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Mapping Three Guanine Oxidation Products along DNA Following Exposure to Three Types of Reactive Oxygen Species

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Abstract

Reactive oxygen and nitrogen species generated during respiration, inflammation, and immune response can damage cellular DNA, contributing to aging, cancer, and neurodegeneration. The ability of oxidized DNA bases to interfere with DNA replication and transcription is strongly influenced by their chemical structures and locations within the genome. In the present work, we examined the influence of local DNA sequence context, DNA secondary structure, and oxidant identity on the efficiency and the chemistry of guanine oxidation in the context of the *Kras* protooncogene. A novel isotope labeling strategy developed in our laboratory was used to accurately map the formation of 2,2-diamino-4-[(2-deoxy-β-D-erythropentofuranosyl)amino]-5(2H)-oxazolone (Z), 8-oxo-7,8-dihydro-2'-deoxyguanosine (OG), and 8-nitroguanine (8-NO₂-G) lesions along DNA duplexes following photooxidation in the presence of riboflavin, treatment with nitrosoperoxycarbonate, and oxidation in the presence of hydroxyl radicals. Riboflavin-mediated photooxidation preferentially induced OG lesions at 5' guanines within GG repeats, while treatment with nitrosoperoxycarbonate targeted 3'-guanines within GG and AG dinucleotides. Little sequence selectivity was observed following hydroxyl radical-mediated oxidation. However, Z and 8-NO₂-G adducts were overproduced at duplex ends, irrespective of oxidant identity. Overall, our results indicate that the patterns of Z, OG, and 8-NO₂-G adduct formation in the genome are distinct and are influenced by oxidant identity and the secondary structure of DNA.

Graphical abstract

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INTRODUCTION

Reactive oxygen and nitrogen species (e.g. hydrogen peroxide, hydroxyl radical, superoxide, peroxynitrite, singlet oxygen) can be induced in living cells upon exposure to ionizing radiation and also form endogenously as a result of aerobic metabolism, immune response, and inflammation (1,2). Oxidative degradation of DNA is implicated in aging, cancer, and neurodegenerative diseases (1–9). Among the four DNA bases, guanine has the lowest redox potential and is preferentially targeted for oxidation (10,11). Interestingly, oxidative guanine lesions can be found at nucleobases remote from the site of initial one-electron oxidation as a result of electron hole migration along DNA duplex to the sites of lowest ionization potential, e.g. the 5' guanines of GG and GGG repeats (10,12–15) and endogenously methylated ^{Me}CG dinucleotides (16).

Guanine oxidation gives rise to a variety of products, including 8-oxo-7,8-dihydro-2'-deoxyguanosine (OG), spiroiminodihydantoin (Sp), 5-guanidinohydantoin (Gh), 2-amino-5-[2-deoxy-β-D-erythro-pentofuranosyl)amino]-4H-imidazol-4-one (Im) and its hydrolysis product, 2,2-diamino-4-[(2-deoxy-β-D-erythropentofuranosyl)amino]-5(2H)-oxazolone (Z) (17–23), while guanine nitration in the presence of peroxynitrite produces 8-NO₂-G (24). These nucleobase adducts are associated with specific biological outcomes as a result of their distinct base pairing characteristics, sequence-dependent formation, and structural recognition by regulatory and DNA repair proteins (11,25–28). However, our understanding of sequence-dependent formation of specific DNA lesions is incomplete because of the limitations of experimental methodologies employed in previous studies.

Sequence specificity for DNA oxidation has been traditionally determined by PAGE of alkali-generated DNA fragments to reveal the sites of nucleobase damage and thus have provided little information about lesion structures (29). Oxidative DNA lesions have been broadly classified as “Fpg sensitive lesions” and “piperidine sensitive lesions” based on their propensity to be cleaved by repair proteins and alkali treatment, respectively (30–32). More recently, a novel methodology based in isotope labeling of DNA-mass spectrometry (ILD-MS) developed in our laboratory has enabled structural characterization and quantification

of oxidative nucleobase lesions originating from specific sites within DNA (16,33–36). We have previously used ILD-MS to show that photooxidation-induced OG and Z lesions preferentially form at guanine bases adjacent to 5-methylcytosine, an epigenetic DNA modification frequently found at CpG repeats of gene promoter sequences (16).

The main goal of the present investigation was to map the formation of structurally defined oxidative DNA lesions within a frequently mutated region of the *K-ras* protooncogene (codons 10–15) following photooxidation in the presence of riboflavin, treatment with nitrosoperoxycarbonate, and exposure to hydroxyl radicals. To our knowledge, this study is the first to map sequence-dependent formation of specific guanine oxidation and nitration products (OG, Z, and 8-NO₂-G) following treatment with reactive oxygen and nitrogen species. These results are important for our understanding of DNA oxidation chemistry and will be helpful in establishing a link between ROS exposure, DNA damage, mutations, and epigenetic regulation of antioxidant response.

METHODS

Chemicals.

Ammonium acetate, acetonitrile, methanol, ascorbic acid, sodium bicarbonate, hydrogen peroxide, nuclease P1, alkaline phosphatase, 8-bromo-dG, and riboflavin were purchased from Sigma-Aldrich (Milwaukee, WI). Sodium phosphate and isopropyl alcohol were from Fisher Scientific (Hanover Park, IL). Peroxynitrite was obtained from Cayman Chemicals (Ann Arbor, MI). ¹⁵N₃,¹³C₁-dG and ¹⁵N₃,¹³C₁-dG phosphoramidites were synthesized at Rutgers University as described elsewhere (37,38).

Preparation of Nucleoside Adduct Standards and Isotopically Labeled Standards.

Z, OG and their ¹⁵N₃,¹³C₁-labeled analogues were synthesized from 2'-deoxyguanosine (dG) and ¹⁵N₃,¹³C₁-dG, respectively (39). 8-NO₂-G was prepared by reacting 8-bromo-dG (Sigma-Aldrich) with a 10-fold molar excess of sodium nitrite in DMF (24,40). The reaction mixture was refluxed for 24 hours at 150 °C under nitrogen atmosphere, concentrated under vacuum, dissolved in 100 mM triethylammonium acetate (pH 7), filtered, and separated by HPLC (39). Molar concentrations of 8-NO₂-G solutions were determined by UV spectrophotometry ($\epsilon_{400} = 9.14 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (41). ¹⁵N₃,¹³C₁-8-NO₂-G was prepared analogously starting with ¹⁵N₃,¹³C₁-8-bromo-dG. ¹⁵N₃,¹³C₁-8-bromo-dG was generated by treating ¹⁵N₃,¹³C₁-dG with aqueous bromine (42), followed by purification by SPE. ¹⁵N₃,¹³C₁-8-bromo-dG was converted to ¹⁵N₃,¹³C₁-8-NO₂-G by reaction with sodium nitrite and purified as described above (24,40). Structural identities of all synthetic standards were confirmed by HPLC-UV, NMR, and tandem mass spectrometry.

DNA Oligodeoxynucleotides.

Synthetic DNA 18-mers (G₁G₂ACTG₃G₄TG₅G₆CG₇TAG₈G₉C) were prepared by solid phase synthesis, and stable isotope tag was introduced at specified positions (G₁, G₃, G₄, G₅, G₆, G₇, G₈, or G₉ as shown in Table 1). Their sequence was derived from a frequently mutated region of the *K-ras* protooncogene (codons 10–15). All oligodeoxynucleotides were prepared by standard solid phase chemistry on an Applied Biosystems ABI 394 instrument

(Foster City, CA). $^{15}\text{N}_3$, $^{13}\text{C}_1$ -dG was introduced using 1,7,NH $_2$ - ^{15}N -2- ^{13}C -dG phosphoramidite (37,38). Synthetic DNA oligomers were purified by HPLC as described previously (43). The identity and the purity of each DNA strand (> 99%) were confirmed by HPLC-ESI-MS (Table 1). Isotopically labeled DNA oligomers (200 μM) were annealed the corresponding complementary strands in 10 mM Tris-HCl buffer containing 50 mM sodium chloride (pH 8). UV melting curves were generated with a Cary 100 spectrophotometer using a 20 μM dsDNA solution in 10 mM sodium phosphate buffer containing 50 mM NaCl (pH 7). The samples were heated from 30 $^\circ\text{C}$ to 90 $^\circ\text{C}$ at a rate of 0.5 $^\circ\text{C}/\text{min}$ with a 10 min hold time, and UV $_{260}$ data was recorded every 0.2 $^\circ\text{C}$.

Exonuclease Sequencing.

To confirm the location of the $^{15}\text{N}_3$, $^{13}\text{C}_1$ -dG in each synthetic strand, they were subjected to controlled digestion with phosphodiesterase I or phosphodiesterase II, followed by analysis of the resulting exonuclease ladders by MALDI-TOF (Supplementary Tables S1 – S6) (44).

Riboflavin Mediated Photooxidation.

DNA duplexes (3 nmol, in triplicate) were dissolved in 15 μL of 100 mM NaCl. Oxygenated riboflavin solution was prepared by purging an aqueous solution of riboflavin (62.5 μM , containing 10 mM sodium cacodylate buffer, pH 7) with oxygen. DNA duplexes were combined with riboflavin solution (60 μL) to achieve the final concentrations of 50 μM riboflavin and 40 μM DNA. The reactions were placed into clear glass vials suspended in ice-cold water and irradiated for 20 min with a 60-watt tungsten bulb positioned 5 cm from the vial. Photooxidized DNA samples were either analyzed immediately or transferred to low actinic vials and stored at -80°C .

Nitrosoperoxy carbonate Treatment.

DNA duplexes (3 nmol aliquots, in triplicate) were dissolved in 20 μL of 31 mM sodium bicarbonate/188 mM sodium phosphate, pH 6.9 and cooled on ice. A 5 μL aliquot of 5 mM peroxyxynitrite solution (Cayman Chemical, Ann Arbor, MI) in 50 mM NaOH was added to each sample to achieve the final concentration of 1 mM peroxyxynitrite. Samples were vigorously mixed by vortexing for 30 s and incubated at room temperature for 30 min, followed by DNA precipitation with cold ethanol.

Hydrogen Peroxide/Ascorbic Acid Treatment.

DNA duplexes (3 nmol, in triplicate) were oxidized in the presence of 12 mM ascorbic acid and 3.5 mM of H_2O_2 in 150 mM phosphate buffer (pH 6.8) (45). Samples were incubated at 37 $^\circ\text{C}$ for 4 hours, followed by DNA precipitation with cold ethanol.

Enzymatic Hydrolysis of Oxidized DNA.

DNA samples (2 nmol) were diluted with buffer to reach the final concentration of 25 mM ammonium acetate/2.5 mM zinc chloride/5 mM TEMPO, pH 5.3. DNA hydrolysis was performed in the presence of nuclease P1 (4.5 U) and alkaline phosphatase (25 U) for 2 hours at 37 $^\circ\text{C}$. The completeness of enzymatic hydrolysis was confirmed by HPLC-UV analysis of enzymatic digests (39). Our previous experiments have revealed that under these

conditions, 8-nitro-dG is quantitatively converted to 8-NO₂-G via spontaneous depurination (29). Samples were split into two for separate analyses of Z and OG/8-NO₂-G. Aliquots designated for Z analysis were incubated 18 h at 25 °C in the dark to allow for the conversion of imidazolone to Z (46).

Concentration Dependence Experiments.

Unlabeled DNA duplexes (3 nmol, in triplicate) were irradiated in the presence of riboflavin for 0, 2, 5, 10, 20, 40, and 60 min, treated with increasing concentrations of peroxyxynitrite (0, 0.25, 0.5, 0.75, 1, 2, 5, and 10 mM), or incubated with H₂O₂/ ascorbate (0, 1, 2.5, 5, 7.5, 10, 15, and 20 mM) as described above. Following DNA precipitation with cold ethanol, samples were spiked with ¹⁵N₂, ¹³C₁-Z and ¹⁵N₃, ¹³C₁-OG (6 pmol each, internal standards for mass spectrometry) and subjected to enzymatic hydrolysis as described above.

Sample Preparation for Z Analysis by HPLC-ESI-MS/MS.

Z was purified by solid phase extraction (SPE) using 150 mg Carbograph SPE cartridges (Grace, Columbia, MD) Samples were diluted to 1 mL with water and loaded onto the cartridges, which were pre-equilibrated with methanol and water. SPE columns were washed with 1 mL of water, and Z was eluted with 3 mL of 20% methanol in water. SPE fractions containing the analyte were dried under reduced pressure and re-dissolved in 20 µL of water prior to HPLC-ESI-MS/MS analysis.

Sample Preparation for 8-oxo-dG and 8-nitro-G Analysis by HPLC-ESI-MS/MS.

OG and 8-NO₂-G were isolated from DNA hydrolysates using off-line HPLC. A Synergi Hydro-RP [4.6 × 250 mm, 4 µm] column (Phenomenex) was eluted at a flow rate of 1 mL/min with a gradient of 10 mM ammonium formate, pH 4.2 containing 6% methanol (solvent A) and 100% acetonitrile (solvent B). Solvent composition was maintained at 0% B from 0 to 32 min, followed by a linear increase to 50% B from 32 to 36 min, and kept at 50% B for 4 min. HPLC fractions containing 8-NO₂-G (16 – 20.4 min) and OG (26.9 – 32.6 min) were collected using an Agilent 1100 fraction collector and immediately placed in – 20 °C freezer to minimize secondary oxidation.

Capillary HPLC-ESI-MS/MS Analysis.

TSQ Quantum mass spectrometer was interfaced with an Agilent Technologies 1100 capillary HPLC. For analysis of Z, a Hypercarb column (0.5 × 100 mm, 5 µm) was eluted with a flow rate of 12 µL/min at a gradient of 3:1 isopropanol: acetonitrile (solvent B) in 0.05% acetic acid (solvent A). HPLC solvent composition was gradually changed as follows: 0 min, 1.5% B; 7.1 min, 10.2% B; 7.6 min, 1.5% B; 16 min, 1.5% B. Typically, the electrospray ionization (ESI) ion source temperature was set to 250 °C, with a spray voltage at 3.1 kV. Quantitative analyses were performed in the selected reaction monitoring mode. The first quadrupole was set to isolate the protonated molecules ([M + H]⁺) of Z (*m/z* 247.1) and ¹⁵N₂, ¹³C₁-Z (*m/z* 250.1), and their fragmentation was induced in the second quadrupole serving as a collision cell. Typical fragmentation energy was 14 V, with a collision gas pressure (Ar) of 1 mTorr. The third quadrupole was set to isolate the product ions corresponding to the neutral loss of deoxyribose and CO₂ ([M + 2H – dR – CO₂]⁺): *m/z* 87.1

for Z and m/z 90.1 for $^{15}\text{N}_2, ^{13}\text{C}_1\text{-Z}$). The lower limit of detection for detection of Z was estimated as 5 fmol ($S/N = 10$).

For HPLC-ESI-MS/MS analysis of OG and $^{15}\text{N}_3, ^{13}\text{C}_1\text{-OG}$, an Agilent Extend C18 column (0.5×150 mm, $3.5 \mu\text{m}$) was eluted isocratically with 13% methanol in 10 mM ammonium formate, pH 4.2. The HPLC flow rate was 11 $\mu\text{L}/\text{min}$, and the column was maintained at 10 °C. The mass spectrometry parameters were optimized to achieve maximum sensitivity. Typically, the source temperature was kept at 50 °C, and the spray voltage was set to 3.1 kV. Quantitative analyses were performed in the selected reaction monitoring mode. The first quadrupole was set to isolate the protonated molecules ($[\text{M} + \text{H}]^+$) of OG (m/z 284.1) and $^{15}\text{N}_3, ^{13}\text{C}_1\text{-OG}$ (m/z 288.1), and their fragmentation was induced in the second quadrupole serving as a collision cell. Typical fragmentation energy was 14 V, with a collision gas pressure (Ar) of 1 mTorr. The third quadrupole was set to isolate the product ions corresponding to the neutral loss of deoxyribose ($[\text{M} + 2\text{H} - \text{dR}]^+$): m/z 168.1 for 8-oxo-dG and m/z 172.1 for $^{15}\text{N}_3, ^{13}\text{C}_1\text{-OG}$. The lower limit of detection for OG was 3 fmol ($S/N = 10$).

For analyses of 8- $\text{NO}_2\text{-G}$, an Agilent Extend-C18 column (0.5×150 mm, $3.5 \mu\text{m}$) was eluted with a flow rate of 14 $\mu\text{L}/\text{min}$ at 25 °C with a gradient of 3:1 methanol:acetonitrile (solvent B) in 5 mM *N,N*-dimethylhexylamine (DMHA), pH 9.2 (solvent A). Solvent composition was changed as follows: 0 min, 3% B; 9 min, 8.4% B; 11 min, 60% B; 14 min, 60% B; 16 min, 3% B; 28 min, 3% B. 8- $\text{NO}_2\text{-G}$ was detected in the negative ion mode. Typically, the ESI source temperature was set to 250 °C, and the spray voltage was -2.7 kV. Quantitative analyses were performed in the selected reaction monitoring mode. The first quadrupole was set to isolate the deprotonated molecules ($[\text{M} - \text{H}]^-$) of 8- $\text{NO}_2\text{-G}$ (m/z 195.0) and $^{15}\text{N}_3, ^{13}\text{C}_1\text{-8-NO}_2\text{-G}$ (m/z 199.0), and their fragmentation was induced in the second quadrupole serving as a collision cell. Typical fragmentation energy was 17 V, with a collision gas pressure (Ar) of 1.3 mTorr. The third quadrupole was set to isolate the product ions at m/z 153.1 for 8- $\text{NO}_2\text{-G}$ and m/z 154.1 for $^{15}\text{N}_3, ^{13}\text{C}_1\text{-8-NO}_2\text{-G}$. The lower limit of detection for 8- $\text{NO}_2\text{-G}$ was estimated as 10 fmol ($S/N = 10$).

ILD-MS experiments to determine sequence-dependent adduct formation.

The extent of adduct formation at the $^{15}\text{N}_3, ^{13}\text{C}_1$ -labeled guanine (X) was calculated using the following equation: % reaction at X = $A_{\text{adduct-dX}} / (A_{\text{adduct-dX}} + A_{\text{adduct-dG}}) \times 100\%$, where $A_{\text{adduct-dX}}$ and $A_{\text{adduct-dG}}$ are the areas under the HPLC-ESI-MS/MS peaks corresponding to the $^{15}\text{N}_3, ^{13}\text{C}_1$ labeled and unlabeled adducts, respectively.

Statistical Analyses.

All statistical analyses were performed at the Biostatistics and Informatics Core of the Masonic Cancer Center, University of Minnesota. One-way analysis of variance (ANOVA) was used to examine the differences in mean percent reactivity among groups. If the overall F-test showed significance, the Bonferroni method was applied to adjust the p-values for pair-wise comparisons between groups. The Bonferroni method was used to maintain the overall level of significance for multiple comparisons. The two-way ANOVA tested for differences in reactivity within and between the groups, while controlling for the effect of

the individual Gs. Again, the Bonferroni method was applied to adjust p-values of pair-wise comparisons. All statistical analyses were conducted in SAS (Statistical Analysis Software) version 9.1. The significance level was set at 5%.

RESULTS

Stable Isotope Labeling Approach.

Our experimental strategy to quantify Z, OG, and 8-NO₂-G adducts originating from specific guanine bases within DNA duplexes relies on the stable isotope labeling approach (ILD-MS) developed in our laboratory (Scheme 1) (16,43). A series of DNA oligomers were constructed representing a region of *K-ras* exon 1 containing frequently mutated codon 12. In each of them, a single guanine base was replaced with a stable isotope tagged guanine (¹⁵N₃, ¹³C₁-dG) (Table 1). Following annealing to the complementary strand, site-specifically labeled DNA duplexes were subjected to riboflavin mediated photooxidation, peroxyxynitrite treatment, or incubated with hydrogen peroxide in the presence of ascorbic acid to induce hydroxyl radicals. The resulting oxidized DNA was enzymatically digested to 2'-deoxyribonucleosides under conditions that led to quantitative release of 8-nitro-G as a free base adduct. Relative amounts of oxazolone, 8-oxo-dG, and 8-nitro-G formed at each site of interest were determined by isotope ratio HPLC-ESI-MS/MS (Scheme 2 and Figure 1). Any nucleobase lesions originating from isotopically labeled G carry the ¹⁵N, ¹³C label and undergo a characteristic mass shift, making it possible to quantify the extent of adduct formation at that site by HPLC-ESI-MS/MS (16). Exact patterns of Z, OG, and 8-NO₂-G formation within *K-ras* derived duplexes were established by sequentially replacing each guanine of interest with ¹⁵N₃, ¹³C₁-dG (Table 1).

Representative HPLC-ESI-MS/MS traces of ¹⁵N₃, ¹³C₁-8-NO₂-G and 8-NO₂-G adducts resulting from nitrosoperoxycarbonate treatment of a site-specifically labeled DNA duplex (GGAGCTG[¹⁵N₃, ¹³C₁-G]TGCGTAGGC) are shown in Figure 1. Signals corresponding to 8-NO₂-G and ¹⁵N₃, ¹³C₁-8-NO₂-G are observed at *m/z* 195.1 → *m/z* 153.1 and *m/z* 199.1 → *m/z* 154.1, respectively (Figure 1A). The extent of adduct formation at the isotopically labeled site can be calculated directly from the areas under the HPLC-ESI-MS/MS peaks corresponding to the labeled and unlabeled 8-NO₂-G (Scheme 1). Site-specific formation of OG and Z adducts was analyzed analogously by following MS/MS transitions *m/z* 284.1 → *m/z* 168.0 (OG), *m/z* 288.1 → *m/z* 172.0 (¹⁵N₃, ¹³C₁-OG), *m/z* 247.1 → *m/z* 87.2 (Z) and *m/z* 250.1 → *m/z* 90.1 (¹⁵N₂, ¹³C₁-Z), respectively (Figure 1B,C) (16). By repeating the experiment using DNA duplexes labeled at specific positions, it was possible to determine the relative amount adduct formation at each guanine of interest along the DNA duplex.

Development of optimal oxidation conditions.

Preliminary experiments were conducted with unlabeled DNA duplexes to identify optimal oxidation conditions that lead to sufficient adduct numbers while maintaining sequence selectivity for DNA oxidation. We define "optimal conditions" as the lowest oxidant concentration that leads to quantifiable amounts of adducts at all positions of DNA duplex, including unfavorable oxidation sites. HPLC-ESI-MS/MS experiments have revealed that following treatment with 1 mM peroxyxynitrite, ~ 4.4% of total DNA strands contained 8-oxo-

dG, ~1.3% of strands carried Z, and ~0.4% contained 8-NO₂-G (Figure 2A). Treatment with 12 mM ascorbic acid/3.5 mM H₂O₂ (45) for 4 hours generated 8-oxo-dG in ~11% of total DNA strands and Z in ~1.5% of strands (Figure 2B). We previously established that photooxidation in the presence of riboflavin for 20 min induced 8-oxo-dG in ~4% of strands and Z in ~2% of strands (16). These conditions were selected for our experiments because they produced sufficient adduct numbers for HPLC-ESI-MS/MS quantitation at all sites, including low reactivity sites, while keeping the overall oxidation levels as low as possible to preserve DNA sequence selectivity.

Sequence-dependent formation of OG, Z, and 8-NO₂-G.

Riboflavin-mediated photooxidation.—Site-specifically labeled DNA 18-mers (Table 1) were subjected to photooxidation in the presence of photosensitized riboflavin, and the extent of OG and Z formation at each site (**G1-G9**) was determined by ILD-MS as described above (Scheme 1, Figure 1). In an event of a random reaction, each of the 13 guanines present in this duplex should give rise to 7.7% of total adducts (100%/13; “uniform reaction” bars in Figure 3A). With an exception of terminal guanine (G1), similar numbers of OG adducts originated from all guanine bases when single stranded *K-ras* oligomer was subjected to photooxidation (Supplementary Figure S1). In contrast, significant sequence effects were observed for double stranded DNA of the same sequence. OG adducts were preferentially formed at G₄ (TGG, 28.1%), G₆ (TGG, 26.2%), and G₉ (AGG, 9.2%), with these locations giving rise to over 63% of the total OG adducts formed (black bars in Figure 3A). By comparison, OG formation at G₁, G₃, G₅, G₇, and G₈ was less efficient (< 5% of total reaction, black bars in Figure 3A). These results indicate that Watson-Crick base pairing with the complementary strand and π - π stacking between neighboring bases are required for the observed sequence specificity. The same guanine bases within GG repeats were also targeted for Z formation, accounting for 12.8, 8.9, and 7.6% of total adducts at G₄, G₆, and G₉, respectively (striped bars in Figure 3A).

The elevated OG and Z formation at G₄ (TGG), G₆ (TGG), and G₉ (AGG) is consistent with low redox potential of these nucleobases (6.52, 6.52, and 6.50 eV, respectively) (47), which are located at 5' guanine in a runs of two Gs (48,49). In contrast, the sites of low reactivity (G₃, G₅, G₇, and G₈) are characterized by higher ionization potentials (7.01, 6.59, 6.63, and 6.91 eV, respectively) (47). One notable exception is the 5'-terminal guanine G₁, which gave rise to over 50% of total Z adducts, but less than 3% of total OG adducts (Figure 3A). This may be rationalized by different fate of guanine radical cations produced at the end of duplexes (see below).

Nitrosoperoxycarbonate mediated oxidation.—We next examined sequence specificity for guanine oxidation in the presence of nitrosoperoxycarbonate, a key reactive species generated from peroxynitrite and carbon dioxide under inflammatory conditions (50–52). The patterns of OG and Z formation following treatment with ONOOCO₂[−] were drastically different from those observed for riboflavin-mediated photooxidation (compare Figures 3A and 3B). While photooxidation targeted G₄ (TGG) and G₆ (TGG) (Figure 3A), OG formation following ONOOCO₂[−] treatment was most efficient at G₃ (AGC, 14.9%) and

G₇ (GGC, 11.8%). These two guanines are characterized by relatively high redox potential (7.01 and 6.63 eV, respectively) (47).

A different trend was observed for Z, which was formed inefficiently at all “internal” guanines examined (G₃, G₄, G₅, G₆, G₈, G₉). Instead, Z was overproduced at duplex ends, with 34.7% of total adducts originating from G₁ (Figure 3B). G₁ was also targeted for oxidation in ss DNA (Fig. S1B). The pattern of 8-NO₂-G formation was similar to that observed for Z, with 46.0% of 8-NO₂-G preferentially formed at G₁ (white bars in Figure 3B). These results reveal striking differences between the patterns of OG and Z adduct formation following treatment with ONOOCO₂⁻.

Hydroxyl radical-mediated oxidation.

Hydroxyl radicals were generated from hydrogen peroxide and ascorbate as described by Nappi and Vass (45). Unlike our results for other two reactive oxygen species, •OH-mediated formation of OG adducts within *K-ras* derived DNA duplexes showed little sequence specificity, with most of the internal Gs giving rise to similar adduct numbers (Figure 3C). The yields of Z were low at all internal guanine bases tested. However, enhanced formation of both OG and Z was observed at duplex ends (24.4% and 26.5% of total adducts at G₁, Figure 3C).

DISCUSSION

Recent studies within the framework of The Cancer Genome Atlas (TCGA) have revealed that *K-ras* is among the most frequently mutated genes in human cancer, with activating mutations of *K-ras* codon 12 and 13 found in colon and rectal carcinoma, uterine endometrial carcinoma, and lung adenocarcinoma (53). Specifically, *K-ras* codon 12 mutations (GGT → TGT, GTT, GAT) are observed in 24 – 56% of adenocarcinomas of the lung (54–56). In normal cells, KRAS protein is essential to the regulation of cellular growth and development (55). However, point mutations within *K-ras* codons 12, 13, or 61 activate this protooncogene, leading to uncontrolled cell growth, a loss of cell differentiation, and malignant transformation (57).

The majority of lung cancer cases (> 80%) are induced by cigarette smoking. It has been hypothesized that smoking-mediated oxidative degradation of DNA contributes to the development of smoking-mediated lung cancer (58,59). Cigarette tar contains phenolic and polyphenolic compounds which can become sources of the superoxide anion radical (O₂^{•-}) via redox cycling (58). O₂^{•-} can undergo dismutation to form hydrogen peroxide (H₂O₂), which is converted to hydroxyl radicals (•OH) via Fe²⁺-mediated Fenton reaction (1). Tobacco smoke also contains high concentrations of nitric oxide (•NO) (>300 ppm) (60,61). •NO combines with O₂^{•-} to form peroxynitrite (ONOO⁻), a potent oxidizing and nitrating agent (62,63). Peroxynitrite can undergo homolysis to •OH and nitrogen dioxide (•NO₂) or can react with carbon dioxide to yield nitrosoperoxycarbonate (ONOOCO₂⁻). The latter rapidly decomposes to carbonate radical (CO₃^{•-}) and •NO₂ (30,51,62,64), which readily react with DNA to form a range of guanine lesions including 8-nitro-G, 8-oxo-dG, and oxazolone (2,65–67).

If formed within *K-ras* codons 12 and 13, oxidative guanine lesions can be misread by DNA polymerases, contributing to initiating mutations within this important protooncogene (6,7,66–68). However, it is not known to what extent codons 12 and 13 are targeted by reactive oxygen species and what nucleobase lesions are formed. Previous studies have relied on gel electrophoresis (30,31,69), next generation sequencing, and nanopore methodologies (70–72) to detect oxidative damage within DNA duplexes. Although these methodologies enable mapping DNA oxidation along broad genomic regions, they provide little information about adduct structures.

The goal of the present work was to quantify the formation of structurally defined DNA lesions (OG, Z, and 8-NO₂-G) at specific guanine bases within *K-ras* derived DNA duplexes (5'-G₁G₂AG₃CTG₄G₅TG₆G₇CG₈TAG₉G₁₀C-3'; codon 12 = G₄G₅T) following treatment with reactive oxygen and nitrogen species. We took advantage of a new stable isotope labeling of DNA-mass spectrometry strategy developed in our laboratory (IDL-MS) because it provides accurate quantitation of specific DNA lesions formed at each site of interest (16,33,34).

Three types of oxidative treatments were chosen for the present study of DNA sequence effects on guanine oxidation: riboflavin-mediated photooxidation, treatment with nitrosoperoxycarbonate, and exposure to hydroxyl radicals generated from H₂O₂/ascorbate. Riboflavin is a typical triplet-excited Type I photosensitizer previously used in many previous studies of DNA oxidation (16,21,48,69,73–77). Nitrosoperoxycarbonate (ONOOCO₂⁻) is a product of peroxynitrite reaction with carbon dioxide and is considered the chemical mediator of inflammation (78–80). Hydroxyl radicals (HO•) are highly reactive species that are formed endogenously via Fenton reaction between hydrogen peroxide and ferrous iron (Fe²⁺) (81). Hydrogen peroxide is a product of superoxide dismutase-mediated reaction of superoxide anions, which is commonly found *in vivo* as a byproduct of oxidative metabolism (23). The three oxidants selected for this study engage in DNA oxidation via distinct mechanisms (Schemes 2–4) and were anticipated to target different sites within DNA duplex.

Riboflavin-mediated photooxidation of *K-ras* derived DNA duplexes resulted in non-random formation of oxidized bases, with both OG and Z showing a preference for the 5'-G in GG repeats in internal regions of the duplex (G₄ and G₆ in Figure 3A) (16,47,82). Photosensitization-mediated oxidation of DNA gives rise to guanine radical cation (G^{•+}), which can undergo nucleophilic addition of water at the C8 position to generate OG or deprotonation to form the guanyl radical (G(-H)•), which gives rise to Z (Scheme 2) (75,83,84). Preferential oxidation of 5'-G in GG runs following photooxidation is likely a result of their relatively low ionization potential, leading to electron hole migration to these sites (48,69). This is consistent with earlier reports that GG repeats are among the most electron-rich sites in B-form of DNA (48) and may act thermodynamic “sinks” for oxidative damage following long-range charge transport from other sites in the helix (GG stacking rule) (12,30,48,69,85).

In addition, Z (but not OG) adducts were overproduced at duplex termini (G1 in Figure 3A), probably a result of preferential deprotonation of G^{•+} to G(-H)• at partially separated duplex

ends. Rokhlenko *et al.* have shown that G:C base pairing in double stranded DNA inhibited the full release of the proton from $G^{\bullet+}$, causing $G(-H)^{\bullet}$ to retain some of the cationic characteristics of $G^{\bullet+}$ (84). These authors reported that the $[G(-H)^{\bullet}:H^+C \leftrightarrow G^{\bullet+}:C]$ equilibrium had a lifetime of ~ 0.22 ms, compared ~ 50 ns for free $G^{\bullet+}$ (84,86). The stabilization of $G^{\bullet+}$ was not observed at duplex ends where the double stranded character of DNA is compromised, resulting in the rapid deprotonation of $G^{\bullet+}$ and its ultimate conversion to Z rather than to OG (Scheme 2, Figure 3A) (30,84,86).

Treatment of site-specifically labeled *K-ras* DNA duplexes with nitrosoperoxycarbonate ($ONOOCO_2^-$) exhibited a different sequence specificity, with G_3 , and G_7 (sites of relatively high redox potential) (49) preferentially forming OG and G_1 giving rise to the greatest numbers of NO_2 -G and Z (Figure 3B). These drastic differences between the patterns of adduct formation may be explained by the chemistry of DNA oxidation in the presence of $ONOOCO_2^-$ and its homolysis products, carbonate radical anion ($CO_3^{\bullet-}$) and nitrogen dioxide ($NO_2^{\bullet}$) (51,79). Electron abstraction from G by $CO_3^{\bullet-}$ produces $G^{\bullet+}$, which can be hydrated to form OG, deprotonated to form Z, or react with $^{\bullet}NO_2$ to form 8- NO_2 -G (Scheme 3) (87–94). As noted previously by Dedon et al. (30). $ONOOCO_2^-$ mediated oxidation does not take place at the sites of lowest redox potential, but rather is dependent on solvent accessibility of guanine bases and the extent of double stranded character.

The hydrophobic interior regions of dsDNA are not solvent accessible, therefore the initial radical attack on dG must occur either in the major (C8 position exposed) or minor (N^2 position exposed) grooves of DNA. Small radical species such as $^{\bullet}OH$, $CO_3^{\bullet-}$, and $^{\bullet}NO_2$ will form hydrogen bonds with the surrounding water. In a bulk solvent, it takes between 1 and 5 ps to break and reform hydrogen bonds allowing solute molecules to randomly diffuse through the solvent (95). Compared to the bulk solvent, the water molecules in the grooves of dsDNA are more ordered, especially in the minor groove (95–98). The re-ordering of hydrogen bonds is slower in the grooves, with more than a 5-fold reduction in speed in the minor groove, in addition to constraints on solute orientation, which can slow down or inhibit reaction of radical with DNA (95,98). The loss of duplex character of DNA at the ends of the strand causes a widening of the grooves which disrupts the water layer which accelerates the ability of solutes to approach the DNA and removed the constraints on orientation (95,97,98). Therefore small radical species such as $CO_3^{\bullet-}$ can easily approach at these locations, resulting in enhanced oxidative nucleobase damage at duplex ends (G_1 in Figure 3B).

In contrast to our results for photooxidation (Figure 3A) and nitrosoperoxycarbonate (Figure 3B), hydroxyl radical-mediated guanine oxidation exhibited little sequence selectivity (Figure 3C) (99). Unlike the former two ROS, $^{\bullet}OH$ does not produce guanine radical cation intermediates ($G^{\bullet+}$), but instead directly adds to the C8 position of dG (Scheme 4). Due to their extreme reactivity, hydroxyl radicals can on average diffuse no more than 70 Å in a cell before reacting with biomolecules (100). Hydroxyl radical-mediated “footprinting” has been exploited to reveal DNA-protein and DNA-small molecule interactions (99,101,102). Hydroxyl radicals can directly add to the C8 position of guanine producing 8-OH- $G^{\bullet}$ radical, which further oxidizes to OG (100,103). Alternatively, $HO^{\bullet}$ can abstract a hydrogen atom from guanine to yield the $G(-H)^{\bullet}$ radical, which can further oxidize to form Z (Scheme 3)

(100,103). Previous reports suggested that DNA sequence has little, if any, effect on $\bullet\text{OH}$ mediated oxidation (102,104,105). Therefore, solvent accessibility to $\bullet\text{OH}$ appears to be the driving force defining the reactivity patterns and leading to the preferential production of both OG and Z at duplex ends (Figure 3C).

Preferential formation of OG rather than Z in the interior regions of the DNA duplex following treatment with H_2O_2 /ascorbate (Figure 3C) may be explained by their mechanism of formation (Scheme 4), which involves $\bullet\text{OH}$ attack at the C8 and the N^2 positions of guanine, respectively. The N^2 position of dG is buried in the minor groove under an ordered layer of water and is therefore less accessible than the C8 position which faces the major groove where the water is less structured and without the constraints to solute orientation (95,97,98). Therefore, in dsDNA, $\bullet\text{OH}$ attack at the C8 position to form 8-OH-G $\bullet$ is more favorable than the abstraction of a hydrogen atom from the N^2 position (Scheme 4), leading to preferential formation of OG (106). In regions of partially single stranded DNA such as duplex ends, the water layer in the minor groove break down, resulting in a lower energy barrier for $\bullet\text{OH}$ attack at guanine N^2 and an increased formation of Z (95–98).

Our observation that OG lesions are preferentially formed at the first guanine of *K-ras* codon 12 under conditions of one electron oxidation (GGT, G₄ in Figure 3A) is of potential significance because this site is commonly mutated in lung cancer (G $\rightarrow$ T) (54,107–109). Site specific mutagenesis and polymerase bypass studies have revealed that OG and its secondary oxidation products primarily cause G to T transversions (25,68,110). Therefore, oxidative damage induced by smoking and inflammation is a possible contributor to the induction of cancer-initiating mutations at *K-ras* codon 12. However, it should be noted that the reactivity of DNA bases towards ROS in cellular systems is anticipated to be mediated by chromatin structure, and the mutational spectra in tumors will be further affected by mutant selection for growth.

In addition, our results suggest that Z and 8-NO₂-G adducts are formed in preference to OG in genomic regions with reduced double stranded character, e.g. in DNA that is undergoing replication, actively transcribed regions, and at guanines paired with abasic sites. Previous studies have shown that 8-NO₂-G preferentially pairs with dA during DNA replication, giving rise to G to T transversions, while Z causes G to C transversions (66,89,111,112). This may lead to increased mutagenesis within actively transcribed regions, unless DNA repair machinery is recruited to remove the damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Z	Z, 2,2-diamino-4-[(2-deoxy- β -D-erythropentofuranosyl)amino]-5(2H)-oxazolone
OG	8-oxo-7,8-dihydro-2'-deoxyguanosine
8-NO₂-G	8-nitroguanine
MeC	5-methyl-2'-deoxycytidine
ILD-MS	isotope labeling of DNA-mass spectrometry
SPE	solid phase extraction
DMHA	<i>N,N</i> -dimethylhexylamine

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Figure 1.

HPLC-ESI-MS/MS analysis 8-NO₂-G (A) OG (B), and Z (C) in enzymatic hydrolysates of peroxynitrite-treated DNA duplex (G₁G₂AG₃CTG₄[¹⁵N₃,¹³C₁-G₅]TG₆G₇CG₈TAG₉G₁₀C). ¹⁵N-labeled signals correspond to adduct formation at G₅ as compared to unlabeled adducts generated elsewhere in the sequence.

Figure 2. Concentration-dependent formation of OG, Z, and 8-NO₂-G in dsDNA subjected to oxidation in the presence of peroxynitrite (A) and H₂O₂/ascorbate (B).

Figure 3.

Distribution of OG and Z lesions along *K-ras*-derived DNA duplex following riboflavin mediated photooxidation (A), treatment with nitrosoperoxycarbonate (B) and hydrogen peroxide/ascorbate (C).

Scheme 1.

Strategy for quantitation of oxidative guanine lesions originating from specific sites within DNA sequence.

Scheme 2.
Guanine oxidation in the presence of riboflavin.

Scheme 3.
Guanine oxidation in the presence of ONOOCO_2 .

Scheme 4.
Guanine oxidation in the presence of hydroxyl radicals.

Table 1.

DNA sequences used in the present study.

ID	Sequence	Observed MW	Calculated MW
[¹⁵ N ₃ , ¹³ C ₁]- <i>Kras</i> -G1	[¹⁵ N ₃ , ¹³ C ₁ -G]GAGCTGGTGGCGTAGGC	5639.9	5640.7
[¹⁵ N ₃ , ¹³ C ₁]- <i>Kras</i> -G3	GGA[¹⁵ N ₃ , ¹³ C ₁ -G]CTGGTGGCGTAGGC	5639.7	5640.7
[¹⁵ N ₃ , ¹³ C ₁]- <i>Kras</i> -G4	GGAGCT[¹⁵ N ₃ , ¹³ C ₁ -G]GTGGCGTAGGC	5640.0	5640.7
[¹⁵ N ₃ , ¹³ C ₁]- <i>Kras</i> -G5	GGAGCTG[¹⁵ N ₃ , ¹³ C ₁ -G]TGGCGTAGGC	5639.9	5640.7
[¹⁵ N ₃ , ¹³ C ₁]- <i>Kras</i> -G6	GGAGCTGGT[¹⁵ N ₃ , ¹³ C ₁ -G]GCGTAGGC	5640.0	5640.7
[¹⁵ N ₃ , ¹³ C ₁]- <i>Kras</i> -G7	GGAGCTGGTG[¹⁵ N ₃ , ¹³ C ₁ -G]CGTAGGC	5640.0	5640.7
[¹⁵ N ₃ , ¹³ C ₁]- <i>Kras</i> -G8	GGAGCTGGTGGC[¹⁵ N ₃ , ¹³ C ₁ -G]TAGGC	5640.0	5640.7
[¹⁵ N ₃ , ¹³ C ₁]- <i>Kras</i> -G9	GGAGCTGGTGGCGTA[¹⁵ N ₃ , ¹³ C ₁ -G]GC	5640.3	5640.7
(-)- <i>Kras</i>	GCCTACGCCACCAGCTCC	5365.0	5365.5