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Peroxynitrite Mediated Oxidation of Purine Bases of Nucleosides and Isolated DNA

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Reaction of nitric oxide with superoxide anion produces the highly reactive species peroxynitrite ($ONOO^-$). This compound has been shown to be a strong oxidant of lipids and proteins. However, no data are available on its effect on DNA, with the exception of the induction of strand breaks. We report the result of studies on the reactions of peroxynitrite with the adenine and guanine moieties of nucleosides and isolated DNA. The samples were analyzed for 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo), 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5-(2*H*)-oxazolone (oxazolone) and 8-oxo-7,8-dihydro-2′-deoxyadenosine (8-oxo-dAdo). The effects of peroxynitrite treatment were compared with those of ionizing radiation in aerated aqueous solution, chosen as a source of hydroxyl radicals. At the nucleoside level, both oxidizing conditions led to the formation of oxazolone and 8-oxo-dAdo. In addition, evidence was provided for the formation of the 4R* and 4S* diastereoisomers of 4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine. The latter dGuo oxidation products were chosen as markers of the release of singlet oxygen ($^1O_2$) upon reaction of peroxynitrous acid with hydrogen peroxide. Oxidation of purine bases was then studied within isolated DNA. A significant increase in the level of 8-oxo-dGuo, oxazolone and 8-oxo-dAdo was observed within double stranded DNA upon exposure to γ-radiation. Oxazolone and 8-oxo-dAdo were formed upon peroxynitrite treatment but no significant increase in the amount of 8-oxo-dGuo was detected. These results showed that peroxynitrite exhibits oxidizing properties toward purine moieties both in nucleosides and isolated DNA. However, the significant differences in the oxidative damage distribution within DNA observed after exposure to γ radiation by comparison with peroxynitrite treatment questions the involvement of hydroxyl radicals as the main oxidizing species released by decomposition of peroxynitrous acid.

Key words: peroxynitrite, nitric oxide, superoxide anion, DNA lesions, hydroxyl radicals, singlet oxygen.

Abbreviations: 4-OH-8-oxo-dGuo, 4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine; 8-oxo-dAdo, 8-oxo-7,8-dihydro-2′-deoxyadenosine; 8-oxo-dGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; dAdo, 2′-deoxyadenosine; dGuo, 2′-deoxyguanosine; HPLC-EC, high performance liquid chromatography coupled to electrochemical detection; NO, nitric oxide; NQS, 1,2-naphthoquinone-4-sulfonic acid; oxazolone, 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5-(2*H*)-oxazolone; Rt, retention time.

INTRODUCTION

Peroxynitrite ($ONOO^-$) is known to be produced by the reaction of nitric oxide (NO) with superoxide anion ($O_2^{\cdot-}$),[1] a process which was recently

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proposed to be of biological interest.[2] Peroxynitrite has also been proposed to be involved in amyotrophic lateral sclerosis,[3] atherosclerosis[4,5] and neurodegeneration.[6,7] In addition, peroxynitrite is produced *in vitro* by macrophages[8] and neutrophils.[9] In addition to these endogenous productions, peroxynitrite could be provided by exogenous sources like cigarette smoke.[10] $ONOO^-$ is also produced upon exposure of nitrates to far-UV light and ionizing radiation.[11]

One major consequence of the cellular formation of peroxynitrite would be the release of hydroxyl radicals (HO°) or HO°-like species upon decomposition.[2,12] The pKa of peroxynitrite anion is 6.8.[11] Therefore, the protonated molecule is expected to be the major form under physiological conditions. The half-life time of peroxynitrous acid (HOONO) is 1s at pH 7 and 37°C, and its decomposition produces highly reactive species. The reactivity of peroxynitrite and/or its decomposition products towards biomolecules has been mainly studied on proteins[13,14,15] and membrane lipids.[16,17] Treatment of double-stranded DNA with peroxynitrite was also recently reported to induce oligonucleotide strand breaks.[18,19] However, damage to nucleobases produced by the oxidizing species released during the decomposition of peroxynitrous acid has not yet been characterized. The identification of such lesions would be a major information for a better understanding of the mutagenic properties of peroxynitrite. In addition, data are needed on the mechanism involved in peroxynitrite mediated-oxidation of nucleobases within DNA. For instance, such results could allow the identification of the actual DNA oxidizing species released within macrophages upon activation. Indeed, a recent study showed an increase in the amount of several oxidative lesions of thymine and guanine.[20] It was proposed that peroxynitrite could be the oxidant involved in this process. In the present study, emphasis was placed on the mechanistic aspects of the oxidation reactions of purine moieties by peroxynitrite. This was based on the determination of the main oxidative damage induced by $ONOO^-$ to the base moieties of 2'-deoxyribonucleosides and isolated DNA. The results were compared with the effect of ionizing radiation to assess the role of hydroxyl radicals.

MATERIALS AND METHODS

Chemicals

2'-Deoxyadenosine (dAdo) and 2'-deoxyguanosine (dGuo) were purchased from Pharma-Waldorf (Geneva, Switzerland). Sodium nitrite and manganese (IV) oxide were Merck products (Darmstadt, Germany). Calf thymus DNA and 30% hydrogen peroxide were obtained from Sigma (St Louis, MO) and 1,2-naphthoquinone-4-sulfonic acid (NQS) from Aldrich (Milwaukee, WI). 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dGuo) was prepared by using the method reported by Lin *et al.*[21] 8-Oxo-7,8-dihydro-2'-deoxyadenosine (8-oxo-dAdo) was prepared by γ irradiation of dAdo in aqueous aerated solution. The 4S* and 4R* diastereoisomers of 4-hydroxy-8-oxo-4,8-dihydro-2'-deoxyguanosine (4-OH-8-oxo-dGuo) were prepared by photosensitization of 2'-deoxyguanosine by methylene blue upon exposure to visible light.[22] All compounds were characterized by UV, mass and ^1H NMR spectroscopies.

Synthesis of Peroxynitrite

The synthesis of sodium peroxynitrite was achieved by oxidation of nitrous acid with hydrogen peroxide.[23] An aqueous solution (100 mL) containing 10 mmoles of sodium nitrite (690 mg) and 10 mmoles of hydrogen peroxide was cooled to 1°C in an ice bath. Then, 10 mL of cold 1 M hydrochloric acid was added under vigorous stirring. This was immediately followed by addition of 15 mL of cold 1 M sodium hydroxide and the solution turned yellow. The concentration of the sodium peroxynitrite solution was determined to be 30 mM by measuring the UV absorption at 302 nm ($\varepsilon_{302} = 1670\ M^{-1}.cm^{-1}$ in 1 M

sodium hydroxide). An aliquot (20 mL) of this solution was treated with 1 g of MnO_2 under stirring for 5 min, and subsequently filtered to remove the solid fraction. Hydrogen peroxide (2.06 mL of a 30% w/w solution) was added to a second 20 mL aliquot of the sodium peroxynitrite solution.

Oxidation of Nucleosides and Isolated DNA by Peroxynitrite

Samples of 2'-deoxyguanosine and 2'-deoxyadenosine (20 mg) were solubilized in 10 mL of each of the three solutions described above. The pH was monitored by using a 537 pH meter (WTW, Weilheim, Germany) and adjusted to 7.5 by addition of 1 M HCl. After 1 min, the pH had spontaneously decreased to 5 and the sample was neutralized by addition of 1 M sodium hydroxide. A control experiment was made with 10 mL of a sodium hydroxide solution (pH 12) containing 1 mmole of hydrogen peroxide. All the solutions were analyzed for 8-oxo-dGuo and 8-oxo-dAdo by high performance liquid chromatography coupled to electrochemical detection (HPLC-EC) without further treatment. The samples were freeze-dried prior to the NQS assay for guanidine residues (*vide infra*) in order to remove hydrogen peroxide.. All experiments were resumed with a preliminary addition of 100 μg of Chelex 100 resin (Biorad, Richmond, CA).

DNA samples (500 μL of a 1 $mg.ml^{-1}$ solution diluted in 10 mL of peroxynitrite solution) were treated under similar conditions. Two additional control experiments were made. A sample (500 μL) of the concentrated solution of DNA was diluted with water to a volume of 10 mL. An identical fraction of DNA was diluted in 10 mL of pH 12 sodium hydroxide and then neutralized by addition of 1 M HCl. All samples were then treated identically. A 3 M aqueous solution of pH 4.5 sodium acetate (1 mL) and 25 mL of cold ethanol were added. The samples were placed at –30°C overnight and then centrifuged for 45 min at 6000 rpm in a cold (– 20°C) Heraeus-Christ centrifuge (Osterode, Germany). The DNA pellet was resuspended in 100 μL of water. All experiments were duplicated and the reported results are the average of the two measurements.

Exposure of Aerated Solutions of Nucleosides and DNA to γ-Radiation

A 2 $mg.ml^{-1}$ solution (5 mL) of nucleoside (dGuo or dAdo) was placed in a Pyrex tube. The sample was then exposed, under constant air bubbling, to the γ-rays emitted by a ^{60}Co source providing 50 $Gy.min^{-1}$. The sample was removed from the source after increasing periods of exposure and 1 mL aliquot fractions were collected. A similar procedure was used for the irradiation of 5 mL of a 200 $\mu g.mL^{-1}$ DNA solution. Aliquot fractions (1 mL) were collected after irradiation periods of 0, 30 and 60 s, respectively. DNA was precipitated from either 400 μL or 250 μL of the latter solution for digestion into nucleosides and NQS assay, respectively, by addition of 0.1 volume of 3 M sodium acetate aqueous solution (pH 4.5) and 2.5 volumes of cold ethanol. After homogenization, the samples were stored at –30°C overnight and the DNA was spun down by centrifugation for 45 min at –20°C. The residue was resuspended in 100 μL of water.

DNA Digestion

The DNA samples (100 μL) were digested by addition of 10 μL of P1 10X buffer (300 mM pH 5.3 sodium acetate, 1 mM $ZnSO_4$) and 10 μL (10 units) of the nuclease P1 solution (Boerhinger, Mannheim, Germany). The samples were held at 37°C for 2 h. Dephosphorylation of the resulting nucleotides was achieved by addition of 12 μL of 10 X phosphatase alkaline buffer (500 mM, pH 8, Tris-HCl, 1 mM EDTA), and 3 μL (3U) of alkaline phosphatase solution (Boerhinger, Mannheim, Germany). After incubation for 1 h at 37°C, the samples were neutralized by addition of 0.1 M HCl.

HPLC-EC Analysis of 8-oxo-dGuo and 8-oxo-dAdo

The HPLC system consisted of a model 2150 LKB pump (Pharmacia LKB Biotechnology, Uppsala, Sweden) connected to a SIL-9A autosampler (Shimadzu, Kyoto, Japan) equipped with an Interchrom HC18-25F octadecylsilyl silica gel column (250 × 4.6 mm i.d.) (Interchim, Montluçon, France). The eluents were [87/13] and [83/17] v/v mixtures of 50 mM sodium citrate (pH 5) and methanol for the analysis of 8-oxo-dGuo and 8-oxo-dAdo, respectively. The amperometric electrochemical detection was performed with a model LC-4B/LC-17A(T) system (Bioanalytical Systems, West Lafayette, IN). The detection potential was set at +650 mV and +850 mV for the detection of 8-oxo-dGuo and 8-oxo-dAdo, respectively. The amount of 8-oxo-dGuo (retention time Rt: 6.0 min) or 8-oxo-dAdo (Rt: 8.2 min) was calculated by calibrating the detector with 5 ng of the authentic products. For DNA analysis, unmodified nucleosides were monitored by a Gilson Model 111b UV detector (Gilson, Middleton, WI) set at 280 nm. The amount of DNA analyzed was inferred from the height of the dGuo peak.

Measurement of Guanidine Released from Oxazolone

Typically, 50 µL of solution (nucleoside or DNA) was placed in a polypropylene vial together with 50 µL of 1 M sodium hydroxide. After homogenization, the solution was held at 70°C for 30 min in a water bath. Then, the sample was removed and cooled down to room temperature. An aqueous solution of NQS (10 µL, 8 mg/mL) was added and the resulting solution incubated for 10 min in a water bath at 70°C. After cooling to room temperature, the sample was neutralized by addition of 50 µL of 1 M hydrochloric acid. The solution was then analyzed with the HPLC system described previously, using a fluorescence detection provided by a F-1050 fluorimeter (Hitachi, Tokyo, Japan) with the excitation and emission wavelengths set at 355 and 405 nm, respectively. The eluent was a [90:10] v/v mixture of a 25 mM aqueous solution of ammonium formate and methanol. The amount of the reaction product of guanidine (Rt: 11.3 min) was inferred from a calibration curve established with derivatized authentic guanidine. The level of guanine modification was estimated by using the amount of DNA determined in the HPLC-EC assay (*vide supra*).

Chromatographic Analysis of 4-OH-8-oxo-dGuo

2′-Deoxyguanosine samples treated by either the crude NaOONO synthesis mixture or the NaOONO solution containing 100 mM H_2O_2 were analyzed for 4-OH-8-oxo-dGuo by HPLC on a 250X4.6 mm i.d. (particle size: 5 µm) LiChrospher 100 NH_2 (Merck, Darmstadt, Germany) amino silica gel column. The isocratic eluent was a [80:20] v/v mixture of acetonitrile and a 100 mM aqueous solution of ammonium formate. The flow rate was 1 $ml.min^{-1}$. The detection was provided by a Waters 990 diode array UV detector (Waters Associates, Mildford, MA). Under these conditions, the 4R* and 4S* diastereoisomers of 4-OH-8-oxo-dGuo were not separated (retention time: 17.45 min). Oxidized dGuo samples were also analyzed by gas chromatography coupled to mass spectrometry. Aqueous solutions containing either aliquot fractions (1 µmole) of oxidized dGuo or 50 nmoles of pure 4-OH-8-oxo-dGuo in aqueous solution were freeze-dried and derivatized in sealed vials by 100 µL of a [50:50] v/v mixture of acetonitrile and *bis*(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylsilyl chloride (silylation grade reagents, Pierce, Rockford, IL). Samples were held at 110°C for 25 min in a heating block. They were subsequently injected (injection volume: 1 µL) in the splitless mode at 210°C, on a HP 5890 serie II gas chromatograph (Hewlett-Packard, Les Ulis, France) equipped with a capillary column (0.25 mm, 30 m) coated with a 0.25 µm film of

methylsiloxane substituted with 5% phenylsiloxane (HP5-MS, Hewlett-Packard). The constant flow rate was 3.2 mL.min^{-1}. The temperature of the GC oven was raised from 70°C to 275°C at a rate 20°C.min^{-1}, and left at the latter temperature for 3.75 min. Positive ions were detected by a HP 5972 mass spectrometer (Hewlett-Packard, Les Ulis, France) using the electron impact ionization mode. A chromatogram was performed in the SCAN mode with 500 pmole of silylated authentic 4R* and 4S* diastereoisomers of 4-OH-8-oxo-dGuo to determine the retention time and the mass spectrum of each diastereoisomer. The pentasilylated derivatives of the two diastereoisomers of 4-OH-8-oxo-dGuo were well separated with a retention time of 11.15 min and 11.31 min. All other chromatograms were recorded in the single ion monitoring mode (detected ions at m/z = 269, 356, 384, 456 and 500).

RESULTS

Decomposition of dGuo and dAdo Upon Exposure to γ-Radiation in Aerated Aqueous Solution

8-Oxo-dGuo[24,25] and the recently characterized 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)-amino]-5-(2*H*)-oxazolone (oxazolone) (Figure 1)[26,27] were chosen as markers of oxidative damage to dGuo. The formation of 8-oxo-dAdo (Figure 1), which is the main HO° mediated oxidation product of dAdo,[28] was monitored in the solutions of the latter nucleoside. 8-oxo-dAdo and 8-oxo-dGuo were measured by a sensitive HPLC-EC method, as previously reported.[29,30] The assay for the

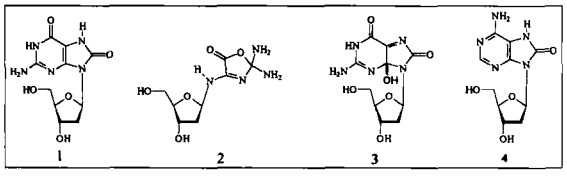

FIGURE 1 Structure of 8-oxo-dGuo (1), oxazolone (2), 4-OH-8-oxo-dGuo (3) and 8-oxo-dAdo (4).

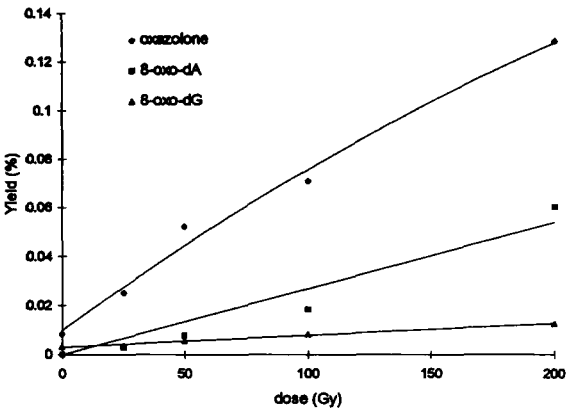

FIGURE 2 Formation of oxidative base damage to 2'-deoxyguanosine and 2'-deoxyadenosine upon exposure to γ radiation of aerated solutions of the nucleoside.

oxazolone is based on the quantitative release of a guanidine residue upon alkaline treatment. Guanidine is further converted into a fluorescent compound by treatment with 1,2-naphthoquinone-4-sulfonic acid.[26] Then, the reaction product is detected by a spectrofluorimeter at the output of the HPLC column. The formation of the three oxidized 2'-deoxynucleosides was monitored in aerated solutions of either dGuo or dAdo exposed to γ-radiation (Figure 2). The rate of formation of 8-oxo-dGuo, oxazolone and 8-oxo-dAdo was found to be in a 1/10/5 ratio, respectively. It should be mentioned that the three lesions were present in a very low amount in the commercial batch of nucleosides (0.003%, 0.025% and 0.003% for 8-oxo-dGuo, oxazolone and 8-oxo-dAdo, respectively).

Oxidation of dGuo and dAdo by Peroxynitrous Acid

Peroxynitrous acid is not a stable molecule. Consequently, it was generated *in situ* by acidification of an alkaline solution containing both sodium peroxynitrite and nucleosides. A spontaneous decrease of the pH was observed after it had been adjusted to 7.5. This can be rationalized in terms

of formation of nitric acid by isomerization of the protonated form of the peroxynitrite anion, which reported pKa values are in the 6.8–7.9 range.[11] This indicates that peroxynitrous acid was actually produced in our experimental workup. As for γ-radiolysis experiments, 8-oxo-dGuo, oxazolone and 8-oxo-dAdo were measured in the various samples. Major attention was paid to possible artefactual oxidation reactions induced by hydrogen peroxide remaining from the sodium peroxynitrite synthesis. Consequently, acidification of an alkaline 100 mM (the starting concentration used in the NaOONO synthesis) H_2O_2 aqueous solution of nucleoside was conducted as a control experiment. The role of hydrogen peroxide in the solution of peroxynitrite was further assessed by treating dGuo and dAdo with a sodium peroxynitrite solution either containing a known amount of H_2O_2 (100 mM) or from which hydrogen peroxide had been preliminary removed by MnO_2 treatment.[23] The role of possible metal contaminants was checked by resuming the experiments in the presence of an efficient chelating resin. No significant differences were observed, and the results of these two sets of experiments were averaged.

A first observation is that no significant amount of 8-oxo-dGuo was produced in any of the experiments (Table 1). On the other hand, oxazolone and 8-oxo-dAdo were found to be generated upon peroxynitrite treatment of dGuo and dAdo, respectively. In contrast, oxazolone was not generated upon hydrogen peroxide treatment alone and the yield of 8-oxo-dAdo under similar conditions was only 15% of that induced by peroxynitrite treatment. In agreement with these observations, the yield of oxazolone and 8-oxo-dAdo was not significantly modified either when the peroxynitrite solution was preliminary treated by MnO_2 or when 100 mM H_2O_2 was added to it. Altogether, these results show that the formation of oxazolone and 8-oxo-dAdo is really induced during the decomposition of peroxynitrous acid.

The yields of 8-oxo-dGuo, oxazolone and 8-oxo-dAdo produced upon peroxynitrite treatment of the nucleosides were found to be in a 1/25/6 ratio, respectively. It should be added that HPLC analysis of all the samples indicated no detectable decrease in the amount of normal nucleoside, indicating a very low degradation yield. It is therefore likely that the three oxidized derivatives studied are among the major reaction products.

2′-Deoxyguanosine samples treated by either the crude peroxynitrite solution or the peroxynitrite solution in which 100 mM hydrogen peroxide had been added, were analyzed for the presence of the 4R* and 4S* diastereoisomers of 4-hydroxy-8-oxo-4,8-dihydro-2′-deoxyguanosine (4-OH-8-oxo-dGuo, Figure 1), the specific singlet oxygen oxidation products of dGuo.[22] Two assays, including HPLC with UV detection and GC-MS, were applied to the detection of 4-OH-8-oxo-dGuo.[26,31] Elution profile on a HPLC amino column of the fractions treated with NaOONO solution exhibited peaks corresponding to products coeluting with the authentic 4R* and 4S* diastereoisomers of 4-OH-8-oxo-dGuo (Figure 3a). The UV absorption spectra of these compounds were identical to those of the 4R* and 4S* diastereoisomers of 4-OH-8-oxo-dGuo, exhibiting

TABLE 1 Yield (%) of formation of oxidative base damage to 2′-deoxyguanosine and 2′-deoxyadenosine in nucleoside solutions treated with: H_2O_2: 100 mM hydrogen peroxide; HOONO: 30 mM sodium peroxynitrite solution; HOONO H_2O_2: 30 mM sodium peroxynitrite and 100 mM hydrogen peroxide solution; HOONO MnO_2: 30 mM sodium peroxynitrite solution treated with MnO_2.

treatment	oxazolone	8-oxo-dGuo	8-oxo-dAdo
H_2O_2	0.04	0.01	0.04
HOONO	1.00	0	0.22
HOONO H_2O_2	0.94	0.01	0.22
HOONO MnO_2	1.23	0.01	0.24

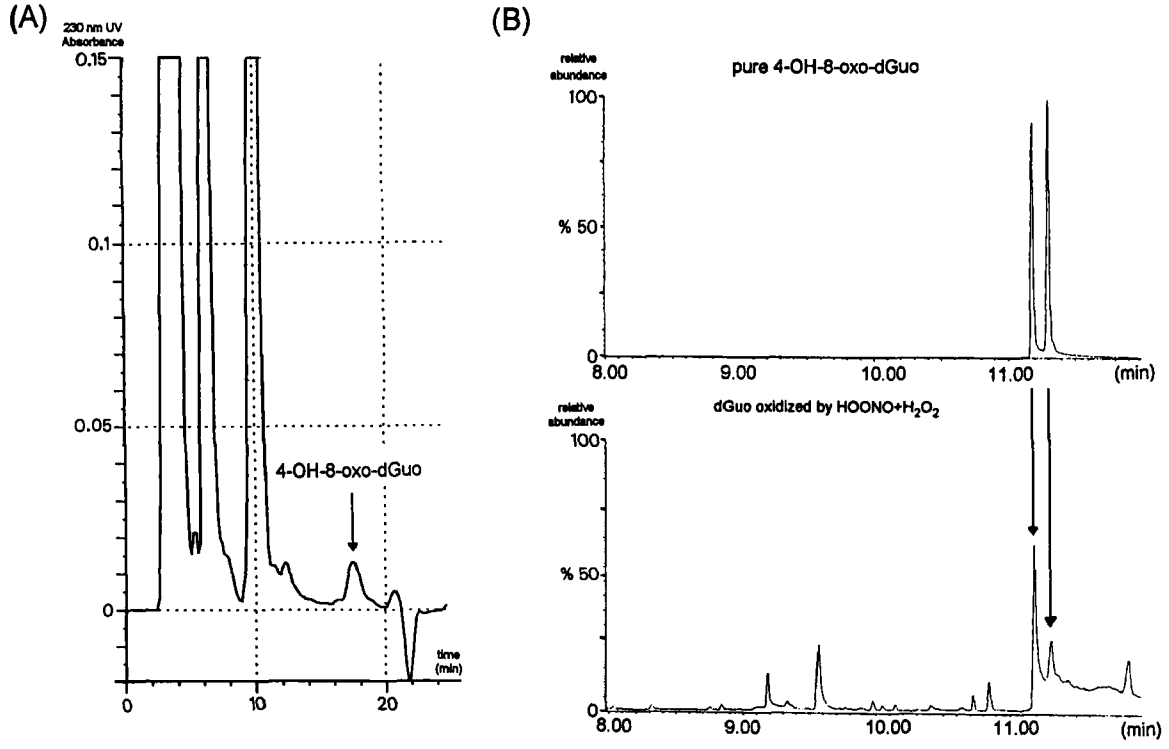

FIGURE 3 Chromatographic analyses of the dGuo solutions treated by peroxynitrite and hydrogen peroxide for the 4R* and 4S* diastereoisomers of 4-OH-8-oxo-dGuo. (a) HPLC on an amino column with UV detection at 230 nm (b) GC-MS in the selected ion monitoring mode.

a maximum centered around 230 nm. GC-MS analysis of the same samples confirmed the presence of 4-OH-8-oxo-dGuo. The mass spectra of the pentasilylated diastereoisomers of 4-OH-8-oxo-dGuo obtained by analysis performed in the SCAN mode were in agreement with published data.[31] The fastest eluting compound exhibits a major peak at m/z = 384, corresponding to the loss of the 2-deoxyribose moiety and a methyl fragment. The mass spectrum of the second diastereoisomer shows a base peak at m/z = 500, corresponding to the loss of a $NH(Si(CH_3)_3)_2$ molecule. Three other minor unidentified ions (269, 356 and 456) were observed in a characteristic relative abundance in the spectrum of each of the two diastereoisomers. The five ions mentioned above were used for the single ion monitoring analysis. On the basis of these chromatographic and spectrometric features, the two diastereoisomers of 4-OH-8-oxo-dGuo were unambiguously detected in the dGuo samples oxidized by either the crude peroxynitrite solution or the peroxynitrite solution in which 100 mM H_2O_2 had been added (Figure 3b). Based on the intensity of the peaks obtained for 500 nmoles of pure mixture of the 4R* and 4S* diastereoisomers of 4-OH-8-oxo-dGuo, the overall yield was estimated to be 0.14% and 0.25% for HOONO and HOONO + H_2O_2 treatment, respectively.

Oxidative Damage Induced in Isolated DNA upon Exposure to γ-Radiation and Peroxynitrite in Aerated Aqueous Solution

The rate of formation of 8-oxo-dGuo, oxazolone and 8-oxo-dAdo was measured within isolated

TABLE 2 Modification rate in DNA samples (expressed in lesions per 10^5 normal bases). Calf thymus DNA: untreated concentrated DNA solution; control pH 7: aliquot of the DNA concentrated aqueous in water prior to ethanol precipitation; control pH 12: aliquot of the DNA concentrated aqueous solution diluted in an aqueous solution of sodium hydroxide (pH 12), followed by neutralization and ethanol precipitation. 0 Gy, 25 Gy and 50 Gy: calf thymus DNA samples exposed to γ-radiation with an overall dose of 0, 25 and 50 Gy, respectively; H_2O_2: 100 mM hydrogen peroxide treatment; HOONO: 30 mM sodium peroxynitrite treatment; HOONO H_2O_2: 30 mM sodium peroxynitrite and 100 mM hydrogen peroxide treatment HOONO MnO_2: treatment by a 30 mM sodium peroxynitrite solution treated with 1 g MnO_2.

sample	oxazolone	8-oxo-dGuo	8-oxo-dAdo
calf thymus	5	3	0
control pH 7	119	25	0
control pH 12	121	25	0
0Gy	84	17	0
25 Gy	267	504	25
50 Gy	522	876	42
H_2O_2	135	557	132
HOONO	182	108	90
HOONO H_2O_2	260	191	119
HOONO MnO_2	162	23	59

DNA in aerated aqueous solution upon treatment with γ-radiation, hydrogen peroxide and peroxynitrite (Table 2). Additional control experiments were made in order to determine possible artefactual oxidation induced by the experimental workup. For this purpose, an aliquot of the starting DNA solution was diluted in water (pH 7) and precipitated. A second sample was diluted in an aqueous solution of sodium hydroxide (pH 12), neutralized and precipitated. They were both analyzed for 8-oxo-dGuo, oxazolone and 8-oxo-dAdo and the results were compared with those obtained for calf thymus DNA from the starting concentrated solution. 8-Oxo-dGuo and oxazolone were detected in a higher amount in the diluted samples than in concentrated DNA solution. This is indicative of a significant artefactual oxidation of dGuo, identical in both control samples. In contrast, no detectable formation of 8-oxo-dAdo was observed. DNA samples were exposed to γ rays with overall doses of 25 and 50 Gy. With a 50 Gy dose, the amount of 8-oxo-dGuo, oxazolone and 8-oxo-dAdo were found to be 876, 522 and 47 modified bases per 10^5 nucleosides, respectively, compared to 17, 84 and 0 for the control aliquot of the radiation experiments. This clearly indicates that the three lesions were generated upon γ radiolysis of DNA, in agreement with previous works on the radiation-induced formation of 8-oxo-dGuo and 8-oxo-dAdo.[25,28]

The artefactual oxidation of guanine residues due to the experimental workup complicated the estimation of the formation rate of 8-oxo-dGuo and oxazolone. No attempts to decrease this phenomenon were made because addition of antioxidants could interfere with the reactions of interest. No detectable increase in the level of 8-oxo-dGuo was observed in DNA treated with peroxynitrite solution from which H_2O_2 had been removed by MnO_2. On the other hand, exposure of DNA to 100 mM hydrogen peroxide, alone or added to the sodium peroxynitrite solution, induced a significant formation of 8-oxo-dGuo. Therefore, the formation of 8-oxo-dGuo upon treatment of DNA with the crude peroxynitrite solution is likely to be due to the presence of traces of unreacted hydrogen peroxide. In contrast, oxazolone was found to be produced upon peroxynitrite treatment. Exposure of DNA to 100 mM H_2O_2 induced a slight increase in the yield of oxazolone by comparison with the control experiments. A higher yield of oxazolone was observed upon treatment with crude peroxynitrite. Therefore, the latter observation could not be

completely accounted for by oxidation due to the presence of hydrogen peroxide. This result was confirmed by the observation that preliminary treatment of the peroxynitrite solution with MnO_2 did not inhibit the formation of oxazolone. The formation of oxazolone within DNA exposed to either HOONO or H_2O_2 is in agreement with the high value found in samples treated with a mixture of both oxidants. A similar result was obtained for 8-oxo-dAdo, which was formed upon treatment of DNA by 100 mM H_2O_2 and/or 30 mM sodium peroxynitrite. The latter observation cannot be accounted for by the presence of remaining hydrogen peroxide since pre-treatment of the peroxynitrite solution by MnO_2 did not inhibit the formation of 8-oxo-dAdo. The corrected values for the level of oxazolone and 8-oxo-dAdo are 42 and 59 lesions per 10^5 nucleosides, respectively, while no significant formation of 8-oxo-dGuo was observed.

DISCUSSION

This work clearly establishes that peroxynitrite induces oxidative damage to purine moieties both in nucleosides and isolated DNA. A comparison with the effects of ionizing radiation was made to assess the role of hydroxyl radical since the latter species is produced by water radiolysis[32] and was proposed to be present among the decomposition product of peroxynitrous acid.[12] Both γ-radiation and peroxynitrite treatment appeared to exhibit similar oxidizing properties at the nucleoside level. In both cases, 8-oxo-dGuo was produced in a very low yield in contrast to oxazolone and 8-oxo-dAdo. These results confirm that either peroxynitrous acid or its decomposition products exhibit oxidative properties similar to those of hydroxyl radicals, as already observed by using deoxyribose, DMSO and 2,2'-azino-*bis*-(3-ethylbenzthiazoline sulphonate) as substrates,[2,33] and 5,5-dimethyl-1-pyrroline-*N*-oxide as a spin trapping agent.[34] It should be mentioned that transition metals do not seem to be involved in these oxidation reactions since the addition of chelating resin did not affect the distribution of oxidation products.

It has been recently reported that peroxynitrous acid reacts with hydrogen peroxide to produce singlet oxygen.[35] Among deoxyribonucleosides, dGuo reacts specifically with singlet oxygen, leading to the formation of specific products characterized as the 4R* and 4S* diastereoisomers of 4-OH-8-oxo-dGuo.[22,36,37] The latter compounds were detected in dGuo samples oxidized by either the crude NaOONO synthesis mixture or the NaOONO solution in which 100 mM H_2O_2 had been added. As expected, the rate of formation increased when H_2O_2 was added to the reaction mixture. However, the yield of singlet oxygen products of dGuo was low in both experiments, representing approximately 10% of that of oxazolone. The low amount of singlet oxygen produced was confirmed by the observation that addition of hydrogen peroxide to the peroxynitrite solution did not modify the yield of neither the oxazolone nor 8-oxo-dAdo. Significant conversion of peroxynitrite into singlet oxygen would have partly inhibited the formation of the latter oxidative damage since they are not produced by reaction of the nucleosides with $^1O_2$.

The similarity between the action of HO° and peroxynitrous acid on purine nucleosides does not apply to isolated DNA. Hydroxyl radicals generated by γ irradiation induced the formation of 8-oxo-dAdo, 8-oxo-dGuo and oxazolone. On the other hand, only a significant production of 8-oxo-dAdo and oxazolone was observed upon peroxynitrite treatment of DNA. Moreover, the ratio between the yield of oxazolone and 8-oxo-dAdo was found to be 10-fold lower in DNA exposed to °OH radicals than in peroxynitrite treated samples. This difference in products distribution might indicate that the conditions of the peroxynitrite reaction inhibit the formation of the guanine intermediates leading to 8-oxo-dGuo and oxazolone within DNA. However, the latter oxidized nucleosides are mainly generated via a reducing and an oxidizing radical, respectively. It is

thus unlikely that the redox reaction conditions could simultaneously prevent the formation of the these two intermediates and of the corresponding final products. This is confirmed by the significant formation of 8-oxo-dGuo and oxazolone in DNA samples treated by a mixture of peroxynitrite and hydrogen peroxide, showing that the peroxynitrite solution does not inhibit the formation of these two guanine oxidation products. An alternative explanation for the difference in the distribution of oxidation products induced by ionizing radiations on one hand and peroxynitrite on the other hand is that hydroxyl radicals are not the main reactive species involved in the decomposition of the latter compounds.[38] This would be in agreement with kinetic considerations[33] and theoretical calculations[39] showing that isomerization of peroxynitrous acid into nitric acid via HO° and °NO_2 formation followed by radical recombination is not a favored pathway. Several other species, including HOONO in a *trans* configuration, an excited vibrational state of the latter molecule and (NO_2° °OH) solvated pair of radicals, have been proposed to account for the oxidizing properties of peroxynitrite.[40–42] However, the reactive compounds involved in the oxidation of purine DNA bases remain to be identified. It can be added that the lack of a detectable formation of 8-Oxo-dGuo rules out the significant contribution of one-electron transfer mechanism in the peroxynitrite mediated oxidation of DNA. Indeed, the latter mechanism has been shown to generate 8-Oxo-dGuo as a major oxidative damage within DNA.[43]

These results, which unambiguously establish that peroxynitrous acid is able to oxidize DNA nucleobases, may have implications in the study of the mutagenicity of nitric oxide. The mutagenic properties of NO were inferred from the observation of mutations and chromosome aberrations in lung cells of rats exposed to nitric oxide.[44] Nitric oxide is a rather unreactive compound and requires a preliminary oxidation before it can react with biomolecules.[45,46] The 'activated species of nitric oxide' which may be produced in cells remain to be further characterized. Compounds likely to react with DNA include nitronium (NO_2^+) and nitrosonium (NO^+) ions, or higher nitric oxides (N_2O_3, NO_2). Peroxynitrite is another possible deleterious derivative which was found to be more cytotoxic than nitric oxide itself.[47] Peroxynitrite has been recently proposed to be involved in the DNA oxidation of activated macrophages.[20] This was based on the observation of the increase in the rate of three DNA lesions, including 5-hydroxymethyluracil, 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 8-oxo-7,8-dihydroguanine. The lack of detectable formation of the latter compound upon direct exposure of isolated DNA to peroxynitrite indicates that another pathway may be involved.

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