Base Modification and Strand Breakage in Isolated Calf Thymus DNA and in DNA from Human Skin Epidermal Keratinocytes Exposed to Peroxynitrite or 3-Morpholinosydnonimine

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Exposure of isolated calf thymus DNA and human skin epidermal keratinocytes to peroxynitrite or the peroxynitrite generator, 3-morpholinosydnonimine (SIN-1), led to extensive DNA base modification. Large increases in xanthine and hypoxanthine, possible deamination products of guanine and adenine, respectively, and in 8-nitroguanine were observed, but only small changes in some oxidized base products were seen. This pattern of damage suggests that hydroxyl radicals were not major contributors to base modification caused by peroxynitrite, as $OH^{\bullet}$ is known to cause multiple oxidative modifications to all four DNA bases. Instead, it seems that reactive nitrogen species play a much greater role in the mechanism of base damage, producing both nitration and deamination of purine bases when DNA or whole cells are exposed to peroxynitrite. If this pattern of damage is unique to peroxynitrite, it might act as a marker of cellular damage by this species *in vivo*.

Introduction

Many pathological conditions including ischemia/reperfusion, inflammation, and sepsis may induce tissues to simultaneously produce an excess of both superoxide ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$). $NO^{\bullet}$ and $O_2^{\bullet-}$ produced by activated phagocytes may play a role in the multistage carcinogenesis process, triggered by chronic infection and inflammation (*1*, *2*). It has been reported that $NO^{\bullet}$ can induce damage by leading to deamination of DNA bases, resulting in mutation (*3*, *4*).

Both $O_2^{\bullet-}$ and $NO^{\bullet}$ contain unpaired electrons and can react at near diffusion limited rates to form the peroxynitrite anion ($ONOO^-$). The reaction rate constant has been determined to be 6.7 ($\pm$0.9) $\times$ 10^9 M^{-1} s^{-1} (*5*). Peroxynitrite is stable at alkaline pH, but decomposes to nitrate at physiological pH after protonation to form peroxynitrous acid (ONOOH) ($pK_a = 6.8$). The half-life of ONOOH is extremely short, but it may be able to diffuse some distance on a cellular scale (100 μm) and it may even cross cell membranes (*6*).

It has become clear that peroxynitrite is a powerful, toxic oxidizing and nitrating agent that can be produced *in vivo* (*7*−*11*). It may initiate reactions currently thought to be characteristic of hydroxyl radical ($OH^{\bullet}$), nitryl cation (NO_2^+), and the nitrogen dioxide radical ($NO_2^{\bullet}$) (*7*, *9*, *12*, *13*). Many different oxidative pathways exist due to the chemical nature of the O−O bond of peroxynitrous acid, which may react as if it was cleaved either homolytically into $OH^{\bullet}$ and $^{\bullet}NO_2$ or heterolytically into OH^- and NO_2^+. Some evidence for $OH^{\bullet}$ formation has been obtained by ESR spin trapping (*14*) and by aromatic hydroxylation experiments (*13*, *15*). However, production of $OH^{\bullet}$ via simple homolytic fission of peroxynitrous acid is thought to be unlikely due to thermodynamic constraints (*7*). Peroxynitrite also oxidizes sulfhydryl groups and induces membrane lipid oxidation (*16*−*18*). It nitrates tyrosine residues in proteins to form 3-nitrotyrosine (*6*, *19*), which has been measured as a putative marker of peroxynitrite-mediated damage in inflamed tissues (*19*−*23*). The postulated ability of peroxynitrous acid to cross cell membranes (*6*) means it may penetrate into the cell nucleus where it might induce damage to DNA. Yermilov et al. (*24*, *25*) have shown that $ONOO^-$ can rapidly react with guanine under physiological conditions to form 8-nitroguanine. It has also been reported that peroxynitrite induces strand breaks in plasmid DNA and oxidative damage in isolated DNA *in vitro* (*26*, *27*) as well as being mutagenic (*28*).

In this study we show that $ONOO^-$ not only causes extensive base modification to isolated DNA but also produces significant base modification and causes strand breakage to DNA when human keratinocytes are exposed to $ONOO^-$ or to the $ONOO^-$ generator 3-morpholinosydnonimine (SIN-1).[1] We also show that the pattern of damage caused by peroxynitrite suggests that free $OH^{\bullet}$ radicals are not involved but that reactive nitrogen species seem to play the major role in DNA base modification.

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[1] Abbreviations: GC-MS: gas chromatography−mass spectroscopy; FAPy-adenine: 4,6-diamino-5-formamidopyrimidine; FAPy-guanine: 2,6-diamino-5-formamidopyrimidine; 5-OH,Me-uracil: 5-(hydroxymethyl)uracil; 5-OH,Me-hydantoin: 5-(hydroxymethyl)hydantoin; SIN-1: 3-morpholinosydnonimine, BSTFA: bis(trimethylsilyl)trifluoracetamide, TMS: trimethylchlorosilane; HBSS: Hank's balanced salt solution; CDTA: *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS: sodium dodecyl sulfate.

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Materials and Methods

Materials. Chemicals were of the highest quality available from Sigma Chemical Co. (Poole, Dorset, U.K.), from the BDH Chemical Co. (Gillingham, Dorset, U.K.) and/or Aldrich Chemical Co. (Milwaukee, WI). Calf thymus DNA was Sigma type I.

SIN-1 was purchased from Molecular Probes Inc. (Eugene, OR). 8-Azaadenine, 6-azathymine, diaminopurine, 8-bromoadenine, 5-hydroxyuracil (isobarbituric acid), 4,6-diamino-5-formamidopyrimidine (FAPy-adenine), 2,5,6-triamino-4-hydroxypyrimidine, and 5-(hydroxymethyl)uracil were purchased from Sigma. 8-Hydroxyguanine was purchased from Aldrich (Poole, Dorset, U.K.). 8-Hydroxyadenine and FAPy guanine were synthesized (courtesy of Dr. H. Kaur, King's College London) by, respectively, treatment of 8-bromoadenine with concentrated formic acid (95%) at 150 °C for 45 min with purification by crystallization from water, as described in ref 29, and treatment of 2,5,6-triamino-4-hydroxypyrimidine with concentrated formic acid with purification by crystallization from water, as described in ref 30. Thymine glycol was synthesized by reaction of 5-methyluracil with OsO_4 for 1 h at 60 °C, and excess OsO_4 was removed by freeze-drying (*31*). 8-Nitroguanine was synthesized by reaction of peroxynitrite (5 mM) with guanine (3 mg) under constant stirring and was purified by preparative HPLC (*24*). Purity of synthesized standards was assessed by mass spectrometry, and all were found to be >99% pure. 2-Hydroxyadenine, 5-hydroxycytosine, and 5-(hydroxymethyl)hydantoin were gifts from Dr. M. Dizdaroglu (National Institute of Standards and Technology, Gaithersburg, MD).

Cellu.Sep dialysis membranes with a relative molecular mass cut off of 3500, silylation grade acetonitile, and bis(trimethylsilyl)trifluoracetamide (BSTFA) (containing 1% trimethylchlorosilane, TMS) were obtained from Pierce Chemical Co. (Rockford, IL). Distilled water passed through a purification system (Elga, High Wycome, Bucks, U.K.) was used for all purposes.

Alamar blue was from Alamar Biosciences (Sacramento, CA). Dulbecco's modified Eagle's medium was from Flow Laboratories (Mclean, VA), and fetal calf serum was from Hyclone Laboratories (Logan, UT).

DNA Damage Induced by Peroxynitrite. Peroxynitrite was synthesized in a quenched flow reaction system, by mixing H_2O_2 (0.6 M) with $NaNO_2$ (0.6 M) under acidic conditions. The $ONOO^-$ was trapped in a NaOH solution (1.2 M), in which it was stable for several weeks. Peroxynitrite was used from the liquid layer formed by freeze fractionation, and excess H_2O_2 was destoyed by granular MnO_2 to eliminate possible base modification by species derived from this oxidant. Prior to each experiment, $ONOO^-$ was quantified spectrophotometrically at 302 nm using a molar extinction coefficient of 1670 M^{-1} cm^{-1} (typical concentrations were 200−300 mM), and thawed $ONOO^-$ solutions were kept on ice (*6*). The reactions were carried out at room temperature (~22 °C). Peroxynitrite at various concentrations was added to a reaction mixture (final volume 1.0 mL) containing sodium phosphate buffer (0.1 M; pH 7.0) and calf thymus DNA (0.2 mg). Solutions were mixed rapidly during addition of $ONOO^-$ for approximately 10 s. The maximum volume of the $ONOO^-$ solution added was 10 μL which gave a final pH of 7.2. Experiments were repeated in reaction mixtures of pH 6.0 and 8.0 to determine the pH dependence of reactivity. Control experiments were performed using the same concentrations of decomposed peroxynitrite (dilution of peroxynitrite in phosphate buffer at pH 7.4 results in rapid decomposition and generated a solution which was used as the control).

Cell Culture. A spontaneously immortalized line of human skin epidermal keratinocytes (HaCaT), derived from adult trunk skin and maintaining features of normal epidermal growth and differentiation, was used (*32*). The cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Exposure of Human Keratinocytes to Peroxynitrite and SIN-1. Cells of approximately 90% confluency were used. Growth medium was removed and cells were washed twice with sterile, filtered PBS. A Hank's balanced salt solution (HBSS) was used for the exposure experiments. HBSS did not affect cell viability, measured using the AlamarBlue assay (*33*). Stock solutions of both peroxynitrite and SIN-1 were made up immediately prior to experiments. Peroxynitrite exposures were to cells in suspension. Cells were removed from plates using trypsin and resuspended in HBSS (~5 million/mL). Peroxynitrite (1 mM) was added directly to cells in suspension followed by constant mixing for 10 s (final pH 7.2). SIN-1 (1 mM) in HBSS was added to cell plates and a 60 min incubation at 37 °C followed. SIN-1 incubated at 37 °C for 24 h before addition to cells was the control for SIN-1 experiments. After incubation, the solutions were removed and cells were washed twice in filtered PBS.

AlamarBlue Assay for Cell Viability. The AlamarBlue assay (*33*) was performed as previously published (*34*) and is a measure of cell viability based on mitochondrial dehydrogenase activity.

Isolation of DNA from Cultured Mammalian Cells. DNA isolation from cells and assessment of RNA contamination were carried out as previously described (*34*).

Analysis of DNA Base Modification by Gas Chromatography−Mass Spectroscopy. Derivatized samples were analyzed by GC-MS (Hewlett-Packard 5890II gas chromatograph interfaced with a Hewlett-Packard 5917A mass selective detector). Preparation, hydrolysis, derivatization, and analysis of samples were performed as described previously (*35*, *36*) with the following modifications. The injection port and the GC-MS interface were kept at 250 and 290 °C, respectively. Separations were carried out on a fused silica capillary column (12 m × 0.2 mm i.d.) coated with cross-linked 5% phenylmethylsiloxane (film thickness 0.33 μm) (Hewlett-Packard). Helium was the carrier gas with a flow rate of 0.93 mL/min. Derivatized samples (1.0 μL) were injected into the GC injection port using a split ratio of 8:1. Column temperature was increased from 125 to 175 °C at 8 °C/min after 2 min at 125 °C, then from 175 to 220 °C at 30 °C/min and held at 220 °C for 1 min, and finally from 220 to 290 °C at 40 °C/min and held at 290 °C for 2 min. Selected-ion monitoring was performed using the electron-ionization mode at 70 eV with the ion source maintained at 185 °C.

Quantitation of modified bases was achieved by relating the peak area of the compound with the internal standard peak area and applying the following formula:

$$\text{concn (nmol/mg of DNA)} = A/A_{\text{IST}} \times [\text{IST}] \times (1/K)$$

where K = relative molar response factor" for each damaged base, A = peak area of product, A_{IST} = the peak area of internal standard, and [IST] = concentration of the internal standard (5 nmol/mg of DNA). K constants were calculated from the slopes of the calibration curves constructed using known concentrations of internal standards and authentic compounds.

Measurement of 8-Nitroguanine. DNA samples were hydrolyzed with 60% (v/v) formic acid for 45 min at 150 °C. Hydrolysates were then analyzed by UV-HPLC. The HPLC system consisted of a pump (ACS isocratic pump 351, Applied Chemical Systems, Macclesfield, Cheshire, U.K.), Rheodyne injection valve, Model 7125 (Fisons, Loughborough, Leicestershire, U.K.), and a stainless steel column (250 × 4.6 mm) packed with Nucleosil RP-C18, 5 μm (Fisons, Loughborough, Leicestershire, U.K.). The detection was carried out by means of a UV detector (ACS, Macclesfield, Cheshire, U.K.) at 396 nm, as previously reported by Yermilov et al. (*25*). An integrator (Data system 450 PCIP version 3.94, Kontron Instruments, Watford, U.K.) linked to a PC was used to monitor the current signal at a chart speed of 0.25 cm/min. The mobile phase (pH 6.0) consisted of ammonium formate buffer (20 mM) which was pumped through the system at a flow rate of 1.0 mL/min. Levels were quantified using a standard curve generated using the synthesized standard which was checked for purity using mass spectroscopy.

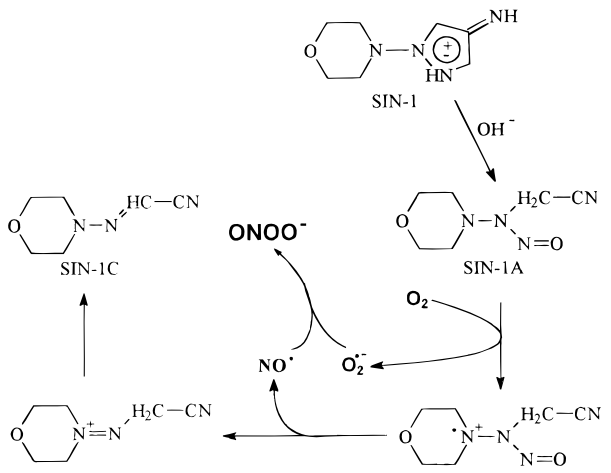

Figure 1. The generation of peroxynitrite ($ONOO^-$) from SIN-1. SIN-1 is stable at acidic pH but breaks down at neutral pH. In the process, several intermediates are formed including both superoxide ($O_2^{\bullet-}$) and nitric oxide ($^{\bullet}NO$), resulting in $ONOO^-$ formation. At physiological pH, SIN-1 decomposition has been shown to generate about 1 μM/min $ONOO^-$.

Fluorometric Detection of DNA Strand Breaks. DNA strand breaks were detected using the alkaline unwinding/ethidium bromide assay in which the fluorescent dye binds selectively to double-stranded DNA (*37*, *38*).

Cells after exposure were washed with PBS and then pelleted and resuspended to achieve a 5−10 million cell/mL concentration. Aliquots of the cell suspension (0.2 mL) were placed in 12 tubes labelled T (total), P (test), or B (blank) in groups of 4. To each was added 0.2 mL of cell lysis and chromatin disruption buffer (urea, 9.0 M; NaOH, 10 mM; CDTA, 2.5 mM; SDS, 0.1%), and tubes were incubated at 0 °C for 10 min to allow total disruption of chromatin. To tubes P and B, 0.1 mL of alkaline unwinding solution A (0.45 volume of cell lysis buffer in 0.2 M NaOH) and 0.1 mL of alkaline unwinding solution B (0.4 volume of cell lysis buffer in 0.2 M NaOH) were added without mixing. During the subsequent incubation at 0 °C for 30 min, the alkali diffuses into the viscous lysate to give a final pH of about 12.8. Before the alkali solutions were added to tube T, 0.4 mL of denaturation prevention solution was added (glucose, 1 M; mercaptoethanol, 14 mM), ensuring that the DNA is never exposed to a denaturing pH (∼11.0). All tubes were then incubated for 60 min at 15 °C, and denaturation was stopped by addition of 0.4 mL of denaturation prevention solution and chilling to 0 °C. To each tube is added 1.5 mL of ethidium bromide solution (ethidium bromide, 6.7 μg/mL; NaOH, 13.3 mM) and their fluorescence is read at room temperature (excitation: 520 nm; emission: 590 nm).

The extent of DNA unwinding after a given time of exposure of cell extracts to alkali is calculated from the formula:

$$\% \text{ DS DNA} = (P - B)/(T - B) \times 100$$

Results

Studies with Isolated DNA. The use of gas chromatography−mass spectroscopy enabled us to demonstrate that both $ONOO^-$ and the $ONOO^-$ generator SIN-1 (Figure 1) cause damage to all four DNA bases. However, oxidized base products did not increase very much in amount, whereas, levels of xanthine, hypoxanthine, and nitrated guanine increased dramatically (Table 1). Furthermore, generation of damaged products appeared to be dependent on the exposure environment, in particular pH.

Panels A and B of Figure 2 show the increases in modified pyrimidine and modified purine bases, respectively, when isolated DNA is exposed to $ONOO^-$ at physiological pH. Small but significant increases in 8-hydroxyguanine, 5-OH-hydantoin, thymine glycol (*trans*), 5-OH,Me-uracil, and the ring opened products FAPy-adenine and FAPy-guanine were observed. No significant changes in 5-OH-uracil, 5-OH-cytosine, 5-OH,Me-hydantoin, 2-hydroxyadenine, and 8-hydroxyadenine were apparent. By contrast, there were very large rises in xanthine, hypoxanthine, and 8-nitroguanine (Table 1; Figure 3) when DNA was exposed to $ONOO^-$. Figure 3 also shows the pH dependence of xanthine, hypoxanthine, and 8-nitroguanine generation by $ONOO^-$. Product generation on exposure to $ONOO^-$ (1 mM) appears to be dependent on pH. 8-Hydroxyguanine formation is optimal at pH 6.0, while xanthine and hypoxanthine formation is favored at pH 7.4, and 8-nitroguanine yields are maximal at pH 8.0 (Figure 4). No changes in base damage were observed when DNA was treated with decomposed peroxynitrite, indicating that changes in products were dependent on peroxynitite and not due to other constituents of the solution.

Table 1. Levels of DNA Base Modification Products in Control DNA and in DNA Exposed to Peroxynitrite (1 mM) at pH 7.4[a]

base product	levels in control DNA (nmol/mg of DNA)	levels in DNA exposed to $ONOO^-$ (1 mM) (nmol/mg of DNA)
5-OH-hydantoin	0.36 ± 0.20	1.06 ± 0.07*
5-OH-uracil	0.11 ± 0.02	0.14 ± 0.01
5-OH,Me-uracil	0.03 ± 0.01	0.15 ± 0.06*
thymine glycol	0.77 ± 0.07	1.86 ± 0.11*
hypoxanthine	1.33 ± 0.11	5.96 ± 0.56*
xanthine	1.83 ± 0.21	303.85 ± 26*
FAPy-adenine	0.21 ± 0.02	0.38 ± 0.04*
8-OH-adenine	0.49 ± 0.03	0.52 ± 0.06
FAPy-guanine	0.45 ± 0.03	0.68 ± 0.05*
8-OH-guanine	0.21 ± 0.01	1.23 ± 0.07*
8-Nitro-guanine	0.87 ± 0.07	123.52 ± 7.90*

[a] Control DNA was commercial calf thymus DNA. Data are plotted as the mean ±SD, $n = 4$. *Significant increase over control, $p \leq 0.05$).

Studies with Keratinocytes. Exposure of human keratinocytes to $ONOO^-$ (1 mM) or SIN-1 (1 mM, 60 min) led to small but significant increases in DNA strand breakage. SIN-1 produced a slightly greater extent of strand breakage than $ONOO^-$ ($ONOO^-$: 21.0 ± 2.6%; SIN-1: 29.0 ± 3.5% strand breakage). These levels of the compounds did not affect cell viability measured just before DNA extraction, but further incubation for 24 h led to small reductions in viability which were most apparent in $ONOO^-$ exposed cells ($ONOO^-$: 81% viable; SIN-1: 92% viable compared to control cells: 100% viable). Thus, neither $ONOO^-$ nor SIN-1 appears lethal at the concentrations used in our experiments.

Levels of oxidative DNA base modification in the control (see Materials and Methods) cells were low, as expected. Exposure of cells to $ONOO^-$ and SIN-1 produced small but significant rises in 8-OH-guanine, FAPy-guanine, and FAPy-adenine and in 5-OH-hydantoin (Figure 5). However, as in the experiments with calf thymus DNA, the most striking increases were in xanthine, hypoxanthine, and 8-nitroguanine (Figure 5). No significant changes in 5-OH,Me-hydantoin, 5-OH-uracil, 5-OH,Me-uracil, 5-OH-cytosine, thymine glycol, or 8-OH-adenine were observed. Levels of 8-nitroguanine of about 8 nmol/mg of DNA correspond to 2−3 8-nitroguanines per 10^3 DNA bases, about the same as the level observed when DNA is exposed to 100 μM $ONOO^-$ *in vitro* (*25*).

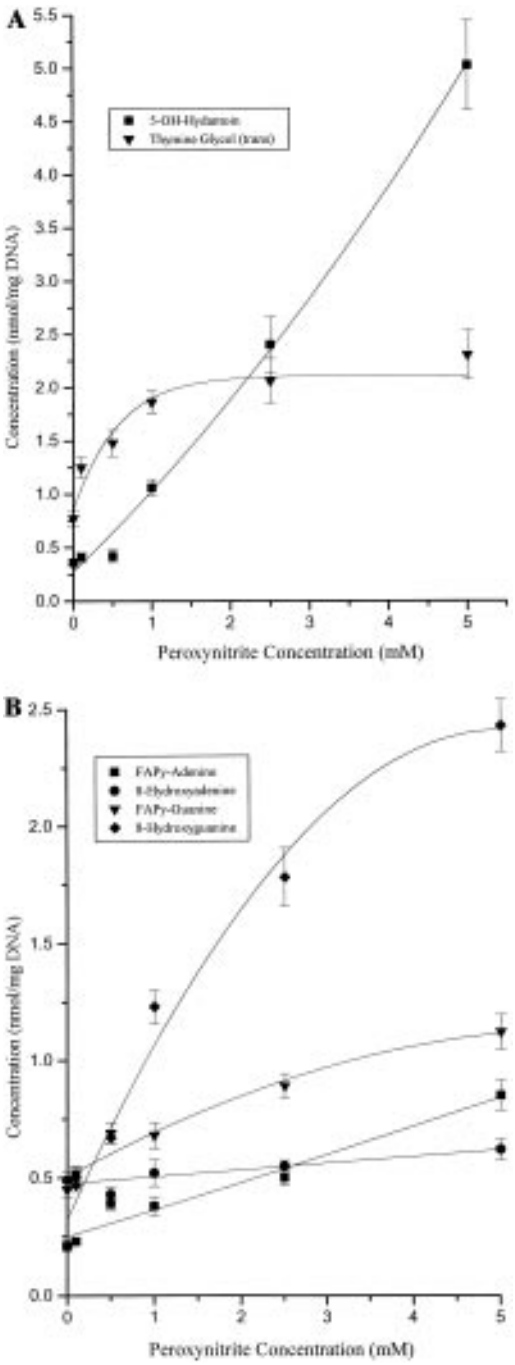

Figure 2. Effect of increasing peroxynitrite concentrations on the generation of oxidized base products. The increase in modified bases derived from pyrimidine bases is shown in (A) and purine bases in (B). Experiments were conducted as in the Materials and Methods section. Data points are means of three separate experiments plotted ±SD ($n = 4$).

Discussion

In this study we have demonstrated that the exposure of DNA to peroxynitrite *in vitro* yields increases in products derived from all four DNA bases and leads to DNA strand breakage. The use of GC-MS allowed us to identify rises in hydroxylated products, for example, 8-OH-guanine, and in the possible deamination products of guanine and adenine, xanthine and hypoxanthine respectively. Large rises in 8-nitroguanine were also observed in agreement with ealier studies (*24*, *25*) and quantified by the use of HPLC. Although the reaction mechanism for the formation of 8-nitroguanine is unknown, studies have suggested that purine nitration at the C-8 position requires either an amino or a hydroxyl group at the C-2 position and only hydrogens at the C-8 carbon and N-7 nitrogen. This would explain why no nitrated adenine product was observed. Our results agree with previous studies (*24*) that the formation of 8-nitroguanine is optimal at a pH close to 8, suggesting that either a heterolytic cleavage of peroxynitrite to form a nitryl cation (NO_2^+) or a high energy intermediate derived from *trans* peroxynitrite ($pK_a = 7.9$) could be involved (*7*). This would be similar to the mechanism of tyrosine nitration and other phenolic compounds by peroxynitrite, which is also seen to be optimum at pH ~8 (*12*, *19*, *21*). Another possibility is that the nitrogen dioxide radical ($NO_2^{\bullet}$) is involved.

The question as to whether $OH^{\bullet}$ is a significant mediator of $ONOO^-$-dependent damage has been repeatedly debated (*6*−*10*, *12*−*15*). Kinetic and thermodynamic calculations suggest that the hydroxyl radical-like reactivity of peroxynitrous acid is mediated by a vibrationally excited intermediate that does not physically separate into free $OH^{\bullet}$ and nitrogen dioxide (*7*). Our data show that only low levels of products characteristic of $OH^{\bullet}$ attack upon DNA were observed when DNA was exposed to peroxynitrite (Figure 2). This would suggest that that $OH^{\bullet}$ radicals are not major contributors to the DNA damage, since $OH^{\bullet}$ is known to produce a wide range of base modifications which results in generation of significant amounts of all the products measured here (*36*, *39*−*43*). The low level of hydroxylated (and ring opened) products observed in our data might result from a mechanism by which a high energy intermediate form of peroxynitrous acid reacts with the target molecule (i.e., guanine) to promote homolytic cleavage of the attacking species (*7*) (Figure 6). Since our paper was submitted, Douki and Cadet (*44*) have reported their inability to find 8-OH-guanine in DNA exposed to 30 mM $ONOO^-$. A possible explanation of this discrepancy is that, at such high levels, $ONOO^-$ leads to oxidative loss of 8-OH-guanine (*45*). In any case, we are all agreed that 8-OH-guanine, like the other oxidized bases, is at most a minor product (*44*, *45*).

The most striking increases in base damage products were in xanthine and hypoxanthine, which can arise as deamination products of guanine and adenine, respectively (*46*, *47*). Deamination could involve $ONOO^-$ itself or other species derived from it, and deamination has been observed in NO-stressed cells (*47*).

When human skin epidermal keratinocytes are exposed to either peroxynitrite or SIN-1, the percentage of double stranded DNA measured after alkali treatment decreases and so the number of strand breaks in the DNA must have risen (*37*, *38*). The extent of DNA strand breakage is greater in the cells exposed to the $ONOO^-$ generator, SIN-1. This may be due to the fact that the majority of $ONOO^-$ added as such reacts with other cell components, for example, lipid membranes and cytosolic constituents, before it can enter the nucleus. From the amount of 8-nitroguanine formed, it seems that only about 10% of the added $ONOO^-$ gave rise to nitrating species close to DNA. SIN-1, in contrast, generates $ONOO^-$ at a of rate 1 μM min^{-1} over the 1 h incubation period, perhaps allowing a greater chance of $ONOO^-$ reaching the DNA. Strand breakage or base modification do not appear to be lethal either directly after the experiment or after 24 h. Strand breakage was accompanied by increases in many modified base products, suggesting that both are due to the same mechanism. The pattern of base damage

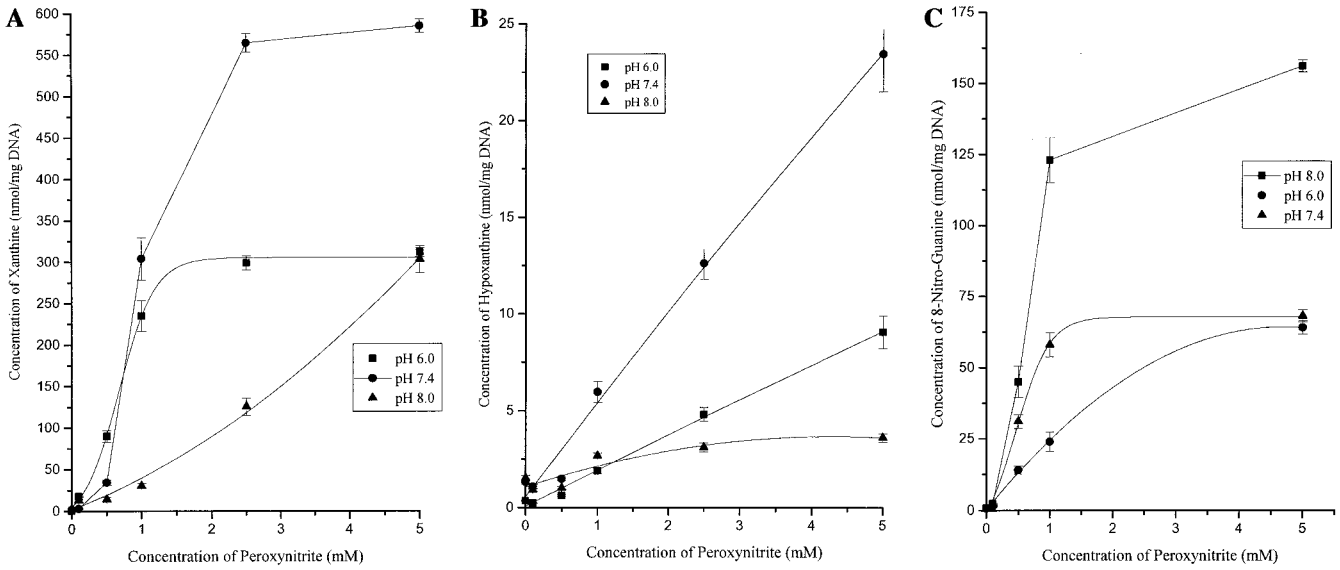

Figure 3. (A) Effect of increasing peroxynitrite concentrations on the generation of xanthine at different pH's. Experiments were conducted as in the Materials and Methods section. Data points are means of three separate experiments plotted ±SD ($n = 4$). (B) Effect of increasing peroxynitrite concentrations on the generation of hypoxanthine at different pH's. (C) Effect of increasing peroxynitrite concentrations on the generation of 8-nitroguanine at different pH's.

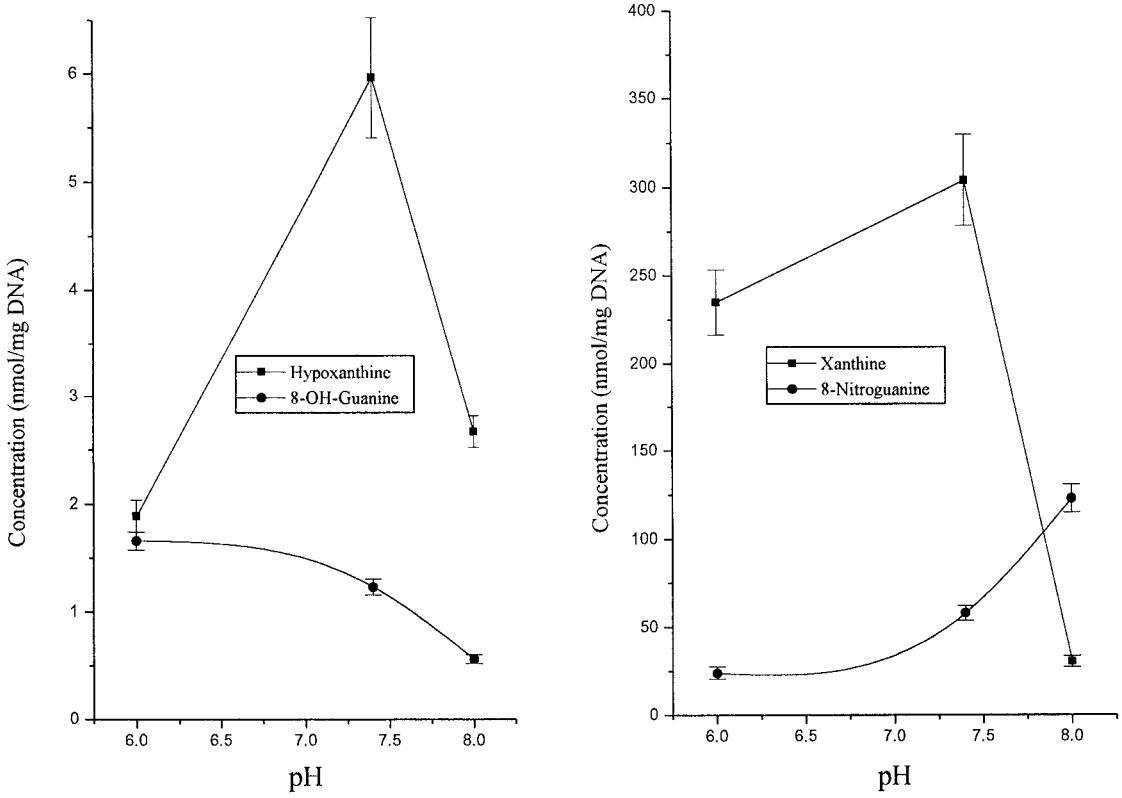

Figure 4. pH dependence of hypoxanthine, xanthine, 8-OH-guanine, and 8-nitroguanine formation by peroxynitrite (1 mM). Data points are mean ±SD ($n = 4$).

is similar to that seen when isolated DNA was exposed to $ONOO^-$. The levels of only a few oxidized bases increased and these changes were small. This pattern of damage is again not suggestive of $OH^\bullet$ attack upon DNA (see above). Increases in the putative deamination products xanthine and hypoxanthine and in 8-nitroguanine indicate attack by reactive nitrogen species. All the products identified and quantified were observed in DNA isolated from untreated cells (including some 8-nitroguanine) and probably arose due to physiological levels of oxidative stress in these cells. The largest increases over background levels were observed in the products xanthine, hypoxanthine, and 8-nitroguanine, which is similar to that seen in isolated DNA exposed to $ONOO^-$. Deamination of guanine and adenine could cause DNA strand breakage due to the relative instability of xanthine and hypoxanthine. Changes in DNA resulting from deamination reactions are consistant with the types of mutations observed in many cancers, e.g., in the p53 gene (*1*, *46*). Deamination of unmethylated cytosine residues *in vivo* produces uracil and its removal by uracil glycosylase (48) can lead to abasic site formation (*49*). Misrepair would produce a G:C → A:T transition, which has been implicated in familial Alzheimer's disease (*50*).

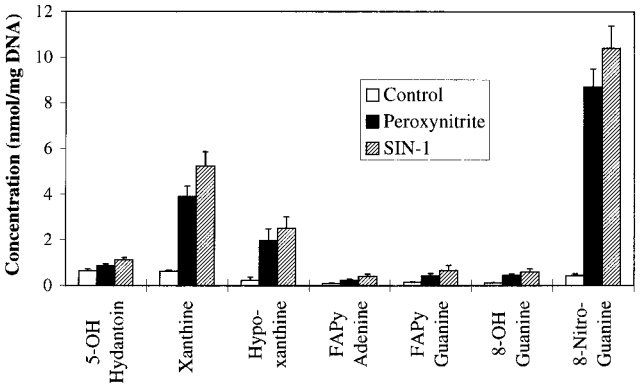

Figure 5. Effect of peroxynitrite (1 mM) and SIN-1 (1 mM; 60 min) on the generation of modified DNA bases in human skin epidermal keratinocytes. Data points are means of three separate experiments plotted with SD from mean.

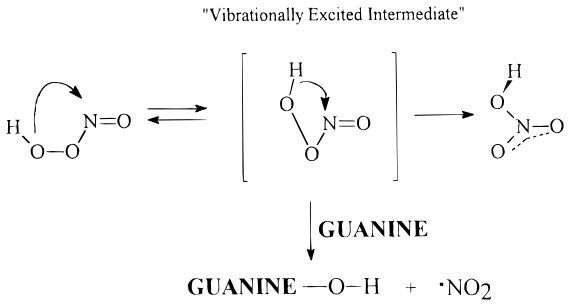

Figure 6. Proposed mechanism of 8-OH-guanine by peroxynitrite. Transfer of the hydroxyl group proceeds via a vibrationally excited intermediate which results from vibrations of the N−O−O bond angle and lengthening of the O−O bond. When this intermediate oxidizes a target molecule, it promotes homolytic cleavage of peroxynitrous acid to leave 8-OH-guanine and $\cdot NO_2$.

Overall, we have shown that the pattern of DNA damage produced by peroxynitrite in isolated DNA and in keratinocyte cells is both complex and extensive. Deamination and nitration reactions appear to be the dominant mechanisms of damage inflicted by $ONOO^-$. However, we have shown that it is also capable of producing a small number of products similar to those produced by $OH^\bullet$ attack on DNA. The pattern of damage observed suggests that reactive nitrogen species rather than reactive oxygen species are responsible for by far the greater amount of damage to DNA in cells generating $ONOO^-$. It is possible that the pattern of damage is unique to peroxynitrite and could be used as a "fingerprint" of peroxynitrite-induced DNA damage *in vivo*. If this is true, it would not only provide vital information on whether peroxynitrite is involved in pathogenic processes but would also go some way to help devise possible ways of protecting cells against damage by $ONOO^-$. Another such "fingerprint" may be the formation of a peroxynitrite−guanine adduct in DNA (*51*).

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References

(1) Ohshima, H., and Bartsch, H. (1994) Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat. Res.* **305**, 253−264.

(2) Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7915−7922.

(3) Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuru, R. K., Misra, M., Dunams, T. M., Cebula, T. A., Koch, W. H., Andrews, A. W., Allen, J. S., and Keefer, L. K. (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **254**, 1001−1003.

(4) Nguyen, T., Brunson, D., Crespi, C. L., Penman, B. W., Wishnok, J. S., and Tannenbaum, S. R. (1992) DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3030−3034.

(5) Huie, R. E., and Padmaja, S. (1993). The reaction of NO with superoxide. *Free Radical Res. Commun.*, **18**, 195−199.

(6) Beckman, J. S., Chen, J., Ischiropoulos, H., and Crow, J. P. (1994) Oxidative chemistry of peroxynitrite. *Methods Enzymol.*, **233**, 229−240.

(7) Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* **5**, 834−842.

(8) Pryor, W. A., and Squadrito, G. L. (1995) The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am. J. Physiol.* **12**, L699−L722.

(9) Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. M., and Freeman, B. A. (1990) Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1620−1624.

(10) Ischiropoulos, H., Zhu, L., and Beckman, J. S. (1992) Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.* **298**, 446−451.

(11) Wang, J. F., Komarov, P., Sies, H., and DeGroot, H. (1991) Contribution of nitric oxide synthase to luminol-dependent chemiluminescence generated by phorbol-ester-activated Kupffer cells. *Biochem. J.* **279**, 311−314.

(12) van der Vliet, A., Eiserich, J. P., O'Neill, C. A., Halliwell, B., and Cross, C.E. (1994) Tyrosine modification by reactive nitrogen species: A closer look. *Arch. Biochem. Biophys.* **319**, 341−349.

(13) Kaur, H., Whiteman, M., and Halliwell, B. (1996) Peroxynitrite-dependent aromatic hydroxylation and nitration of salicylate and phenylalanine. Is the hydroxyl radical involved? *Free Radical Res.*, in press.

(14) Augusto, O., Gatti, R. M., and Radi, R. (1994) Spin-trapping studies of peroxynitrite decomposition and of 3-morpholinosydnonimine N-ethylcarbamide autooxidation: direct evidence of metal-independent formation of free radical intermediates. *Arch. Biochem. Biophys.* **310**, 118−125.

(15) van der Vliet, A., O'Neill, C. A., Halliwell, B., Cross, C. E., and Kaur, H. (1994) Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite. Evidence for hydroxyl radical production from peroxynitrite. *FEBS Lett.* **339**, 89−92.

(16) Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266**, 4244−4250.

(17) Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide annd nitric oxide. *Arch. Biochem. Biophys.* **288**, 481−487.

(18) van der Vliet, A., Smith, D., O'Neill, C. A., Kaur, H., Darley-Usmar, V., and Cross, C. E. (1994) Interaction of peroxynitrite with human plasma and its constituents: Oxidative damage and antioxidant depletion. *Biochem. J.* **303**, 295−301.

(19) Beckman, J. S., Ischiropoulos, H., Zhu, L., van der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J. C., and Tsai, M. (1992) Kinetics of superoxide dismutase- and iron-catalyzed nitration of phenolics by peroxynitrite. *Arch. Biochem. Biophys.* **298**, 438−445.

(20) Beckman, J. S, Ye, Y. Z., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M., and White, C. R. (1994) Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol. Chem. Hoppe-Seyler* **375**, 81−88.

(21) Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J. C., Smith, C. D., and Beckman, J. S. (1992) Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch. Biochem. Biophys.* **298**, 431−437.

(22) Haddad, I. Y., Pataki, G., Hu, P., Galliani, C., Beckman, J. S., and Matalon, S. (1994) Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *J. Clin. Invest.* **94**, 2407−2413.

(23) Kaur, H., and Halliwell, B. (1994) Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. *FEBS Lett.* **350**, 9−12.

(24) Yermilov, V., Rubio, J., Becchi, M., Friesen, M. D., Pignatelli, B., and Ohshima, H. (1995) Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite *in vitro*. *Carcinogenesis* **16**, 2045−2050.

(25) Yermilov, V., Rubio, J., and Ohshima, H. (1995) Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination. *FEBS Lett*. **376**, 207−210.

(26) King, P. A., Anderson, V. E., Edwards, J. O., Gustafson, G., Plumb, R. C., and Suggs, J. W. (1992) A stable solid that generates hydroxyl radical upon dissolution in aqueous solutions: Reactions with proteins and nucleic acids. *J. Am. Chem. Soc.* **114**, 5430−5432.

(27) Salgo, M. G., Stone, K., Squadrito, G. L., Battista, J. R., and Pryor, W. A. (1995) Peroxynitrite causes DNA nicks in plasmid pBR322. *Biochem. Biophys. Res. Commun*. **210**, 1025−1030.

(28) Juedes, M. J., and Wogan, G. N. (1996) Peroxynitrite-induced mutation spectra of pSP189 following replication in bacteria and in human cells. *Mutat. Res*. **349**, 51−61.

(29) Dizdaroglu, M., and Bergtold (1986) Characterization of free radical-induced base damage in DNA at biologically relevant levels. *Anal. Biochem*. **156**, 182−188.

(30) Cavelieri, L. F., and Bendich, A. (1950) The ultraviolet absorption spectra of pyrimidines and purines. *J. Am. Chem. Soc*. **72**, 2587−2592.

(31) Dizdaroglu, M., Holwitt, E., and Hagan, M. P. (1986) Formation of cytosine glycol and 5,6-dihydroxycytosine in deoxyribonucleic acid on treatment with osmium tetroxide. *Biochem. J*. **235**, 531−536.

(32) Boukamp, P., Petrussevska, R. T., Breitreutz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell. Biol*. **106**, 751−771.

(33) Sun, W., Wu, R., and Last, J. A. (1994) Coordinated expression of a 45 kD protein and ozone toxicity in a human bronchial epithelial cell line. *Am. J. Respir. Cell Mol. Biol*. **10**, 673−682.

(34) Spencer, J. P. E., Jenner, A., Chimel, K., Aruoma, O. I., Cross, C. E., Wu, R., and Halliwell, B. (1995) DNA strand breakage and base modification induced by hydrogen peroxide treatment of human respiratory tract epithelial cells. *FEBS Lett*. **374**, 233−236.

(35) Spencer, J. P. E., Jenner, A., Aruoma, O. I., Evans, P. J., Kaur, H., Dexter, D. T., Jenner, P., Lees, A. J., Marsden, D. C., and Halliwell, B. (1994) Intense oxidative DNA damage promoted by L-DOPA and its metabolites. Implications for neurodegenerative disease. *FEBS Lett*. **353**, 246−250.

(36) Aruoma, O. I., Halliwell, B., and Dizdaroglu, M. (1989) Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase., *J. Biol. Chem*. **264**, 13024−13028.

(37) Ryberg, B. (1975) The rate of strand separation in alkali of DNA of irradiated mammalian cells. *Radiat. Res*. **61**, 274−287.

(38) Kohn, K., Erichson, L. C., Ewig, R. A. G., and Friedman, C. A. (1976) Fractionation of DNA from mammalian cells by alkaline elution. *Biochemistry* **15**, 4629−4646.

(39) Halliwell, B., and Aruoma, O. I. (1991) DNA damage by oxygen-derived species. *FEBS Lett*. **281**, 9−19.

(40) Halliwell, B., and Dizdaroglu, M. (1992) The measurement of oxidative damage to DNA by HPLC and GC/MS techniques. *Free Radical Res. Commun*. **16**, 75−87.

(41) Dizdaroglu, M. (1992) Oxidative damage to DNA in mammalian chromatin. *Mutut. Res*. **275**, 331−342.

(42) Halliwell, B., and Aruoma, O. I. (1993) *DNA and Free Radicals*, Ellis Horwood, London.

(43) von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, Taylor and Francis, London.

(44) Douki, H., and Cadet, J. (1996) Peroxynitrite mediated oxidation of purine bases of nucleosides and isolated DNA. *Free Radical Res*. **24**, 369−380.

(45) Uppu, R. M., Cueto, R., Squadrito, G. L., Salgo, M. G., and Pryor, W. A. (1996) Reactions of peroxynitrite with 2′-deoxyguanosine, 7,8-dihydro-8-oxo-2′-deoxyguanosine, and calf-thymus DNA. *Free Radical Biol. Med*. **21**, 407−411.

(46) Liu, R. H., and Hotchkiss, J. H. (1995) Potential genotoxicity of chronically elevated nitric oxide. A review. *Mutat. Res*. **339**, 73−89.

(47) deRojas-Walker, T., Tamir, S., Ji, H., Wishnock, J. S., and Tannenbaum, S. R. (1995) Nitric oxide induces oxidative damage in addition to deamination in macrophage DNA. *Chem. Res. Toxicol*. **8**, 473−477.

(48) Wallace, S. S. (1988) AP endonucleases and DNA glycosylases that recognize oxidative DNA damage. *Environ. Mol. Mutagen*. **12**, 431−477.

(49) Sagher, D., and Strauss, B. (1983) Insertion of nucleotides opposite apurinic/apyrimidinic sites in deoxyribonucleic acid during in vitro synthesis: Uniqueness of adenine nucleotides. *Biochemistry* **22**, 4518−4526.

(50) Goate, A., Charter-Harlin, M.-C., Mullen, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Pericak-Vance, M., Roses, A., Williamson, R., Rossor, M., Owen, M., and Hardy, J. (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* **349**, 704−706.

(51) Douki, T., Cadet, J., and Ames, B. N. (1996) An adduct between peroxynitrite and 2′-deoxyguanosine: 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine. *Chem. Res. Toxicol.* **9**, 3−7.

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