4.6 mm, 5 μm column was used with the following isocratic conditions: 50 mM ammonium formate (pH = 5.5) buffer at a flow rate of 1.0 mL/min.

Reaction of Xanthine with Peroxynitrite or NO_2^-/HOCl Mixture

A. with peroxynitrite

To a solution of xanthine (0.34 mg, 2.25μmol) in 0.5 N HCl (0.2 mL) was added 11 μL of peroxynitrite (200 mM in 0.67 N NaOH and 50 mM ammonium formate) and 989 μL of 0.67 N NaOH containing 50 mM ammonium formate with vigorous stirring at room temperature for 2 min. Final pH of the reaction was 7.0 with a final volume of 1.2 mL. For reactions with higher amounts of peroxynitrite, the volume of peroxynitrite was increased and that for the NaOH solution

was decreased accordingly with the final volume unchanged.

B. with NO_2^-/HOCl mixture

To a solution of xanthine (0.34 mg, 2.25 μmol) in 0.5 N HCl (0.2 mL) was added 828 μL of a solution of $NaNO_2$ (3.7 mM in 0.4 M potassium phosphate buffer, pH 12.8), followed by 172 μL of a solution of NaOCl (13.1 mM in 0.4 M potassium phosphate buffer, pH 12.8) with stirring. Final pH of the reaction mixtures was 7.0. For reactions with higher amounts of nitryl chloride, the concentrations of $NaNO_2$ and NaOCl were increased accordingly with a final volume of 1.2 mL. A 120 μL aliquot of the reaction mixture was analyzed by HPLC using system 1 at 376 nm.

Reaction of dG Reaction with Nitryl Chloride

Typically, to a solution containing 2′-dG and $NaNO_2$ dissolved in 0.5 mL of potassium phosphate buffer (final concentration 0.1 M, pH 7.0) was added a solution containing NaOCl (0.24 M) with stirring at room temperature for 5 min. The reaction mixture was adjusted to pH 4.0 or 5.5 and an aliquot of the reaction mixture was analyzed by HPLC using system 1 at 376 nm or using system 2 at 393 nm.

Reaction of 8NX or 8NG with Excess Nitryl Chloride

To a solution containing 8NX or 8NG (43.5 μg, 0.22 μmol) in 1.0 mL of 0.2 M potassium phosphate buffer (pH 7.0) was added 87 μL of a solution of $NaNO_2$ (63 mM or 126 mM) and 23 μL of a solution of NaOCl (0.24 M) with stirring. Final pH of the reaction mixture was 7.0. The entire reaction mixture was analyzed by HPLC using system 2 at 376 and 393 nm.

Reaction of 8NX or 8NG with HOCl

To a solution containing 8NX or 8NG (43.5 μg, 0.22 μmol) in 1.087 mL of 0.2 M potassium phosphate buffer (pH 7.0) was added 23 μL of a solution of NaOCl (0.24 M) with stirring. Final pH of the reaction mixture was 7.0. The entire reaction mixture was analyzed by HPLC using system 2 at 376 and 393 nm.

Reaction of 8NX or 8NG with Peroxynitrite

To a solution containing 8NX or 8NG (43.5 μg, 0.22 μmol) in 1.083 mL of 0.2 M potassium phosphate buffer (pH 7.0) was added 27 μL of peroxynitrite (200 mM in 0.67 N NaOH) with stirring. Final pH of the reaction mixture was 7.0. The entire reaction mixture was analyzed by HPLC using system 2 at 376 and 393 nm.

Reaction of 8NX or 8NG with H_2O_2

To a solution containing 8NX or 8NG (43.5 μg, 0.22 μmol) in 1.083 mL of 0.2 M potassium phosphate buffer (pH 7.0) was added 27 μL of H_2O_2 (200 mM) with stirring. Final pH of the reaction mixture was 7.0. The entire reaction mixture was analyzed by HPLC using system 2 at 376 and 393 nm.

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Key Words

DNA; 2′-Deoxyguanosine; Hypochlorous acid; Nitration; Nitrite; 8-Nitroguanine; 8-Nitroxanthine; Nitryl chloride; Peroxynitrite.

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