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Peroxynitrite-induced oxidation and nitration products of guanine and 8-oxoguanine: Structures and mechanisms of product formation ☆

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Abstract

Peroxynitrite induces DNA base damage predominantly at guanine (**G**) and 8-oxoguanine (**8-oxoG**) nucleobases via oxidation reactions. Nitration products are also observed, consistent with the generation of radical intermediates that can recombine with the $^{\cdot}NO_2$ formed during peroxynitrite degradation. The neutral **G** radical, **G**$^{\cdot}$, reacts with $^{\cdot}NO_2$ to yield 8-nitroguanine (8-nitroG) and 5-nitro-4-guanidinohydantoin (**NI**), while for **8-oxoG** we have proposed a reactive guanidinylidene radical intermediate. The products generated during peroxynitrite-mediated 8-oxoG oxidation depend on oxidant flux, with dehydroguanidinohydantoin (**DGh**), 2,4,6-trioxo-[1,3,5]triazinane-1-carboxamidine (**CAC**) and **NO_2-DGh** predominating at high fluxes and spiroiminodihydantoin (**Sp**), guanidinohydantoin (**Gh**) and 4-hydroxy-2,5-dioxo-imidazolidine-4-carboxylic acid (**HICA**) predominating at low fluxes. Both product sets are observed at intermediate fluxes. It is therefore important in model systems to ensure that the relative concentrations are well controlled to minimize competing reactions that may not be relevant in vivo. Increasingly sophisticated systems for modeling peroxynitrite production in vivo are being developed and these should help with predicting the products most likely to be formed in vivo. Together with the emerging information on the genotoxic and mutational characteristics of the individual oxidation products, it may be found that the extent of tissue damage, mutational spectra and, hence, cancer risk may change as a function of peroxynitrite fluxes as different product combinations predominate.

Keywords: Peroxynitrite; Guanine; 8-Oxoguanine; 8-Nitroguanine; Dehydroguanidinohydantoin; 2,4,6-Trioxo- [1,3,5]triazinane-1-carboxamidine; Spiroiminodihydantoin; 4-Hydroxy-2,5-dioxo-imidazolidine-4-carboxylic acid; Nitric oxide; DNA damage; Oxidative damage

Nitric oxide ($^{\cdot}NO$) plays a pivotal role in many biological processes, and is produced primarily by the enzyme nitric oxide synthase (NOS), of which there are three isoforms, namely, endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). The expression, regulation, synthesis, and role of NOS and $^{\cdot}NO$ in physiologic processes such as vasodilation, neurotransmission, inhibition of platelet aggregation, and in proper immunologic function, have been extensively studied and previously reviewed [1–8]. $^{\cdot}NO$ plays a dual role in human physiology: both eNOS and nNOS produce relatively low $^{\cdot}NO$ levels which modulate processes such as vasodilation and platelet aggregation in the cardiovascular system, and long term potentiation and neurotransmission in the nervous system. In the immune system, $^{\cdot}NO$ is produced by iNOS in higher amounts and is used by macrophages as a cytotoxic agent against engulfed pathogens. More recently, nitric oxide has been shown also to arise under certain conditions via reduction of nitrite by deoxygenated hemoglobin [9,10].

Since $^{\cdot}NO$ is toxic at levels higher than normal physiological levels, its production must be tightly controlled both spatially and temporally to minimize undesired effects. This balance, however, can be disrupted in certain pathophysiologic states. During chronic gastrointestinal inflammatory

☆ Peroxynitrite is used to denote the sum of the conjugate acid (HOONO) and base ($ONOO^-$) of oxoperoxonitrate (1−). Specific reference to either species is indicated by use of HOONO or $ONOO^-$.

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conditions, such as the inflammatory bowel diseases [11–13], and chronic *Helicobacter pylori* infection [14,15], exuberant $^{\cdot}NO$ production by activated macrophages is believed to be an important tissue-damage mediator, and to potentiate the increased cancer risk associated with these conditions. In fact, significant increases in the $^{\cdot}NO$-derived 8-nitroguanine (**8-NO_2G**) and 8-oxoguanine (**8-oxoG**) products (vide infra), have been demonstrated in gastric tissue from *H. pylori* infected individuals, and these mutagenic lesions may play an important role in *H. pylori* related carcinogenesis. Thus, understanding the connection between $^{\cdot}NO$ and these outcomes requires consideration of the fate of $^{\cdot}NO$ in biological systems, and the capacity of the associated intermediates to react with various cellular components, especially DNA, since damage to this informational molecule can lead to mutations and ultimately carcinogenesis.

$^{\cdot}NO$ itself is relatively unreactive toward DNA, but it can be converted into several highly reactive species. These include nitrous anhydride (N_2O_3), nitrogen dioxide (NO_2), nitryl chloride (NO_2Cl), and peroxynitrite ($ONOO^-$),[1] and the chemistry underlying the formation and reactivity of these species has been recently reviewed [16]. In particular, our group has been interested in understanding the contribution of peroxynitrite-induced DNA damage to the cytotoxicity and carcinogenesis associated with pathological states in which $^{\cdot}NO$ is overproduced. Toward this objective, we have been characterizing the peroxynitrite-induced DNA-damage products and, through collaborative efforts, determining how they interact with the replication and repair machinery in vitro and in vivo to establish their individual genotoxic and mutagenic profiles. We have also been supplementing these biological studies with experiments aimed at understanding the mechanism by which the various products form, and have gained some key chemical insights that are discussed later. Of particular relevance toward connecting the chemistry of formation with the biological significance of the products, we have established that both product distribution and identity are dramatically affected by peroxynitrite fluxes. Specifically, two distinct product sets dominate at low and high peroxynitrite fluxes, respectively, raising the intriguing possibility that peroxynitrite may induce significantly different mutational spectra and genotoxic profiles at different production rates in chronically inflamed tissues.

This review summarizes the progress in identifying the major peroxynitrite-induced DNA-damage products, with emphasis on the nucleobases guanine (**G**) and 8-oxoguanine (**8-oxoG**). The quantitative aspects related to these substances are also of considerable interest [17], but treatment of these is beyond the scope of this review. The chemical insights gained from our studies with peroxynitrite, together with those of other groups using different systems to study oxidation of these bases, have been synthesized to highlight how seemingly-different oxidants ultimately result in similar or identical intermediates and final products. Finally, some biological aspects of these reaction products are also discussed.

[1] *Abbreviations used:* SIN-1, 3-morpholinosydnonimine; MnTMPyP, manganese (III) 5,10,15,20-tetrakis(*N*-methylpyridyl)porphyrin; **Iz**, 2,5-diamino-4H-imidazol-4-one; **Oz**, 2,2,4-triamino-5-(2H)-oxazolone; **HICA**, 4-hydroxy-2,5-dioxo-imidazolidine-4-carboxylic acid.

A brief review of peroxynitrite decomposition chemistry

Peroxynitrite chemistry is complex due to dependence on a combination of pH and carbon dioxide concentrations. In the absence of CO_2, peroxynitrite will decay ($t_{1/2} \sim 1$ s) via an acid-catalyzed pathway. At physiologic pH, both $ONOO^-$ and its conjugate acid, ONOOH ($pK_a = 6.8$) exist, and the latter decomposes via homolysis to give the hydroxyl ($HO^{\cdot}$) and nitrogen dioxide ($^{\cdot}NO_2$) free radicals, each in approximately 25–28% yield [18–20]. In the presence of physiologic CO_2 levels (~1–1.3 mM), decay occurs almost exclusively via a CO_2-catalyzed pathway. $ONOO^-$ reacts with CO_2 ($k_2 = 3–5 \times 10^4\ M^{-1}\ s^{-1}$) to give peroxynitrosocarbonate, which undergoes homolysis to give the $CO_3^{\cdot -}$ and $^{\cdot}NO_2$ free radicals, each in ~30–33% yields [21–25]. Peroxynitrite reactivity with various substrates can be induced by these radical species, but both $ONOO^-$ and ONOOH are reactive in their own right. $ONOO^-$ is known to act as a nucleophile in reactions with CO_2 [23,24], aldehydes [26] and ketones [27], while ONOOH mediates ebselen oxidation [28]. Overall, in solutions buffered at physiologic pH and containing CO_2, the major reactive species are expected to be $CO_3^{\cdot -}$, $^{\cdot}NO_2$, and $ONOO^-$, along with minor amounts of ONOOH and $HO^{\cdot}$. Together, these reactive intermediates determine the spectrum of oxidation products observed when peroxynitrite reacts with DNA.

Peroxynitrite-induced DNA oxidation products

Peroxynitrite induces both sugar-phosphate backbone damage and base damage. Direct backbone damage at reasonably low peroxynitrite concentrations is manifested as single strand breaks ([29,30]; for detailed reviews, see [31,32]), and H-atom abstraction from the deoxyribose moiety by the peroxynitrite-derived radicals outlined above is believed to be the initiating event [31]. The formation of base propenals when deoxynucleotides are treated with peroxynitrite supports this hypothesis [33]. Interestingly, CO_2 dramatically suppresses peroxynitrite-induced sugar damage, both at the nucleoside and DNA levels [33,34]. In DNA, while the total damage (sugar and base) remains unchanged, independent of CO_2, proportionately more base damage occurs in the presence of CO_2 [34].

In this review, we focus exclusively on base damage, and specifically on **G** and its oxidation product, **8-oxoG**, since these are the most reactive toward peroxynitrite [35], and possibly the major contributors to peroxynitrite-derived genotoxic and mutagenic lesions.

Peroxynitrite-mediated guanine oxidation

G has the lowest reduction potential of the four normal DNA bases ($E^{o} = 1.29$ V [36]), and its reaction with the peroxynitrite-derived $HO^{\cdot}$ ($E^{o} = 1.9$–2.1 V [37]) and $CO_3^{\cdot-}$ ($E^{o} = 1.5$ V [38]) radicals peroxynitrite is thermodynamically favorable. Furthermore, the bimolecular rate constants for reaction of these radicals with **G** (7.8×10^9 $M^{-1}s^{-1}$ for $HO^{\cdot}$ [39]) and $7 \times 10^7\,M^{-1}s^{-1}$ for $CO_3^{\cdot-}$ [40] dictate extremely fast kinetics for the initial oxidation event. The **G** radical cation, $\mathbf{G}^{\cdot+}$, is initially produced and subsequently converted into several nitration and oxidation products that have been structurally characterized by us and other groups, as summarized in Fig. 1.

Nitration products

The first nitration product to be identified was 8-nitroguanine, **8-NO_2G** [41]. This modified base, when formed in the context of oligonucleotides [34] or double stranded DNA [42], depurinates with a half-life on the order of hours under simulated physiologic conditions. Thus, **8-NO_2G** formation in vivo may be an important source of apurinic sites arising from peroxynitrite production.

Later, we identified 5-nitro-4-guanidinohydantoin, **NI**, as a novel peroxynitrite-induced **G** nitration product [43]. Unlike **8-NO_2G**, this compound has a stable glycosidic bond, and persists either as the free nucleoside or nucleotide in DNA. **NI** represents the only peroxynitrite-induced guanine nitration product characterized to date in which the pyrimidine ring has been disrupted.

There is a single report of a peroxynitrite addition product, 4,5-dihydro-5-hydroxy-4-(nitrosooxy)guanine [44]. Apparently, this compound does not spontaneously depurinate, and appears to be stable despite the favorable elimination of nitrous acid to extend the π-conjugation in the pyrimidine ring. Furthermore, several C8-substituted 5-hydroxypurines are known to be hydrolytically unstable, readily undergoing hydration at the C6 position to yield hydantoin products [45–48]. The extent to which the C8-substitution impacts the rate of this reaction has not been fully explored, but the above issues need to be reconciled before acceptance of this proposed structure.

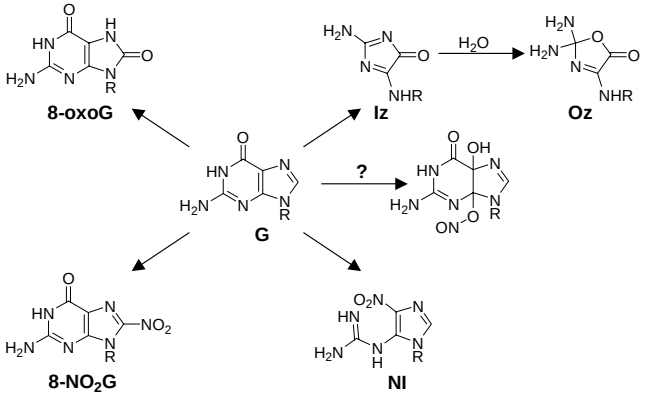

Fig. 1. Summary of the peroxynitrite-induced **G** oxidation and nitration products.

Oxidation products

Two main oxidation products are formed during the peroxynitrite-induced oxidation of **G**, namely, **Iz** [49] and **8-oxoG** [30]. **Iz**, which had previously been identified as a product of Type I **G** photooxidation, undergoes hydrolysis to **Oz** at physiologic pH [50]. Thus, in tissues under oxidative stress in which **Iz** is initially formed, **Oz** is the product that is expected to accumulate.

The proposed formation of **8-oxoG** from the reaction of **G** with peroxynitrite was initially controversial, with some investigators using fairly high ratios of peroxynitrite to **G** claiming no formation of **8-oxoG** [42,49] and others using lower ratios reporting its formation [30]. This apparent discrepancy was finally resolved when Pryor and co-workers demonstrated that peroxynitrite reacted preferentially with **8-oxoG** over **G** [51]. When relatively high concentrations of peroxynitrite are used, the resulting **8-oxoG** formed is rapidly consumed in subsequent reactions with excess peroxynitrite. Thus, a compound previously identified as 4-hydroxy-7,8-dihydro-8-oxoG was isolated from the reaction of **G** with peroxynitrite [49,52]. However, further work by our group and others has since established that, first, this product is in fact spiroiminodihydantoin, **Sp** [47,53,54] and, second, that it most likely arises from further oxidation of **8-oxoG**.

*Peroxynitrite-mediated **8-oxoG** oxidation*

In addition to the inherently interesting chemistry underlying peroxynitrite-mediated **G** oxidation, we have also been interested in elucidating the potential biological importance of these reactions. Several findings coincided to influence our decision to focus on the reaction between peroxynitrite and **8-oxoG** as a potentially important source of biologically relevant DNA lesions.

First, **8-oxoG** has been detected, in amounts ranging from 4 to 11 in 10^7 bases, in genomic DNA isolated from tissues not under chronically elevated levels of oxidative stress [55]. Reliable measurements of levels in chronically inflamed tissues are lacking, but presumably these will be higher than the levels mentioned above, thus making **8-oxoG** an even more likely target for oxidative damage in DNA. Second, **8-oxoG** is inherently more reactive than **G** with peroxynitrite, and in a study by Pryor's group a 10^3-fold excess of **G** over **8-oxoG** protected only half of the **8-oxoG** from reacting with peroxynitrite [51]. Thermodynamically, **8-oxoG** ($E^{o} = 0.74$ V [56]) is more susceptible to oxidation than **G**. Recent studies have also highlighted that the peroxynitrite-derived radicals will rapidly react with **8-oxoG**, and bimolecular rate constants of $8 \times 10^8\,M^{-1}s^{-1}$ [40] and $5 \times 10^6\,M^{-1}s^{-1}$ [57] have been reported for $CO_3^{\cdot-}$ and $^{\cdot}NO_2$, respectively. Furthermore, direct electron transfer from **8-oxoG** to the oxidizing $\mathbf{G}^{\cdot}$ readily occurs with a bimolecular rate constant of

$4.6 \times 10^8\,M^{-1}\,s^{-1}$ [56]. These observations indicate that even with **G** in large excess over **8-oxoG**, oxidation of the latter can still dominate because of (i) the inherent thermodynamic and kinetic favorability of the reaction of **8-oxoG** with peroxynitrite, and (ii) the high propensity for damage initiated on **G** to be transferred to **8-oxoG**. Third, the ability of double stranded DNA to support charge transfer over long distances to sites remote from the initial oxidation site has become well established [58,59], and ultimately sites with the lowest reduction potentials serve as sinks for the migrating positive hole. Thus, in oligonucleotides containing **8-oxoG** in addition to the other normal bases, oxidation occurs preferentially at the **8-oxoG** even when the initial oxidizing event is well removed from this residue [60]. Taken all together, these facts suggest that oxidative damage may preferentially accumulate at **8-oxoG** residues in DNA.

*Studying **8-oxoG** oxidation using authentic peroxynitrite introduced by bolus addition*

In early experiments, bolus addition of preformed peroxynitrite to solutions containing acetylated 8-oxo-(2′-deoxy)guanosine was used as a model system to characterize several reaction products, shown in Fig. 2. The nature of the sugar moiety did not affect the identity of the nucleobase oxidation products, and so both 3′,5′-di-*O*-acetyl-β-D-erythropentofuranosyl- and 2′,3′,5′-tri-*O*-acetyl-β-D-erythropentofuranosyl-**8-oxoG** derivatives were used. Without acetylation, the reaction products are extremely polar, so this modification proved useful by improving their chromatographic properties, and facilitated purification by standard reversed-phase HPLC.

Although the structure of the major product formed over a range of peroxynitrite concentrations was initially assigned tentatively [61], subsequent detailed NMR study of this compound produced by MnTMPyP/$KHSO_5$-mediated oxidation of d(GpT) established it as dehydroguanidinohydantoin, **DGh** [62]. In buffered phosphate/bicarbonate solutions (pH 7.4) it readily hydrolyzes to give oxaluric acid (**OA**) as a relatively stable product [61,62]. Interestingly, in a more physiologic buffer containing Mg^{2+} and bicarbonate, **OA** decomposes to give the urea (**UA**) [63]. This reaction occurs in the context of both nucleoside and oligonucleotide, and suggests that the initial **DGh** lesion can give rise in vivo to several further hydrolysis products that may be of biological significance.

The second most abundant product was identified as 2,4,6-trioxo-[1,3,5]triazinane-1-carboxamidine (**CAC**). This is identical to the major singlet oxygen-derived **8-oxoG** oxidation product [64], which will hydrolyze to cyanuric acid (**CA**) [61,64]. There appear to be two routes to **CAC**, one involving an unstable, uncharacterized intermediate (**CAP**) and the other the further oxidation of **DGh** by peroxynitrite. Interestingly, CO_2, which is known to alter the reactive species produced during peroxynitrite decomposition [23–25], has a profound impact on the product profile with respect to **DGh** and **CAC** formation. When reactions are carried out in the absence of CO_2, **DGh** is the major product up to ~1 mM peroxynitrite, above which **CAC** becomes the major product. We have shown that further oxidation of **DGh** to **CAC** is at least one factor accounting for this phenomenon [61]. In the presence of CO_2, however, **DGh** remains the major product up to 2 mM peroxynitrite, suggesting that the oxidants produced under these conditions

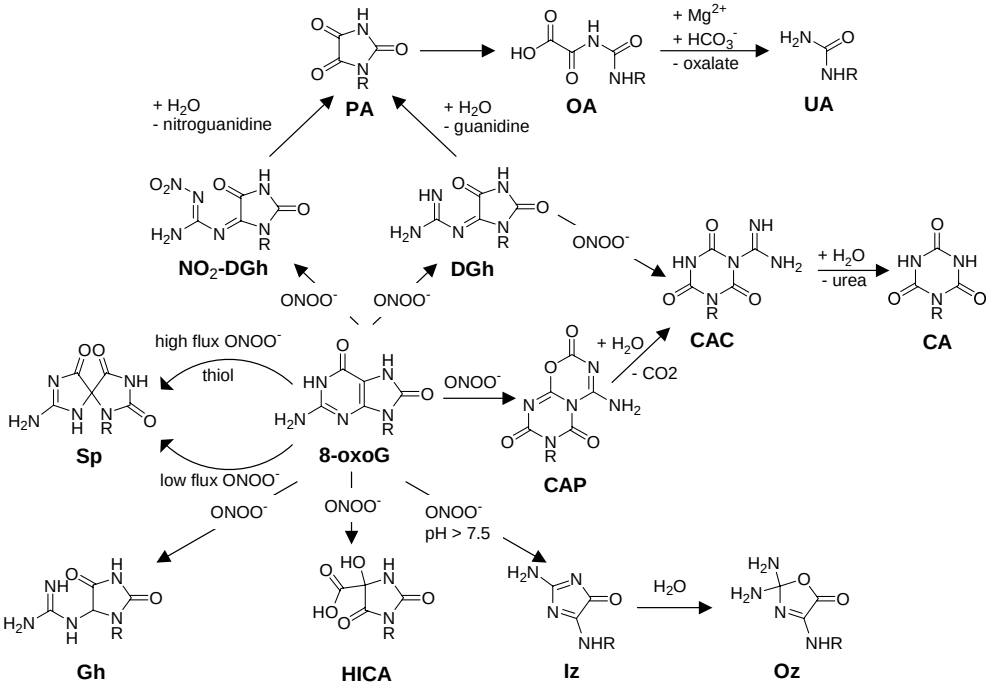

Fig. 2. Summary of the peroxynitrite-induced **8-oxoG** oxidation products.

do not as effectively yield **CAC** (J.C. Niles, J.S. Wishnok, S.R. Tannenbaum, unpublished results).[2]

Two relatively minor products identified in this reaction are parabanic acid (**PA**) and *N*-nitro-dehydroguanidinohydantoin (**NO_2-DGh**) [61,65]. The former is likely an intermediate in the hydrolysis of **DGh** to **OA**, but a direct route to its formation cannot be excluded. **NO_2-DGh** is the only peroxynitrite-induced **8-oxoG**-derived nitration product identified to date, and provides some important insights into the mechanism via which the various reaction products are formed. Like **DGh**, both **PA** and **NO_2-DGh** are hydrolytically unstable and will decompose to **OA**.

The final oxidation product of this reaction is **Iz**, the same product that is formed during peroxynitrite-induced **G** oxidation [49]. It is therefore tempting to conclude that **Iz** formed during the reaction with **G** is arising from secondary oxidation of **8-oxoG**. However, there is a distinct difference in the pH-dependence of **Iz** formation when starting from **G** versus **8-oxoG**. With **G**, **Iz** formation is prominent at pH 7.2, while with **8-oxoG**, **Iz** formation becomes prominent only at pH > 7.5, and increases up to pH 8.5 (J.C. Niles, J.S. Wishnok, S.R. Tannenbaum, unpublished results). Therefore, in the oxidation of **G**, any contribution to **Iz** formation from secondary **8-oxoG** oxidation at pH 7.2 must be minor, indicating that there are at least two paths to **Iz** that are, respectively, **8-oxoG** dependent and independent.

Additionally, it is important to note that **DGh** is not a precursor to **Iz**, as was suggested in earlier literature [66,67]. It is possible under basic conditions in the presence of ammonium hydroxide to convert **DGh** into the *base moiety* of **Iz**, but not the intact nucleoside [61]. Taken together with the fact that **DGh** hydrolyzes exclusively to **OA** [61,62,68] and not **Iz** at near neutral pH, the most logical conclusion is that **DGh** and **Iz** represent distinct fates of an earlier reaction intermediate(s).

In an effort to gain further insights into the mechanism underlying product formation, we attempted reductive trapping of reaction intermediates. Using thiol reductants (glutathione and cysteine), in addition to the expected decrease in overall product yield, a new compound identified as **Sp** was formed [53]. While this strategy failed to trap compounds that could be unambiguously assigned as product precursors, it served together with other work [47,54] to establish that the oxidation product previously identified as 4-hydroxy-7,8-dihydro-8-oxoG [52] is in fact **Sp**.

*Studying **8-oxoG** oxidation using infused authentic and in situ generated peroxynitrite*

While bolus addition of preformed peroxynitrite to reaction mixtures served as a convenient initial model, it clearly does not simulate peroxynitrite formation in vivo. Thus, the possibility that the chemistry and hence the products identified during bolus addition experiments may not accurately reflect the in vivo situation could not be excluded. To test this, we reduced the instantaneous peroxynitrite concentrations in reaction mixtures using two strategies, namely: (i) infusion of preformed peroxynitrite and (ii) in situ peroxynitrite generation using SIN-1, a compound that decomposes in aqueous solution to produce stoichiometric amounts of $O_2^{\cdot-}$ and $^{\cdot}NO$ that combine to produce peroxynitrite at very low fluxes [69–71]. The products formed in these studies are also summarized in Fig. 2.

When peroxynitrite was infused into buffered solutions containing **8-oxoG**, three additional compounds, i.e., **Sp**, guanidinohydantoin (**Gh**) and **HICA** became quantitatively important [48,71]. Delivering the same total amount of peroxynitrite over increasing time periods led to **Sp**/**Gh** becoming the major reaction product [48]. It is important to note that while the **Sp** formed is structurally identical to that isolated during our attempted thiol trapping experiments [53], it arises here via a distinct mechanism, and this is discussed later. Additionally, an inverse, pH-dependent relationship exists between **Sp** and **Gh** formation, with **Sp** being produced predominately at neutral pH and **Gh** being produced predominantly at low pH [47,48].

Interestingly, using SIN-1 to generate lower peroxynitrite fluxes than achievable by infusion leads predominantly to **Sp**/**Gh** and **HICA** formation and the complete absence of bolus addition products [53]. Thus, two distinctly different product sets are obtained depending on whether peroxynitrite is added quickly to **8-oxoG** at high (bolus addition) or slowly at low (SIN-1) fluxes. At intermediate fluxes (infusion) these two pathways compete, yielding a combination of products. These intriguing findings motivated us to explore in greater detail the mechanism(s) of product formation during the peroxynitrite-mediated **G** and **8-oxoG** oxidation, and our studies, together with work from several other groups, have provided additional insights into these complex reactions.

Mechanistic insights into peroxynitrite-induced oxidation of guanine and 8-oxoguanine

Guanine oxidation

Oxidation of **G** has been extensively studied, and it is well established that the initial event, using a variety of oxidant systems, is a one-electron oxidation to produce the **G** radical cation, ($\mathbf{G}^{\cdot+}$) [39]. As previously mentioned, this is carried out efficiently by the peroxynitrite-derived $HO^{\cdot}$ and $CO_3^{\cdot-}$ radicals. $^{\cdot}NO_2$ ($E^{o} = 1.04$ V [37]) the other peroxynitrite-derived radical, however, has been shown experimentally not to induce **G** oxidation [57]. Thus, the initial step in the reaction between peroxynitrite and **G** is one-electron oxidation of **G** to $\mathbf{G}^{\cdot+}$ by either $HO^{\cdot}$ or $CO_3^{\cdot-}$. In our experiments, ~1–1.3 mM CO_2 was used, making $CO_3^{\cdot-}$ the quantitatively more important initiating radical.

Once formed, $\mathbf{G}^{\cdot+}$ ($pK_a = 3.9$) deprotonates at physiologic pH to give the neutral radical, $\mathbf{G}^{\cdot}$ [39], which is

[2] **CAP** is formed under these conditions, but we are unable to accurately quantitate it. Therefore, it has not been included in the total **CAC** formed.

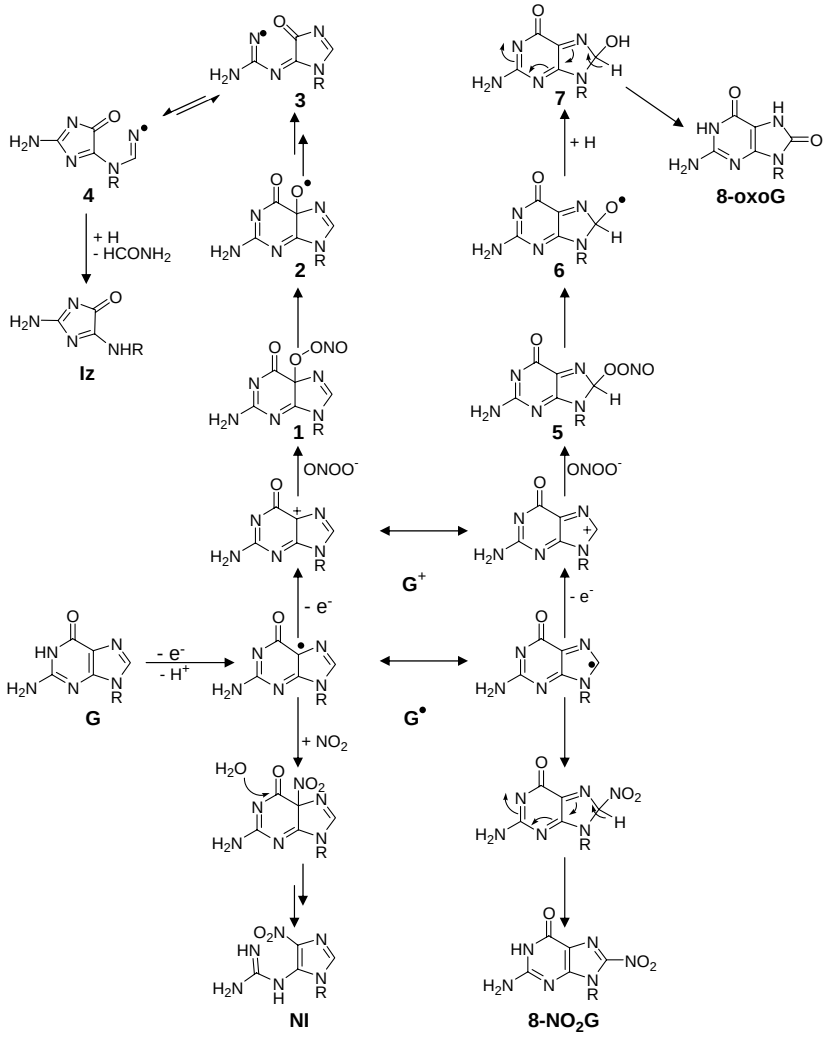

Fig. 3. Proposed mechanism for the peroxynitrite-induced formation of **G**-derived products.

expected to have significant unpaired electron density at O6, C5, and C8 [72]. Formation of **8-NO_2G** and **NI** can be mechanistically rationalized by radical recombination of $^{\cdot}NO_2$ at C8 and C5 of **G**$^{\cdot}$, respectively (Fig. 3) [43,73]. For **8-NO_2G**, the aromatic purine ring is restored by deprotonation at C8, while for **NI**, the purine ring is disrupted to give the aromatic imidazole nucleus [43]. Supporting this proposal is the recent demonstration that **G**$^{\cdot}$ generated independently of peroxynitrite will react with $^{\cdot}NO_2$ to give both **8-NO_2G** and **NI** [73,74].

The mechanism of **Iz** formation is an intriguing aspect of **G** oxidation chemistry, since the same product forms via a variety of oxidants, including $HO^{\cdot}$ [50], MnTMPyP/$KHSO_5$ [67], and peroxynitrite [43,49], yet the source of the exogenous [O] atom incorporated at C4 varies among these oxidants. In $HO^{\cdot}$-mediated oxidations, molecular oxygen provides the C4 [O] atom [50], while for MnTMPyP/$KHSO_5$ and peroxynitrite, HSO_5^- [67] and $ONOO^-$ (J.C. Niles, J.S. Wishnok, S.R. Tannenbaum, unpublished results) are the sources, respectively. These findings highlight two important facts. First, there are multiple, independent paths leading to formation of common intermediates that ultimately converge on the same final product(s). This is true for both **G** and **8-oxoG** oxidation, and applies to all the purely oxidative products. Second, involvement of HSO_5^- and $ONOO^-$ in direct nucleophilic reactions suggests that electrophilic intermediates are produced during the reaction.

We propose that the C5-alkoxide radical intermediate, **2**, is important in **Iz** formation. With peroxynitrite, this intermediate is generated when $ONOO^-$ attacks an electrophilic intermediate, which is likely to be the guanine cation, **G**$^+$ [67] at C5. The weak peroxo bond in the resulting ROONO species, **1**, will undergo homolysis to give the C5-alkoxide radical and $^{\cdot}NO_2$. Indeed, precedent exists for the favorable peroxo bond homolysis in the analogous HOONO [15,16] and $ONOOCO_2^-$ [75,76] species. Pyrimidine ring opening of the C5-alkoxide is proposed to lead to formation of a putative guanidinylidene radical intermediate, **3**, which is in equilibrium with **4**. H-atom abstraction by the latter, followed by hydrolysis, then produces formamide [67] and **Iz**.

The guanidinylidene radical has been proposed as a reactive intermediate to rationalize a critical deficiency in the mechanisms proposed to explain **Iz** formation. First, as outlined earlier and contrary to previously proposed mechanisms, **DGh** is not a direct precursor to **Iz**. Second, despite the fact that the guanidino functional group is a poor nucleophile, requiring elevated temperatures, alkaline pH ($pK_a \sim 12$) and prolonged reaction times (hours to days) for reasonable reaction yields, this chemistry is invoked to account for the rapid formation of **Iz** on the timescale of seconds (for peroxynitrite) to minutes (singlet oxygen, MnTMPyP/$KHSO_5$, and riboflavin) at ambient temperatures and pH ~ 7. This short timescale for **Iz** formation indicates the involvement of an intermediate with very high reactivity, and the guanidinylidene radical, which is expected to have reactivity more comparable to that of aminyl, iminyl, and amidyl radicals [77,78], is a putative candidate. While there is no direct evidence to date for this intermediate, **NO_2-DGh** formation during the oxidation of **8-oxoG** suggests its existence during these reactions (vide infra).

We have also determined that peroxynitrite is the major source of the oxygen atom incorporated into **8-oxoG** during the peroxynitrite-mediated oxidation of **G** (J.C. Niles, J.S. Wishnok, S.R. Tannenbaum, unpublished results). **8-OxoG** was not directly detected under our reaction conditions, so the **8-oxoG**-derived product **CAC** was used as a surrogate marker. We established that **CAC** derived from the reaction of ^{18}O-labeled peroxynitrite with unlabeled **G** and **8-oxoG** contained three and two peroxynitrite-derived [O] atoms, respectively, indicating that the extra label in the **G**-derived product must have been present in the precursor **8-oxoG**. In rationalizing this fact mechanistically, we have proposed that while $ONOO^-$ reacts with $\mathbf{G}^+$ at C5 to give **Iz**, reaction at C8 gives the adduct, **5**, that decomposes via **6** and **7** into **8-oxoG**. Alternatively, elimination of nitrous acid from **5** can directly yield **8-oxoG**. Here again, for the same final product, the pattern of exogenous [O] atom incorporation is dependent on the oxidizing system used. Thus, for riboflavin- (Type I) and singlet oxygen- (Type II) mediated photooxidation, H_2O [79] and O_2 [52] are the sources of this [O] atom, respectively. Furthermore, these findings—along with the formation of **8-NO_2G** and **NI**—add experimental support to C5 and C8 being primary sites of unpaired electron density and/or electrophilicity.

8-Oxoguanine oxidation

As with **G**, the initial step in **8-oxoG** oxidation is the one-electron oxidation by the peroxynitrite-derived radicals to produce the radical cation, $\mathbf{8\text{-}oxoG}^{\cdot+}$ (Fig. 4) [40,56,57]. This reaction is both thermodynamically and kinetically more favorable than **G** oxidation, as discussed earlier. The pK_a of $\mathbf{8\text{-}oxoG}^{\cdot+}$ is 6.6 [56], and thus, at physiologic pH, both the neutral radical and radical cation exist, making it difficult to readily distinguish the reacting species. In addition to the $\mathbf{8\text{-}oxoG}^{\cdot+}$, electrochemical studies indicate that oxidation to a reactive quininoid diimine, $\mathbf{8\text{-}oxoG}^{ox}$ intermediate readily occurs [45,80]. We have included this species as a key reactive intermediate in our proposed model of peroxynitrite-induced **8-oxoG** oxidation. However, we cannot exclude the possibility that $\mathbf{8\text{-}oxoG}^{\cdot}$/$\mathbf{8\text{-}oxoG}^{\cdot+}$ are relevant reactive intermediates in the chemistry proposed below.

For the reaction of **8-oxoG** with peroxynitrite, it is helpful to consider the mechanistic details in the context of high and low peroxynitrite fluxes. The products formed at high fluxes, namely, **DGh**, **NO_2-DGh**, and **CAC**, all incorporate peroxynitrite-derived [O] atoms, while those formed at low fluxes, namely, **Sp**, **Gh**, and **HICA**, all incorporate H_2O-derived [O] atoms [48]. Based on these observations, we have proposed that nucleophiles in solution compete for reaction with $\mathbf{8\text{-}oxoG}^{ox}$. At high and low peroxynitrite fluxes, respectively, reaction of $\mathbf{8\text{-}oxoG}^{ox}$ with $ONOO^-$ and H_2O will occur exclusively. It is only when peroxynitrite is infused that both pathways directly compete, leading to formation of both product sets.

Mechanism of high flux peroxynitrite product formation

In our model, at sufficiently high peroxynitrite concentrations $ONOO^-$ reacts with $\mathbf{8\text{-}oxoG}^{ox}$ at C5 to give **8**, which rapidly undergoes homolytic peroxo bond cleavage to give the C5-alkoxide, **9**. Just as proposed for the analogous intermediate formed during **G** oxidation, pyrimidine ring opening leads to the guanidinilydene radical intermediate, **10**, which has multiple fates, namely: (i) H-abstraction to yield **DGh**, the major reaction product; (ii) combination with $^{\cdot}NO_2$ to give **NO_2-DGh** and, (iii) cyclization via **11** and **12** to give **Iz**. As before, the guanidinylidene radical is proposed as an intermediate to rationalize the formation of several key products. However, the intriguing challenge—of directly observing or specifically trapping this intermediate in both **G** and **8-oxoG** oxidation reactions—remains.

Another issue for further study is the mechanism of **CAC** formation. As mentioned earlier, there are at least two pathways to this product, namely: (i) an indirect route involving further oxidation of **DGh** [61], and (ii) a "direct" route involving formation of a hydrolytically unstable intermediate, **CAP**, that has eluded detailed structural characterization (J.C. Niles, J.S. Wishnok, S.R. Tannenbaum, unpublished results). Based upon a putative $[M\text{-}H^+]^-$ ion produced in ESI-MS along with the fact that it hydrolyzes to **CAC**, we speculate that this product may be **CAP**. Several attempts to trap this species with various amines to isolate a stable urea derivative have proven unsuccessful. Full structural elucidation of this product will be essential for improving our mechanistic understanding of **CAC** formation.

Mechanism of low flux peroxynitrite product formation

At low peroxynitrite fluxes, H_2O dominates as the nucleophile, and attacks $\mathbf{8\text{-}oxoG}^{ox}$ at C5 to give 5-hydroxy-8-oxoG. This is an important intermediate in

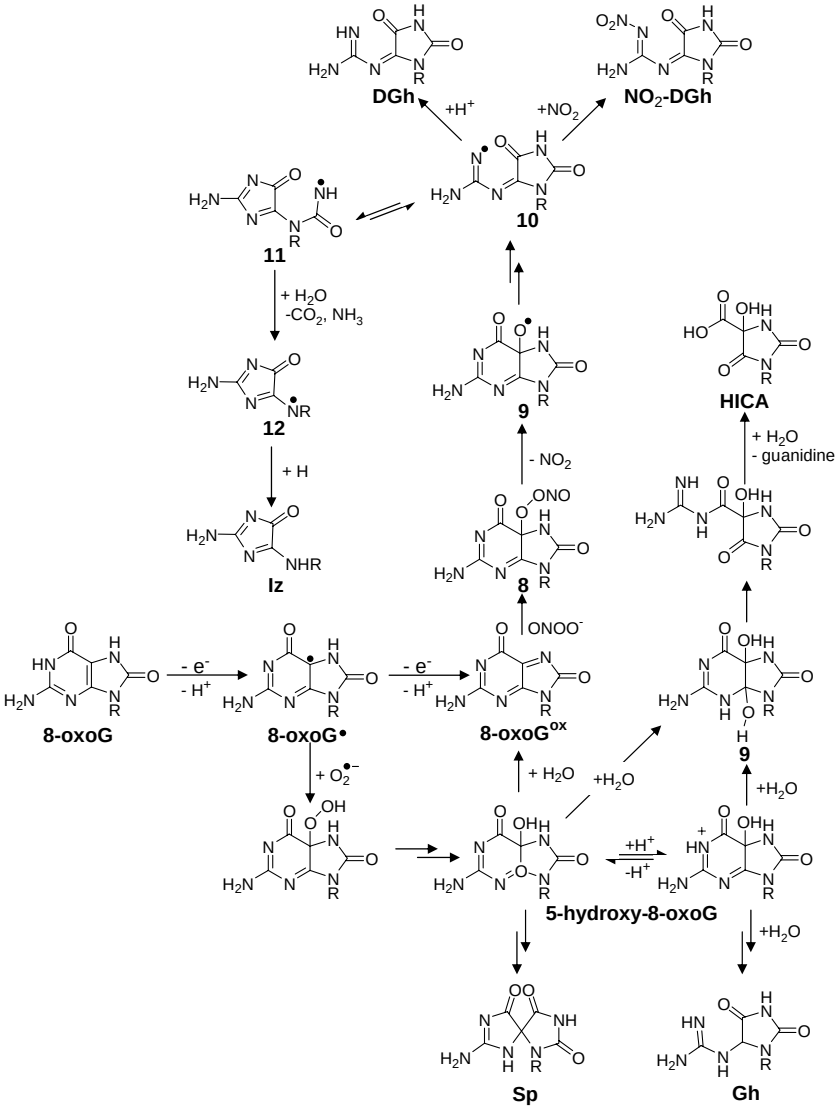

Fig. 4. Proposed mechanism for the peroxynitrite-induced formation of **8-oxoG**-derived products.

several model systems of electrochemical [45,80] and one-electron oxidant (e.g., $IrCl_6^{4-}$, $CoCl_2/KHSO_5$) [47] mediated **8-oxoG** oxidation. This intermediate has a pH-dependent fate, giving rise to **Sp** and **Gh**, respectively, at higher and lower pH [47,48]. By assuming that **Sp** and **Gh** are the major products derived from 5-hydroxy-8-oxoG, we have proposed a simple model in which the latter has a $pK_a \approx 5.8$, and that the protonated intermediate preferentially undergoes hydration at C6 to trigger pyrimidine ring opening, and **Gh** formation. On the other hand, C5–C6 bond migration to form a new C4–C6 bond dominates when 5-hydroxy-8-oxoG is deprotonated, and this yields **Sp** . This scheme is also applicable to the one-electron oxidant system $CoCl_2/KHSO_5$, since the same pH profile with respect to **Sp** and **Gh** formation is observed, further supporting the conclusion that this pH profile is due to inherent physicochemical properties of the participating intermediate(s), and is not dependent on the oxidant used [48].

With respect to **Sp** formation, we reemphasize that this product, which is formed using high peroxynitrite fluxes in the presence of thiol, versus low peroxynitrite fluxes incorporates an O_2- and an H_2O-derived [O] atom, respectively. The outcome in the high flux experiments can arise if **8-oxoG**$^{\cdot+}$**/8-oxoG**$^{\cdot}$ reacts with: (i) O_2 to give the C5-peroxyl radical intermediate, or (ii) O_2^- to give the C5-peroxide. In both cases, the intermediates subsequently undergo reduction to 5-hydroxy-8-oxoG (the same intermediate that arises from attack of **8-oxoG**^{ox} by H_2O), which rearranges to **Sp**, as these studies were conducted at pH = 7.2 [53]. Several factors coincide to favor mechanism (ii). First, O_2^- will rapidly react with **8-oxoG**$^{\cdot+}$**/8-oxoG**$^{\cdot}$ [81,82], with a bimolecular rate constant of $1.3 \times 10^8\ M^{-1}\ s^{-1}$ [82]. While **DGh** was the major product in those studies, it should be noted that potent reductants were not included, and so efficient reduction of the C5-hydroperoxide to 5-hydroxy-8-oxoG is not achieved. This contrasts with our studies in which glutathione and cysteine, both capable of reducing hydroperoxides

to alcohols, are present [53]. Thus, **Sp/Gh** are the expected final products in our system. Second, **8-oxoG**$^{\cdot+}$/**8**$^{\cdot}$**-oxoG** reacts very slowly with O_2 ($k_2 < 10^2\,M^{-1}\,s^{-1}$ [82]) and hence this reaction cannot effectively compete with $O_2^{\cdot-}$ for **8-oxoG**$^{\cdot+}$/**8-oxoG**$^{\cdot}$. Lastly, it is likely that aerobic peroxynitrite decomposition in the presence of thiols leads to $O_2^{\cdot-}$ production [83], thus ensuring that reaction of $O_2^{\cdot-}$ with **8-oxoG**$^{\cdot+}$/**8-oxoG**$^{\cdot}$ effectively out-competes the slow reaction with O_2. Thus, mechanism (ii) is an attractive model for explaining **Sp** formation with incorporation of an O_2-derived [O] atom.

Recognition of **HICA** as a product of peroxynitrite-mediated **8-oxoG** oxidation came only after performing low flux experiments. Isotope labeling indicates that it incorporates three water-derived [O] atoms, and we have proposed that it is formed via hydration of **8-oxoG**^{ox} at both C4 and C5 to give **12**, with subsequent cleavage of the N3–C4 bond and elimination of guanidine to yield **HICA** [71]. Chemically, **HICA** is a minor reaction product in comparison to **Gh/Sp**, and this may in part reflect preferential hydration at C5 over the relatively more hindered C4. However, it may still be important biologically, even if formed at very low levels, if it is a highly persistent mutagenic or toxic lesion.

*Biological implications of peroxynitrite-mediated **G** and **8-oxoG** oxidation*

Thus far, we have emphasized the identity and chemistry of peroxynitrite-induced **G** and **8-oxoG** products at the nucleoside level. However, several studies have shown that they are also produced in single and double stranded oligodeoxynucleotides and calf thymus DNA treated with bolus peroxynitrite. Both **G**-derived nitration products **8-NO$_2$G** [34,42] and **NI** [84] have been reported, albeit the latter at lower levels in double relative to single stranded DNA. The oxidation product **8-oxoG** has also been detected in peroxynitrite-treated plasmid DNA [30], and its further oxidation products identified in oligodeoxynucleotides [85,86]. Taken together, these observations demonstrate that the same products formed at the nucleoside level are produced in DNA, and hence may be important in understanding peroxynitrite's mutagenicity.

Indeed, a strong correlation exists between the preferential reactivity of peroxynitrite with **G** and **8-oxoG** and its ability to induce DNA mutations predominantly at **G** residues. The mutation types and frequencies in initial studies consisted of G:C → T:A (75%), G:C → C:G (12%), and G:C → A:T (14%) and were determined using shuttle vectors treated with high peroxynitrite fluxes [87]. Our studies demonstrating a dramatic shift in the products obtained at high and low peroxynitrite fluxes, prompted further investigations comparing mutational profiles at high (bolus addition), intermediate (infusion), and low (SIN-1 generated) peroxynitrite fluxes. While mutation frequencies were significantly reduced for both infusion and SIN-1 reactions relative to bolus addition, G:C → T:A transversions were still the predominant mutations observed [88]. These results suggest that the DNA-damage products formed at low and high peroxynitrite fluxes may differ, but there is significant overlap in their propensity to induce similar mutations originating at damaged **G** residues.

This conclusion is supported by studies examining the mutational properties of the individual oxidation products introduced site-specifically into oligonucleotides or viral genomes for in vitro and in vivo study, respectively. The sufficiently stable major products of **G** and **8-oxoG** oxidation have been studied, and their mutational spectra are summarized below. **8-OxoG** is known to induce G:C → T:A mutations [89–93]. Of the other **G**-derived products, **Iz** gives rise mainly to G:C → C:G mutations [94], in agreement with a previous preliminary prediction made based on molecular orbital calculations [95]. However, its hydrolysis product **Oz** causes G:C → T:A mutations [86,96]. **NI** gives rise to G:C → T:A (22%), G:C → A:T (19%), and G:C → C:G (9%) mutations [94]. **8-NO$_2$G** depurinates to leave an abasic site, and in systems that conform to the A-rule, is expected to lead to G:C → T:A mutation [97,98]. Recently, this product has also been shown to be a miscoding lesion, directly inducing G:C → T:A mutations predominantly, and G:C → C:G and G:C → A:T mutations and deletions to a lesser extent [99]. Therefore, **8-NO$_2$G** induces mutations directly and indirectly, but the principal mechanism cannot easily be distinguished by examining mutational spectra, as both pathways lead to the same predominant mutation. Of the **8-oxoG** products, **OA** and **UA** are expected to be the major stable products in bolus addition studies. **OA** has been shown to induce G:C → T:A mutations at high frequency [86,100], and this is also true for **UA** [63]. Similarly, **CA** causes predominantly G:C → T:A mutations [86,101]. On the other hand, the major products of low peroxynitrite fluxes, **Gh** and **Sp** will induce both G:C → C:G and G:C → T:A transversions [102,103], with the former as the predominant mutation (98% for **Gh** and 65% for **Sp**, respectively) [103]. Unfortunately, **DGh** and **NO$_2$-DGh** are too unstable for determination of mutational spectra, while the more recently elucidated **HICA** has not been studied. Overall, the strong concordance between the types of mutations induced by the individual products with those observed in aggregate studies lend further support to the conclusion that oxidation of **G** and **8-oxoG** can explain the majority of peroxynitrite-induced mutations.

Conclusions

Peroxynitrite induces DNA base damage predominantly at **G** and **8-oxoG** nucleobases via oxidation reactions that are thermodynamically and kinetically favorable. In addition to purely 'oxidative' products, nitration products result in both cases, consistent with the generation of radical intermediates that can recombine with the $^{\cdot}NO_2$ formed during peroxynitrite degradation. The neutral **G** radical, **G**$^{\cdot}$, is the intermediate

that reacts with $^{\cdot}NO_2$ to yield **8-NO_2G** and **NI**, while for **8-oxoG** we have proposed a reactive guanidinylidene radical intermediate. In addition to rationalizing the formation of **NO_2-DGh**, the putative guanidinylidene radical generated during **G** and **8-oxoG** oxidation also reconciles the formation of **Iz** on the very rapid timescale of peroxynitrite-mediated reactions, and may also be important in other **G** and **8-oxoG** oxidation models.

The products generated during peroxynitrite-mediated **8-oxoG** oxidation are dependent on oxidant flux. At high fluxes, **DGh**, **CAC**, and **NO_2-DGh** are the predominant products, and result from reactions involving attack by the peroxynitrite anion, $ONOO^-$, on an electrophilic intermediate. At low fluxes, **Sp**, **Gh**, and **HICA** are formed, and these arise from H_2O attacking the electrophilic intermediate. At intermediate fluxes, these two pathways compete and both product sets result.

The dramatic shift in product distribution as a function of peroxynitrite flux illustrates the importance of using in vitro model systems that, as closely as possible, recapitulate the physiologic system. Given that peroxynitrite and its derived radicals are all chemically reactive, it is crucial to ensure that the relative concentrations are well controlled to minimize competing reactions that may not be relevant in vivo. In addition to the reaction of **8-oxoG** with peroxynitrite, a similar phenomenon has been observed in experiments with tyrosine, where 3-nitrotyrosine and 3,3′-dityrosine formation dominates at high and low peroxynitrite fluxes, respectively [104–108]. Increasingly sophisticated systems for modeling in vivo peroxynitrite production are being developed [104–108] and these should help with predicting more precisely the most likely in vivo products.

Nevertheless, this product shift with changing peroxynitrite flux is intriguing. Together with the emerging information on the genotoxic and mutational characteristics of the individual oxidation products, it suggests that the extent of tissue damage, mutational spectra and, hence, cancer risk may change as a function of peroxynitrite fluxes as different product combinations predominate. This will require further work to establish how and whether the individual products are repaired, and their frequency in DNA from tissues under oxidative stress. The former goal is readily achievable, but the latter presents an enormous challenge since some of these products are unstable under the conditions required to digest DNA, and the resulting nucleosides and aglycones are highly polar, making chromatographic separation difficult. However, with the rapid improvements in sensitive and specific tandem chromatographic and mass spectrometric methods, this problem too, should be tractable.

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