Nicotine-modulated formation of spiroiminodihydantoin nucleoside via 8-oxo-7,8-dihydro-2′-deoxyguanosine in 2′-deoxyguanosine–hypochlorous acid reaction

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Received 14 January 2002; revised 15 February 2002; accepted 18 February 2002

First published online 6 March 2002

Edited by Barry Halliwell

Abstract Hypochlorous acid (HOCl) is generated by myeloperoxidase of activated neutrophils which kill invading microorganisms, but also cause DNA damage in inflamed tissues. We report here that spiroiminodihydantoin nucleoside (dS), a further oxidized product of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), is formed, in addition to 8-chloro-2′-deoxyguanosine and 8-oxo-dG, by reaction of 2′-deoxyguanosine with HOCl. Presence of low concentrations of nicotine significantly enhanced the yields of these HOCl-modified nucleosides. Our results imply that nicotine may enhance genotoxicity and tissue damage caused by neutrophil activation. dS may also serve as a new biomarker for oxidative DNA damage induced by oxidants such as HOCl.

Key words: Spiroiminodihydantoin; 8-Oxo-7,8-dihydro-2′-deoxyguanosine; 8-Chloro-2′-deoxyguanosine; 2′-Deoxyguanosine; Hypochlorous acid

1. Introduction

Myeloperoxidase is the most abundant protein in neutrophils, accounting for up to 5% of their dry weight [1–3]. This enzyme generates hypochlorous acid (HOCl) as an endogenous product of the respiratory burst from H_2O_2 and Cl^- [4,5]. HOCl generated by myeloperoxidase is of central importance in host defense mechanisms [3]. However, the HOCl formed also has potential to harm normal tissue and contribute to inflammatory injury.

HOCl has been reported to react with nucleic acid bases to form various compounds, including *N*-chloramine, 5-chloro, 5-chloro-6-hydroxy and 5-hydroxy derivatives of cytosine, a 5-chloro-6-hydroxy derivative and thymine glycol from thymine and *N*-chloramine and 8-chloro derivatives and parabanic acid from adenine [6–11]. However, studies of the reaction of guanine with HOCl have been limited, probably due to its complexity [7,9]. Our group recently reported that HOCl reacts with 2′-deoxyguanosine (dG) to form 8-chloro-dG (8-Cl-dG) and 8-oxo-7,8-dihydro-dG (8-oxo-dG) [12]. Moreover, the presence of physiologically relevant concentrations of tertiary amines such as nicotine dramatically increased yields of both 8-Cl-dG and 8-oxo-dG in the dG–HOCl reaction. We have proposed the following mechanisms for the formation of 8-Cl-dG and 8-oxo-dG by HOCl [12]. The Cl atom in HOCl behaves like Cl^+, a strong electrophile, and reacts with dG to form the N7 chlorine adduct cation of dG. This intermediate might then be converted to 8-Cl-dG by migration of chlorine from N7 to C8, or to 8-oxo-dG by reaction with water. Tertiary amines react with HOCl to form reactive Cl^+ adducts (R_3N^+–Cl) that readily release Cl^+ and chlorinate dG at the N7 position, thus enhancing formation of 8-Cl-dG and 8-oxo-dG by HOCl [12].

As the G–463A polymorphism of the *MPO* gene, which strongly reduces myeloperoxidase mRNA expression, is associated with a reduced risk of lung cancer in smokers [13], we have proposed that chlorination damage of nucleic acids and nucleosides by myeloperoxidase and its enhancement by nicotine may be important in the pathophysiology of human diseases associated with tobacco use [12].

During the course of this study, we found that the yields of 8-Cl-dG and 8-oxo-dG account for only about 5% of the consumption of dG by HOCl, suggesting that some other products are formed in the reaction. Recently, our group reported that 8-oxo-dG reacts readily with HOCl to form spiroiminodihydantoin nucleoside (dS) almost exclusively [14]. We have proposed a mechanism for formation of dS by HOCl including Cl^+ addition and/or two-electron oxidation on 8-oxo-dG. Interestingly, the presence of a 1000-fold excess of dG did not inhibit the reaction of 8-oxo-dG with HOCl, indicating that 8-oxo-dG reacts more rapidly than dG with HOCl.

In the present study, we have found that a major product of the reaction of dG with HOCl is dS. We have examined the effects of nicotine on the formation of dS and 8-oxo-dG.

2. Materials and methods

2.1. Materials

dG was obtained from Fluka (Buchs, Switzerland). 8-Oxo-dG and 8-Cl-dG were purchased from Sigma (MO, USA) and Biolog Life Institute (Bremen, Germany), respectively. All other chemicals of reagent grade were purchased from Sigma, Fluka or Aldrich (WI, USA), and used without further purification. Chloride-free sodium hypochlorite (NaOCl) was prepared by a previously reported method [15]. The concentration of NaOCl was determined spectrophotometrically at 290 nm using a molar extinction coefficient of 350 M^{-1} cm^{-1} [16].

2.2. High-performance liquid chromatography (HPLC) conditions

For normal-phase HPLC, the HPLC system consisted of an

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Abbreviations: 8-Cl-dG, 8-chloro-2′-deoxyguanosine; dG, 2′-deoxyguanosine; dS, spiroiminodihydantoin nucleoside; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)02503-6

HP1050 series pumping system (Hewlett Packard, CA, USA) with a Hypersil NH_2 column (H5NH2-25QS, 4.6×250 mm and particle size of 5 μm, Interchim, Montluçon, France). On-line UV spectra were obtained with a Spectra Focus UV-visible photodiode-array detector (Spectra Physics, CA, USA). The eluent used was 20 mM ammonium formate (pH 7.0) and acetonitrile (2/8, v/v). The column temperature was 30°C and the flow rate 1.0 ml/min. For reversed-phase HPLC with UV detection, the HPLC system consisted of an SP 8810 pumping system (Spectra Physics) with an Ultrasphere octadecylsilane column (4.6×250 mm and particle size of 5 μm, Beckman, CA, USA). Samples were analyzed by a Lambda-Max model 481 UV-visible spectrophotometer (Waters, MA, USA) at 260 nm. The eluent used was 20 mM ammonium formate (pH 5.0) containing 10% methanol. The column temperature was ambient and the flow rate 1.0 ml/min. For reversed-phase HPLC with electrochemical detection, the HPLC system consisted of an LC-10AD pumping system (Shimadzu, Kyoto, Japan) with the same octadecylsilane column as above. Samples were analyzed by a Coulochem II electrochemical detector (ESA, MA, USA). The eluent used was 20 mM citric acid–sodium acetate buffer (pH 3.75) containing 12% methanol. The column temperature was 35°C and the flow rate 1.0 ml/min.

2.3. Quantitative procedures

For quantification of dS in all the reaction systems and 8-oxo-dG in an 8-oxo-dG–HOCl system, normal-phase HPLC was used [14]. The concentrations were determined from the integrated peak areas at 245 nm for dS and 295 nm for 8-oxo-dG on HPLC chromatograms and molar extinction coefficients (ε). We calculated the ε values for both diastereomers of dS as 2.95×10^3 M^{-1} cm^{-1} at 245 nm and for 8-oxo-dG as 9.10×10^3 M^{-1} cm^{-1} at 295 nm, on the basis of the reported ε values at 230 nm [14] and on-line UV spectra of dS and 8-oxo-dG. For quantification of dG, 8-Cl-dG and 8-oxo-dG, reversed-phase HPLC with either UV at 260 nm or electrochemical detection was used [12].

2.4. Reaction conditions

For the reaction of dG with HOCl, 500 μM dG was incubated with 0–1000 μM NaOCl in 50 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 min. The reaction was terminated by addition of 0–2 mM *N*-acetylcysteine. The effect of nicotine concentration (0–1000 μM) on the reaction of 500 μM dG with 0–500 μM HOCl was also studied. For the reaction of 8-oxo-dG with HOCl, 50 μM 8-oxo-dG was incubated with 50 μM NaOCl in 50 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 min. The reaction was terminated by addition of 100 μM *N*-acetylcysteine. All experiments were carried out in triplicate.

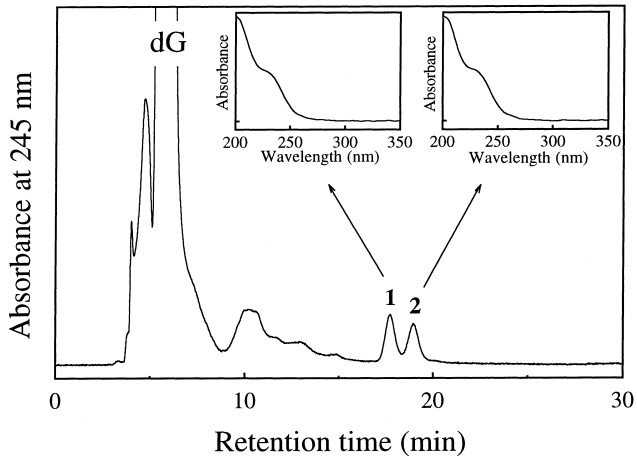


Fig. 1. A normal-phase HPLC chromatogram of a reaction mixture of dG treated with HOCl. dG (500 μM) was incubated with 500 μM NaOCl in 50 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 min. The reaction was terminated by addition of 1 mM *N*-acetylcysteine. The products were separated by an amino-substituted silica column. The eluent was 20 mM ammonium formate (pH 7.0) and acetonitrile (2/8, v/v) and the flow rate was 1.0 ml/min. Insets are on-line detected UV spectra of products 1 and 2.

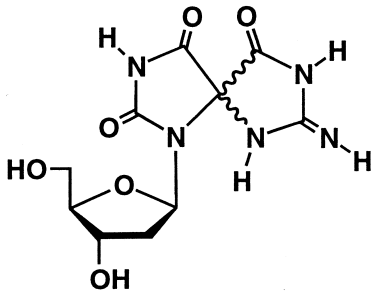


Fig. 2. The structure of 1 and 2, diastereomers of spiroiminodihydantoin deoxyribonucleoside (dS).

3. Results

3.1. Formation of dS from dG with HOCl

When the mixture obtained from the reaction of 500 μM dG with 500 μM HOCl at pH 7.4 and 37°C was analyzed by normal-phase HPLC using an amino-substituted silica column, several peaks were detected on the chromatogram in addition to unreacted dG (Fig. 1). Among the product peaks, two peaks with retention times of 17.7 and 19.0 min (referred to as 1 and 2, respectively) exhibited similar UV spectra with a shoulder near 230 nm (Fig. 1, insets). Recently, our group has reported that the diastereomers of dS are generated as major products in the reaction of 8-oxo-dG with HOCl [14]. The retention times and UV spectra for 1 and 2 in the peaks were identical to those of the major products in the 8-oxo-dG–HOCl system. On the basis of these results, we conclude that the products obtained from the reaction of dG with HOCl are the diastereomers of dS (Fig. 2).

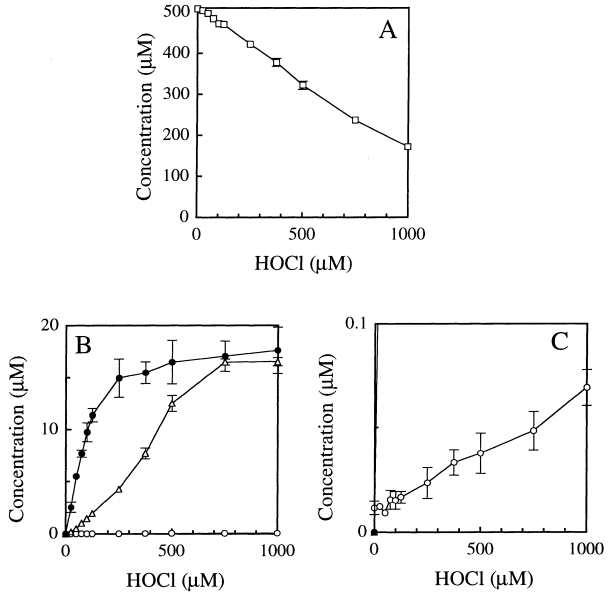


Fig. 3. Effect of HOCl concentration on the reaction of dG with HOCl. A: Concentrations of remaining (unreacted) dG (open squares). B: Formation of dS (closed circles), 8-Cl-dG (open triangles) and 8-oxo-dG (open circles). C: Formation of 8-oxo-dG (an expanded plot of B). dG (500 μM) was incubated with 0–1000 μM NaOCl in 50 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 min. The reaction was terminated by addition of a molar excess of *N*-acetylcysteine. Means ± S.D. ($n = 3$) are presented.

3.2. HOCl dose-dependence in the dG–HOCl system

We investigated the effect of HOCl dose on the reaction of dG with HOCl. The concentrations of dS, 8-Cl-dG, 8-oxo-dG and unreacted dG in the reaction mixture were quantified using HPLC systems with UV or electrochemical detection. Fig. 3A shows the concentrations of unreacted dG after 500 μM dG was treated with various concentrations of HOCl (0–1000 μM) at pH 7.4 and 37°C. The consumption of dG was increased in a dose-dependent manner with increasing concentration of HOCl. Fig. 3B and C show the changes in yield of the products (dS, 8-Cl-dG and 8-oxo-dG). At concentrations of HOCl below 250 μM, the yields of all the products increased dose-dependently, and the dominant product was dS. With 100 μM of HOCl, the concentrations of the products were 9.7 μM dS, 1.5 μM 8-Cl-dG and 0.015 μM 8-oxo-dG, with 36 μM dG consumed. Thus, the yields were 27% dS, 4% 8-Cl-dG and 0.04% 8-oxo-dG relative to the dG consumed. At high concentrations of HOCl (>750 μM), the yield of dS was similar to that of 8-Cl-dG.

3.3. Stability of dS

To examine the stability of dS under physiological conditions, the isolated dS was incubated in 50 mM sodium phosphate buffer at pH 7.4 or 3.0, and 37°C for 4 days. No decomposition of dS was detected in both pHs by normal-phase HPLC analysis (data not shown).

3.4. Effect of nicotine on the formation of dS and 8-oxo-dG in the dG–HOCl system

It has been reported that the yields of 8-Cl-dG and 8-oxo-dG in the reaction of dG with HOCl are increased by addition of nicotine at a biologically relevant concentration [12]. To evaluate the effect of nicotine on dS production in the dG–HOCl (500 μM each) system, the reaction was carried out in the presence of various concentrations of nicotine (0–1000 μM). As shown in Fig. 4A, the dG consumption increased with increasing concentration of nicotine. This indicates that nicotine is an enhancer of the reaction of dG with HOCl. However, addition of a high concentration of nicotine (1000 μM) significantly suppressed the consumption of dG. The yields of dS, 8-Cl-dG and 8-oxo-dG increased dose-dependently with increasing concentration of nicotine, but decreased at the highest concentrations (Fig. 4B). It should be noted that the nicotine concentration giving the maximum

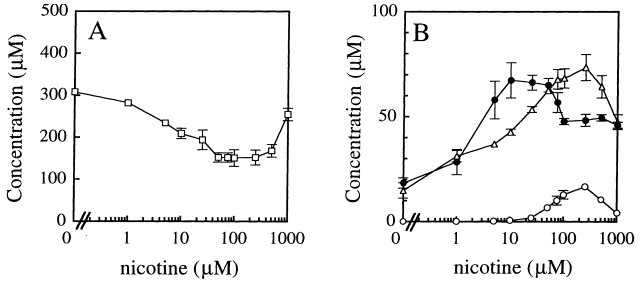


Fig. 4. Effect of nicotine concentration on the reaction of dG with HOCl. A: Concentrations of remaining (unreacted) dG (open squares). B: Formation of dS (closed circles), 8-Cl-dG (open triangles) and 8-oxo-dG (open circle). dG (500 μM) was incubated with 500 μM NaOCl in 50 mM sodium phosphate buffer (pH 7.4) in the presence of 0–1000 μM nicotine at 37°C for 15 min. The reaction was terminated by addition of 1 mM *N*-acetylcysteine. Means ± S.D. ($n=3$) are presented.

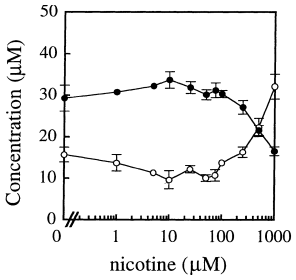


Fig. 5. Effect of nicotine concentration on the yield of dS (closed circles) from the reaction of 8-oxo-dG (open circles) with HOCl. 8-Oxo-dG (50 μM) was incubated with 50 μM NaOCl in 50 mM sodium phosphate buffer (pH 7.4) in the presence of 0–1000 μM nicotine at 37°C for 15 min. The reaction was terminated by addition of 100 μM *N*-acetylcysteine. Means ± S.D. ($n=3$) are presented.

yield of dS (5–50 μM) differed from that for formation of 8-oxo-dG (50–250 μM). The increase in 8-oxo-dG yield was accompanied by a decrease in dS yield (Fig. 4B).

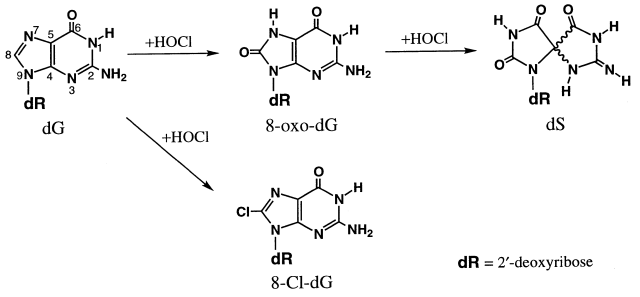
When 100 μM HOCl was incubated with 500 μM dG in the presence of 1, 10 and 100 μM nicotine, the yield of dS was increased 1.2, 1.6 and 2.2 times, respectively, compared to that in the absence of nicotine. The yields of 8-oxo-dG and 8-Cl-dG were also increased up to 220 and eight times, respectively, under these conditions.

3.5. Effect of nicotine on the 8-oxo-dG–HOCl system

It has been reported that 8-oxo-dG reacts readily with HOCl to form dS almost exclusively [14]. To evaluate the effect of nicotine on the reaction of 8-oxo-dG with HOCl, 50 μM 8-oxo-dG was reacted with 50 μM HOCl at pH 7.4 and 37°C in the presence of various concentrations of nicotine (0–1000 μM). dS and unreacted 8-oxo-dG in the reaction mixture were quantified by normal-phase HPLC with UV detection. As shown in Fig. 5, low concentrations of nicotine (5–75 μM) slightly enhanced the conversion of 8-oxo-dG to dS, whereas high concentrations of nicotine (>250 μM) inhibited the reaction.

4. Discussion

In the present study, we established that dS was generated in the reaction of dG with HOCl in addition to 8-Cl-dG and 8-oxo-dG. Although the yield of dS was similar to that of 8-Cl-dG at high doses of HOCl (>750 μM), it was much greater than that of 8-Cl-dG at a low dose (Fig. 3B). When 100 μM HOCl and 500 μM dG were incubated, the yield of dS was 6.6-fold greater than that of 8-Cl-dG and corresponded to 27% of the amount of dG consumed. The yield of 8-oxo-dG remained low (<0.07 μM) over the HOCl concentration range examined, although the yield of 8-oxo-dG increased dose-dependently with HOCl (Fig. 3C). A small amount of nicotine enhanced the formation of dS and 8-Cl-dG and also consumption of dG (Fig. 4A). The yield of dS was increased about three-fold by the addition of micromolar concentrations of nicotine. On the other hand, an increase in the yield of 8-oxo-dG was observed only with concentrations above 50 μM of nicotine (Fig. 4B). This increase in yield of 8-oxo-dG was accompanied by a decrease in dS yield. High concentrations of nicotine suppressed its enhancing effect on the dG–HOCl system. The conversion from 8-oxo-dG to dS by HOCl was inhibited by high concentrations of nicotine



Scheme 1. Proposed reaction pathway for the reaction of dG with HOCl.

(Fig. 5). On the basis of these observations, the most likely reaction pathway appears to be that shown in Scheme 1. HOCl behaving as Cl^+, a strong electrophile, is generated by protonation of OCl^-. Attack on dG by HOCl gives rise to 8-oxo-dG and 8-Cl-dG. A further attack by HOCl on the 8-oxo-dG formed results in formation of dS. Since HOCl reacts with 8-oxo-dG much faster than with dG, almost all of the 8-oxo-dG formed is converted to dS. When a small amount of nicotine is present, the reactions of dG and 8-oxo-dG with HOCl are enhanced since the nicotine–Cl^+ adduct formed reacts with dG and 8-oxo-dG more efficiently. However, a high level of nicotine inhibits the reaction of dG to form 8-Cl-dG and 8-oxo-dG and the reaction of 8-oxo-dG to form dS, possibly because nicotine competes with dG and 8-oxo-dG in the reaction with Cl^+ released from the nicotine–Cl^+ adduct. At medium concentrations (50–250 μM under the present reaction conditions), nicotine inhibits the reaction of 8-oxo-dG with HOCl more effectively than that of dG, resulting in accumulation of 8-oxo-dG in the reaction mixture.

8-Oxo-dG has been used extensively as a biomarker for oxidative DNA damage [17]. However, it may not be an adequate biomarker for oxidative DNA damage caused by HOCl, since it reacts readily with HOCl to form the further oxidized product dS [14]. In the present study, we have shown that dS is stable and is formed in high yields not only from 8-oxo-dG but also from dG. dS, therefore, could be measured as a better biomarker of oxidative DNA damage by HOCl. It has also been reported that several oxidation systems can convert 8-oxo-dG and/or dG into dS [18–20]. Thus, dS may also serve as a good biomarker for oxidative DNA damage caused by other reactive oxygen species.

There is no information currently available with regard to the formation and occurrence of dS in cells and tissues. If dG 5′-triphosphate in the cellular nucleotide pool reacts with endogenous HOCl and other oxygen reactive species, a significant amount of the 5′-triphosphate of dS should be formed. This 5′-triphosphate of dS might be incorporated into DNA and cause mutations. In addition, if dS is formed in DNA, it may be mutagenic, since it has been reported that 2′-deoxyguanosine dG 5′-monophosphate and 2′-deoxyadenosine 5′-monophosphate are incorporated opposite to a lesion including dS by a DNA polymerase causing G·C → C·G and G·C → T·A transversions [21]. HOCl has been reported to be mutagenic in a *Salmonella typhimurium* system and to act as a co-carcinogen in a mouse skin tumor carcinogenesis system [22,23]. In the present study, we found that dS was formed as a major product not only from 8-oxo-dG but also from dG with HOCl and that biologically relevant doses of nicotine increased the yield of dS. Further studies are necessary to elucidate the biological significance of dS and nicotine in relation to HOCl-mediated DNA damage and mutations, especially in smokers.

Acknowledgements: The work reported in this paper was undertaken during the tenure of a Special Training Award granted to T.S. by the International Agency for Research on Cancer. The authors thank Dr. J. Cheney for editing the manuscript and Ms. P. Collard for secretarial assistance.

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