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Perspective

A constellation of reactive intermediates — electrophiles and free radicals — capable of damaging cellular constituents is generated during normal physiological or pathophysiological processes. The consequences of this damage include enhanced mutation rates, altered cell signaling, and events summarized in other articles in this Perspective series. In many cases, the initially generated reactive intermediates convert cellular constituents into second-generation reactive intermediates capable of inducing further damage. Cells have adapted to the existence of reactive intermediates by the evolution of defense mechanisms that either scavenge these intermediates or repair the damage they cause. High levels of damage can lead to cell death through apoptosis or necrosis. This article provides an overview of DNA and protein damage by endogenous electrophiles and oxidants and an introduction to the consequences of this damage. First-generation reactive intermediates Some reactive intermediates are produced as diffusible cofactors in normal metabolic pathways. For example, S-adenosylmethionine is a cofactor for many methylation reactions that are required in biosynthesis or in regulation of gene expression. The latter role requires its presence in the nucleus, where it also can react nonenzymatically with DNA bases to produce a variety of methylated derivatives. Some of these methylated DNA bases are highly mutagenic during DNA replication (see below). Most reactive intermediates are produced as unavoidable consequences of our existence in an aerobic environment (Figure […]

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First-generation reactive intermediates

Some reactive intermediates are produced as diffusible cofactors in normal metabolic pathways. For example, *S*-adenosylmethionine is a cofactor for many methylation reactions that are required in biosynthesis or in regulation of gene expression. The latter role requires its presence in the nucleus, where it also can react nonenzymatically with DNA bases to produce a variety of methylated derivatives. Some of these methylated DNA bases are highly mutagenic during DNA replication (see below).

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Nonstandard abbreviations used: superoxide anion (O_2^-); peroxynitrite ($ONOO^-$); peroxynitrous acid (ONOOH); nitrosoperoxycarbonate ($ONO_2CO_2^-$); nitroxyl anion (NO^-); 8-oxo-7,8-dihydrodeoxyguanosine (8-oxo-dG); 8-oxo-7,8-dihydrodeoxyadenosine (8-oxo-dA); 5-hydroxydeoxycytidine (5-hydroxy-dC); 2′-deoxyadenosine (dA); 2′-deoxycytidine (dC); 2′-deoxyguanosine (dG); 2′-deoxythymidine (dT); malondialdehyde (MDA); 4-hydroxynonenal (4-HNE); pyrimidopurinone (M_1dG); 8-hydroxypropanodeoxyguanosine (HO-PdG); methionine sulfoxide reductase A (MSRA).

Most reactive intermediates are produced as unavoidable consequences of our existence in an aerobic environment (Figure 1). Utilization of O_2 for energy production puts us at risk because of the generation of reactive oxidants as products of O_2 reduction. Mitochondria reduce O_2 to water via cytochrome oxidase as the final step in respiratory electron transport. Although most of the electrons are successfully transferred down the chain, estimates suggest that up to 10% of the reducing equivalents from NADH leak to form superoxide anion (O_2^-) and H_2O_2, which diffuse from the mitochondria (1). Macrophages and neutrophils reduce O_2 to O_2^- via NADPH oxidase as part of the host defense system. Most of the O_2^- generated in inflammatory cells dismutates to form H_2O_2, which is a substrate for myeloperoxidase. O_2^- is also generated in epithelial cells by the action of an NAPDH oxidase complex that is related to the oxidase complex in phagocytic cells but that has a much lower capacity for O_2^- generation (2). The low flux of oxidants in epithelial cells is believed to play a role in signaling.

In addition to serving as a source of H_2O_2, O_2^- can react with ferric ion or nitric oxide to generate more potent oxidants (Figure 1). Reduction of ferric ion produces ferrous ion, which can reduce H_2O_2 to hydroxide ion and hydroxyl radical. Hydroxyl radical has a redox potential of 2.8 V, so it is an extremely strong oxidant capable of oxidizing virtually any molecule it encounters. Inflammatory cells generate copious amounts of NO and O_2^- by the action of inducible NO synthase and NADPH oxidase, respectively. Both species are important components of the host defense system. Chronic activation of the inflammatory response can induce collateral damage in adjacent normal tissue, which contributes to a range of diseases (see below and other articles in this Perspective series).

Reaction of O_2^- with NO produces peroxynitrite ($ONOO^-$). $ONOO^-$ and its protonated form, peroxynitrous acid (ONOOH), react with cellular nucleophiles or oxidize hemeproteins to ferryl-oxo derivatives. Both ONOOH and ferryl-oxo complexes are strong oxidants. ONOOH is a direct oxidant and also undergoes homolysis to produce hydroxyl radical and $NO_2^{\bullet}$ (3, 4). Approximately 25% of these radicals escape the solvent

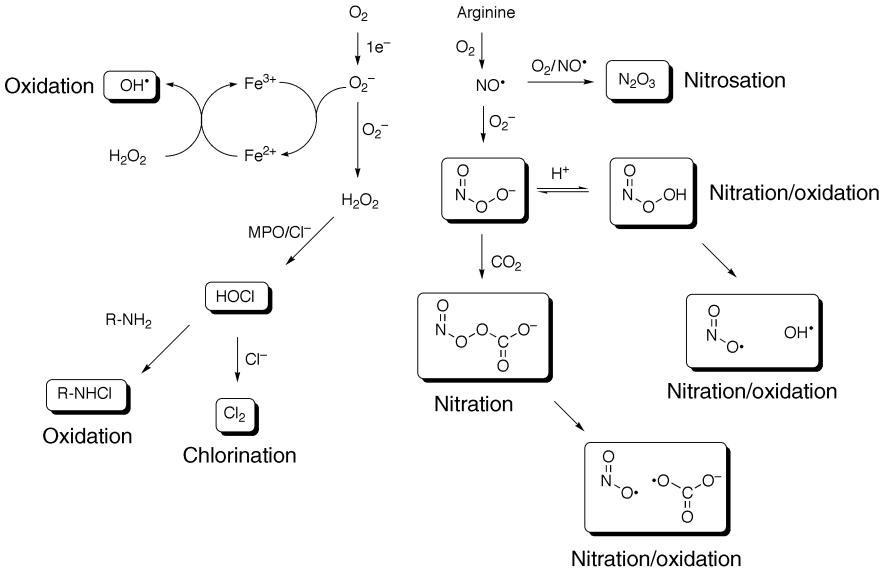

Figure 1
Reactive intermediates generated from superoxide and nitric oxide. MPO, myeloperoxidase.

cage to react with cellular constituents (5). $ONOO^-$ reacts with CO_2 to form a carbonate adduct, nitrosoperoxycarbonate ($ONO_2CO_2^-$) (6). The level of CO_2 in most tissues is sufficient to trap all of the $ONOO^-$ released from inflammatory cells. The carbonate adduct is not as strong an oxidant as $ONOO^-$ or ONOOH, but it is an effective nitrating agent. The mechanism of nitration is believed to involve homolytic scission of $ONO_2CO_2^-$ to carbonate radical, which can either oxidize $NO_2^\bullet$ to NO_2^+ or oxidize DNA or protein to a derivative that then couples with $NO_2^\bullet$ to form a nitro derivative (e.g., tyrosine→tyrosyl radical; guanine→guanyl radical) (7).

In the presence of O_2, NO generates a nitrosating agent that reacts with sulfhydryl groups to form *S*-nitroso derivatives (8). The chemistry of nitrosation is complex, but N_2O_3 appears to be the principal nitrosating agent produced. *S*-nitroso derivatives of proteins can demonstrate altered structural and functional properties depending upon their location in the protein. They also can transfer the nitroso group to other thiol-containing molecules (e.g., glutathione) or react with thiols to form mixed disulfides and release nitroxyl anion (NO^-).

H_2O_2 produced by inflammatory cells oxidizes myeloperoxidase to a higher oxidation state that has a redox potential in excess of 1 V. This higher oxidation state (a ferryl-oxo complex) oxidizes Cl^- to HOCl, which is capable of oxidizing or chlorinating cellular macromolecules (Figure 1) (9). HOCl also reacts with amines to form chloramines or with Cl^- to form Cl_2 gas; the latter chlorinates DNA or protein (10). A similar cascade of reactions is triggered by bromoperoxidase in eosinophils (11). Myeloperoxidase also can oxidize nitrite to $NO_2^\bullet$, which can react with NO to form N_2O_3. This reaction may serve as an alternate pathway of protein nitrosation to the uncatalyzed reaction that produces N_2O_3 (which requires four NOs and O_2) (12).

Second-generation reactive intermediates

The oxidants generated from O_2 reduction react with cellular components to produce a range of unstable oxidation products that can break down to diffusible electrophiles (Figure 2). For example, myeloperoxidase oxidizes α-amino acids to aldehydes; of particular interest is the oxidation of threonine to acrolein (13). Polyunsaturated fatty acid residues in phospholipids are very sensitive to oxidation at the central carbon of their pentadienyl backbone. Oxidation of the pentadienyl functionality by oxidants such as hydroxyl radical and ONOOH leads to a complex series of reactions that generate a range of electrophilic derivatives (Figure 2) (14). These include aldehydes and epoxides that are capable of reacting with protein or nucleic acid. α,β-Unsaturated aldehydes are particularly important oxidation products, because they have two sites of reactivity, which leads to the formation of cyclic adducts or cross-links. Epoxyaldehydes display complex reactivity toward nucleophiles, including the transfer of a two-carbon unit to DNA bases (15). A series of dehydration and oxidation reactions leads to the formation of aldehydes and ketones from carbohydrates (Figure 2) (16). These compounds are capable of reacting with protein nucleophiles to form advanced glycation end products. So, when considering the consequences of the generation of cellular electrophiles and free radicals, one must realize that primary oxidants (e.g., $HO^\bullet$, ONOOH) can give rise to a range of secondary oxidants, free radicals, and electrophiles. Obviously, cells devote considerable energy to protecting themselves from the deleterious effects of this panoply of reactive intermediates, but consideration of these mechanisms is beyond the scope of this Perspective.

Endogenous DNA damage

Exposure to endogenous oxidants and electrophiles leads to increases in damage to cellular macromolecules, including DNA. This section catalogs the major types of DNA damage and how they affect replication. DNA damage can result from reactions with nucleic acid bases, deoxyribose residues, or the phosphodiester backbone. The majority of the literature focuses on damage to bases or degradation of deoxyribose residues (17). Unrepaired DNA lesions accumulate with time and can contribute to the development of age-related diseases.

The consequences of DNA damage have been analyzed in vitro through polymerase bypass and enzymatic repair experiments and in vivo using site-specific mutagenesis experiments in bacterial and mammalian cells (18, 19).

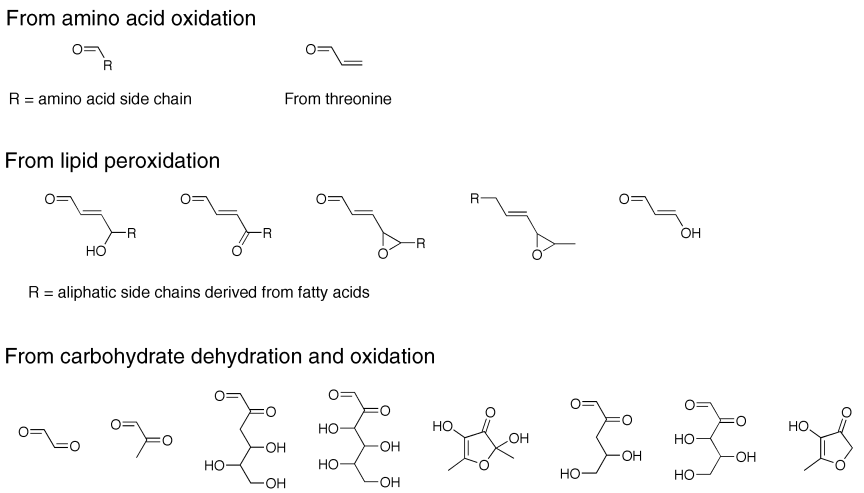

Figure 2
Reactive intermediates derived from amino acid, lipid, and carbohydrate oxidation.

The extent of DNA damage has been evaluated by examination of tissues for adduct content (20). Adducts have been quantified at levels from as low as 1 in 10^9 to as high as 1 in 10^6 nucleotides, corresponding to approximately 3–3,000 adducts per cell. Though there is a great deal of interesting and controversial work defining adduct levels in various tissues, those data are beyond the scope of this Perspective. Due to space limitations, only a few illustrative examples of specific types of damage and their effects are discussed.

Hydroxyl radical–mediated DNA damage

Hydroxyl radical can add to double bonds of DNA bases or abstract hydrogen atoms from either methyl groups or deoxyribose residues (21) (Figure 3). Hydroxyl radical reacts with purines by adding to the 7,8 double bond to generate the 8-oxo-7,8-dihydrodeoxyguanosine (8-oxo-dG) that is more abundant than 8-oxo-7,8-dihydrodeoxyadenosine (8-oxo-dA) (22). Hydroxyl radical also adds to the 5,6 double bond of pyrimidines to produce pyrimidine glycols (23). Alternatively, hydrogen abstraction from the 5-methyl group of thymine can produce a carbon-centered radical that reacts with molecular oxygen to form a hydroperoxide; this hydroperoxide is reduced to hydroxymethyl-dU. Hydrogen-atom abstraction from deoxyribose can lead to single-strand breaks concomitant with the formation of base propenals (21). The latter are substituted enals and can react with nucleophilic sites elsewhere on DNA (see below).

8-Oxo-dG induces transversions to T or C in both in vitro replication experiments and in vivo mutagenesis experiments (Table 1) (24, 25). A→C mutations have been observed as a result of in vitro replication of 8-oxo-dA (26). 8-Oxo-dG and 8-oxo-dA are known to undergo imidazole ring-opening to formamidopyrimidine (FAPy) adducts, which are minor, alkali-labile products of purine oxidation (27). Both 8-oxo-dG and FAPy adducts are repaired by glycosylases in prokaryotic and eukaryotic systems (28).

Hydroxyl radical damage to pyrimidines is also mutagenic. 5-Hydroxydeoxycytidine (5-hydroxy-dC) induces C→T and C→A mutations in vitro and C→T transitions in vivo (29). 5-Hydroxy-dC also deaminates to 5-hydroxy-deoxy-uracil, which codes as T. This provides an additional mechanism for the induction of C→T transitions. Thymidine glycol causes T→C mutations in vivo (30, 31).

Nitric oxide–mediated DNA damage

As discussed earlier, nitric oxide can form ONOOH, $ONO_2CO_2^-$, and N_2O_3. These reactive intermediates can damage DNA directly. ONOOH and $ONO_2CO_2^-$ have been shown to form nitrated nucleosides, the most abundant of which occur on dG (e.g., 8-nitro-dG) (32). 8-Nitro-dG is an unstable adduct, and its glycosidic bond readily hydrolyzes to form an abasic site. Strand cleavage, seen when cells are exposed cells to ONOOH, is presumed to arise from 8-nitro-dG depurination (32). Alternatively, 8-nitro-dG may decompose by reaction with another equivalent of ONOOH to form 8-oxo-dG (33). 8-Oxo-dG itself can be oxidized by ONOOH to form degradation products; 3a-hydroxy-5-imino-3,3a,4,5-tetrahydro-1*H*-imidazo[4,5-d]imidazol-2-one is an intermediate formed from 8-oxo-dG at low ONOOH concentrations (32).

N_2O_3 reacts with the exocyclic amines of 2′-deoxyadenosine (dA), 2′-deoxycytidine (dC), and 2′-deoxyguanosine (dG) to produce nitroso and then diazo intermediates that are hydrolyzed to hypoxanthine, uracil, and xanthine; these are all potentially mispairing lesions. Human cells treated with NO exhibit both G→A transitions and G→T transversions, presumably from uracil and xanthine, respectively (32, 34).

Table 1
Mutagenic consequences of replication of endogenous DNA adducts

Damage type	Mutations	Ref.
Adenine		
8-Oxo-dA	A→C	21
Etheno-dA	A→G	35
Cytosine		
5-Methyl-dC	C→T	48
Etheno-dC	C→A, C→T	35
Guanine		
O^6-methyl-dG	G→T	47
M_1dG	G→T, G→A	46
8-Bromo-dG	G→T	50
8-Oxo-dG	G→A	22
1,N^2-etheno-dG	G→A, G→T	35
Thymine		
Thymine glycol	T→C	30

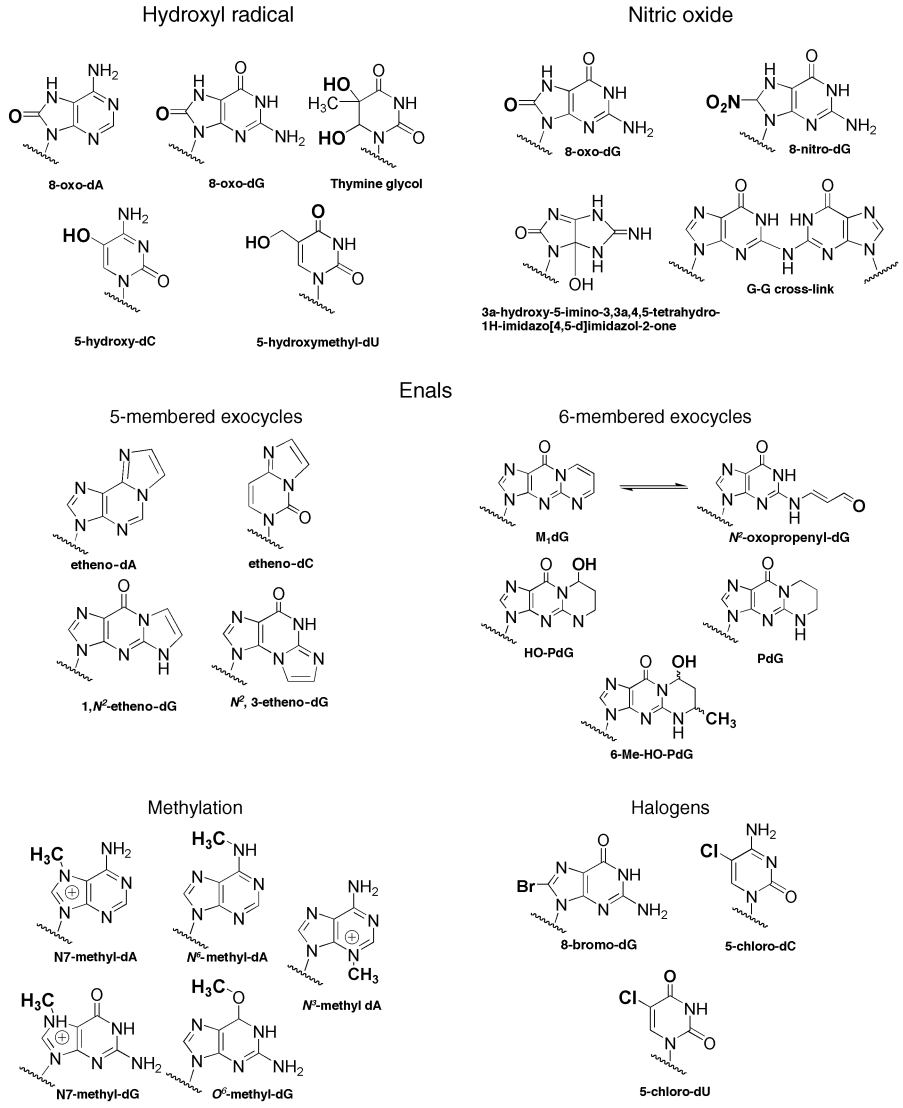

Figure 3
Endogenous products of DNA damage.

Furthermore, adjacent guanines can cross-link by an N_2O_3-mediated conversion of the amine on one guanine to a diazonium ion, followed by the attack of the exocyclic amine of the neighboring dG (32). N_2O_3 also has been shown to induce single-strand breaks on naked DNA and in intact cells.

Enal and exocyclic adducts

Enals can react at the exocyclic amino groups of dG, dA, and dC to form various alkylated products. Some common enals that cause DNA damage are malondialdehyde (MDA), acrolein, crotonaldehyde, and 4-hydroxynonenal (4-HNE). Enals are bifunctional electrophiles that present two reactive sites to DNA. The most common adducts arising from enals are exocyclic adducts such as etheno adducts from dA, dG, and dC; a pyrimidopurinone (M_1dG) adduct from dG; and 8-hydroxypropanodeoxyguanosine (HO-PdG) adducts from dG. Etheno adducts also arise from reaction of exogenous sources such as vinyl chloride, and substituted etheno adducts originate from the 4-HNE derivative 4-oxo-nonenal (35, 36).

Etheno adducts are mispairing and mutagenic in bacterial and mammalian cells, although significant differences in mutagenicity have been described in prokaryotes and eukaryotes with regard to etheno-dA (35). Bacterial and mammalian cells have evolved base excision repair pathways to deal with etheno adducts. 1,N^6-etheno-dA and, to a lesser extent, N^2,3-etheno-dG are excised by *N*-methylpurine-glycosylases, whereas 3,N^4-etheno-dC has been shown to be repaired by a mismatch-specific thymine-DNA glycosylase (37–39).

The M_1dG adduct arises by reaction of MDA with dG residues (40). When positioned in duplex DNA opposite dC, M_1dG undergoes hydrolytic ring-opening to N^2-oxopropenyl-dG (41). Both the ring-closed and the ring-opened adducts are mutagenic in bacteria (G→A and G→T in both cell types) (42). The mutagenic fre-

quency induced by the ring-opened form is lower than that observed for the ring-closed form.

Crotonaldehyde is mutagenic and forms diastereomeric six-membered ring adducts (6-methyl–HO-PdG) in vitro (43). HO-PdG, the structurally related adduct derived from acrolein, is mutagenic in vitro and in vivo (44, 45). The unsubstituted propano-dG (PdG) adduct is mutagenic in vitro and in vivo and causes frameshift mutations (though PdG is not physiologically relevant) (42, 46). Some exocyclic adducts (e.g., M_1dG and HO-PdG) are themselves electrophilic and may react with nucleophilic sites in small molecules, DNA, or protein, thereby forming cross-links (6).

DNA methylation

DNA methylation occurs through the nonenzymatic reaction of bases with *S*-adenosylmethionine and various exogenous methylating agents. The most common sites of damage are on 2′-deoxythymidine (dT), forming O^4-methyl-dT; on dG, forming O^6-methyl-dG and N7-methyl-dG; and on dA, forming N3-methyl-dA, N^6-methyl-dA, and N7-methyl-dA (47). Enzymatic methylation with *S*-adenosylmethionine on cytosine plays a role in mammalian gene regulation and imprinting, which can lead to functional inactivation of genes.

Methylated bases are mispaired in vitro and in vivo and can interfere with the binding of DNA enzymes and regulatory elements. Mutagenic events in *Escherichia coli* and mammalian cells reveal that O^6-methyl-dG mispairs with thymidine to generate G→A transitions (47). O^4-methyl-dT causes T→C transitions, whereas N3-methyl-dA and N^6-methyl-dA promote A→G transitions and abasic site formation (47). Methylation on N7 of purines causes destabilization of the glycosidic bond, and subsequent depurination to an abasic site. A number of glycosylases from prokaryotes and eukaryotes have been isolated that remove alkyl adenine damage. O^6-methyl-dG and alkyl-substituted adenine also have been directly implicated in methylation sensitivity in a variety of cell types (47). Strains with compromised or deleted O^6-methyl-dG repair (such as methylguanine methyltransferase) or alkyl adenine repair (encoded by *AlkA* in *E. coli* or by *aag* in humans) demonstrate higher levels of mutation and cell death when exposed to methylating agents (48).

Halogens

The generation of HOCl provides a pathway for the halogenation of DNA bases. 5-Chloro-dC has been observed as the major product of reaction of HOCl with DNA and has been detected in human tissues (49). Similarly, eosinophil peroxidases produce hypobromous acid (HOBr), which generates 5-bromo-dC and the deamination product 5-bromo-dU (11). Halogenation at the 5 position of pyrimidines may lead to mispairing. Chlorinated products, most notably 8-chloro-purines, have been isolated (50). These lesions are mispairing and cause transitions and transversions (50).

In addition to causing replication errors that lead to genetic disease, DNA damage triggers signaling cascades that slow cell cycle progression or lead to apoptosis. For example, treatment of cultured colon cancer cells with concentrations of MDA that lead to levels of M_1dG of approximately 1 in 10^7 nucleotides induces cell cycle arrest at the G1/S and G2/M transitions (51). The sensors of DNA damage and the signaling pathways that lead to cell cycle arrest and apoptosis are areas of very active investigation.

Amino acid and protein damage

Protein damage that accumulates during oxidative stress is unquestionably more diverse than the spectrum of intermediates that causes it. Modification of polypeptides by reactive species can occur on the peptide backbone, various nucleophilic side chains, and redox-sensitive side chains (Figure 4). For many of the reactive species discussed earlier, the modifications that they carry out on proteins have been characterized, and several of their molecular targets are known.

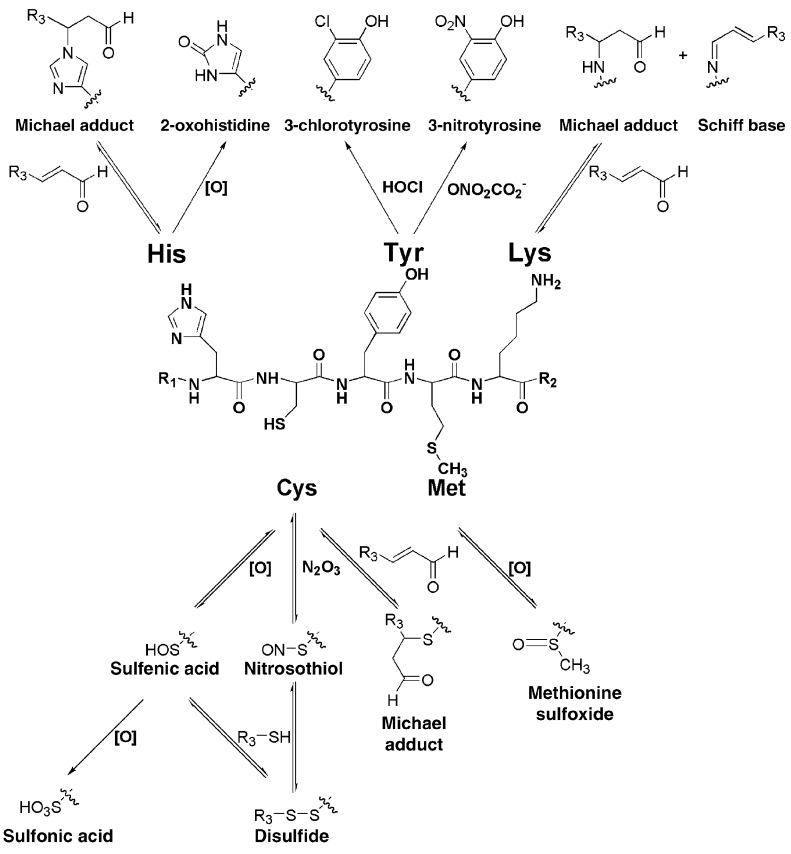

Figure 4
Endogenous damage to amino acid side chains.

In many cases, the chemistry associated with protein damage is dynamic and reversible, although several types of protein adducts accumulate during aging and/or age-related diseases.

Damage to peptide backbones

Free radicals produced during oxidative stress can damage the peptide backbone, resulting in the generation of protein carbonyls. The process is initiated by hydrogen abstraction from the α-carbon in a peptide chain. If two protein radicals are in close proximity, they may cross-link with one another by radical coupling (52). Alternatively, O_2 can attack the α-carbon–centered radical to form peroxide intermediates, leading to rearrangement and subsequent cleavage of the peptide bond to form carbonyl-containing peptides (52). Protein carbonyls also may be generated by the oxidation of several amino acid side chains (e.g., Lys, Arg, Pro) and by the formation of Michael adducts between nucleophilic residues and α,β-unsaturated aldehydes (see below) (53). Carbonyl content can be analyzed by reaction of 2,4-dinitrophenylhydrazine with proteins to form the corresponding hydrazone. As a marker of oxidative damage to proteins, protein carbonyls have been shown to accumulate during aging and age-related disease in a variety of organisms (53). Levels of protein carbonyls are, therefore, a potentially useful indicator of intracellular redox status.

Modification of side chains by intracellular oxidants and radicals

Intracellular oxidants and radicals carry out numerous modifications on the side chains of amino acid residues in polypeptides. Although most amino acids can be modified by various endogenous oxidants, residues commonly modified by $HO^{\bullet}$, O_2^{-}, HOCl, ONOOH, and $ONO_2CO_2^{-}$ include cysteine, tyrosine, and methionine. Reversible oxidation of the sulfhydryl group on cysteine converts it to cysteine sulfenic acid, which can react with thiols or undergo further irreversible oxidation to a sulfinic acid and a sulfonic acid (Figure 4) (54). Thiols with particularly low pK_a's are most susceptible to oxidation. Sulfenic acids in proteins can react with glutathione or other sulfhydryl-containing molecules to form disulfide bonds (54). In the case of glutathione, the formation of the disulfide bond is termed glutathionylation. Disulfide bonds accumulate under oxidizing conditions, but they can be readily reduced to free sulfhydryl groups by the enzyme thioredoxin or by reducing agents (55).

Recent discoveries in the biology of nitrosating agents, such as *S*-nitrosoglutathione (GSNO), have revealed that several proteins are particularly susceptible to *S*-nitrosation at specific cysteine residues. In searching for consensus nitrosation sites, it has been suggested that there are both hydrophobic and acid-base motifs in target proteins adjacent to the site of their modification (56). GSNO, a commonly used exogenous nitrosating agent, has been detected in vivo at micromolar concentrations in brain tissue and can directly transfer NO equivalents to target protein thiols (8). Additionally, other mechanisms of *S*-nitrosation of nucleophilic cysteines (e.g., by N_2O_3) are plausible (8). *S*-nitrosothiols in proteins can react with free sulfhydryl groups to create disulfide bonds and can be reduced by various reductants (8). Although the chemical pathways through which nitric oxide forms *S*-nitrosocysteine are complex, experiments using induced expression of NO synthase isoforms and neuronal NO synthase knockout cells have established that NO production increases levels of overall protein *S*-nitrosation (57, 58). *S*-nitrosation of proteins is potentially a key method that cells use to mediate inflammatory responses and other NO-regulated processes.

Methionine residues in proteins are highly susceptible to oxidation by various reactive intermediates. Upon oxidation, methionine is converted to methionine sulfoxide and can be further oxidized to methionine sulfone (59). Oxidation of methionine residues in proteins can be reversed by methionine sulfoxide reductase A (MSRA), an NADH-dependent enzyme that reduces methionine-*R*-sulfoxide to methionine (59), or by selenoprotein R, an enzyme that acts on the methionine-*S*-sulfoxide stereoisomer (60). It has been proposed that methionine residues in proteins serve protective roles by preventing oxidative damage at other residues, since methionine oxidation is enzymatically reversible (59). In accordance with this hypothesis, mice lacking the *MsrA* gene have decreased life spans, behavioral abnormalities, and increased protein carbonyl levels (61). A separate study using a transgenic *MSRA Drosophila* model supports these observations by demonstrating that transgenic flies that overexpress MSRA live longer, more active lives when compared with wild-type littermates (62).

Oxidants generated during stress can modify the aromatic residues phenylalanine and tyrosine. Phenylalanine is sensitive to oxidation by $HO^{\bullet}$ and transition metals, which cause its conversion to *o*- and *m*-tyrosine. Tyrosine residues can be modified by several oxidants, including HOCl, ONOOH, and $ONO_2CO_2^{-}$ (63). A variety of oxidative modifications can occur to tyrosine side chains in proteins, including formation of *o*,*o*′-dityrosine, 3,4-dihydroxyphenylalanine, 3-nitrotyrosine, and 3-chlorotyrosine. These modifications are likely irreversible, so inactivation of various proteins in this manner could have lasting detrimental effects. Recent reports have documented that 3-nitrotyrosine and 3-chlorotyrosine can be generated ex vivo during sample processing, illustrating the limitations of some quantitative methods that do not use isotopically labeled internal standards that can distinguish in vivo from ex vivo generation (e.g., immunochemical, spectroscopic, and electrochemical detection). This underscores the importance of developing robust analytical methods and validating them in in vivo settings. Indeed, some advances in the mass spectrometric quantification of tyrosine modification have been described (64–66). Application of these methods has demonstrated increases in oxidative damage to aromatic amino acid residues in atherosclerosis and other degenerative dis-

eases, supporting the hypothesis that aromatic amino acid damage contributes to the development and/or progression of various age-related diseases (67, 68).

Modification of proteins by aldehydic intermediates

Modification of amino acids by α,β-unsaturated aldehydes commonly occurs on the nucleophilic residues cysteine, histidine, and lysine (Figure 4) (16, 69). The chemistry that shorter α,β-unsaturated aldehydes undergo when reacting with proteins is slightly different from that of the reactions depicted in Figure 4. For instance, two molecules of acrolein react with the ε-amino group of lysine to form predominantly a cyclic 3-formyl-3,4-dehydropiperidino adduct (16). MDA mainly reacts with lysine residues by Michael addition, but other forms of multimerized MDA have been found to react with lysine as well (16). Michael adducts between 4-hydroxy-2-alkenals and cysteine, histidine, and lysine are formed as depicted, as well as Schiff base adducts that form only following reaction with the amino group on lysine residues (16, 69). The products of 4-hydroxy-2-alkenals can be converted to more stable structures, including cyclic hemiacetals, pyrroles, and various other structures (16, 69). Increased levels of acrolein and 4-HNE protein adducts, abundant products of lipid peroxidation, are found in cardiovascular disease and neurodegenerative diseases (16, 69).

Protein targets of reactive species

Endogenous oxidants and electrophiles modify a host of proteins that run the gamut of biological functions (Table 2). For most protein targets, there is a remarkable specificity in the type and location of the residue(s) modified. An exhaustive description of these targets, the effect that is mediated, and the modifying agent that carries out the damage is not possible due to space limitations. For more information, the reader is referred to other articles that deal with protein targets of individual modifying agents (59, 70, 71). A survey of common protein targets is listed in Table 2 and is expounded below. Where possible, the modifications are described, and the biological consequences are discussed.

Damage to structural proteins

Cell structure is maintained through a variety of cytoskeletal components including tubulin and filamentous proteins. Due to their abundance in specific cell types, these proteins are common targets of a variety of reactive species. Although the consequences of these modifications are still unclear, functional assays have been developed for some damaging agents. Tubulin isoforms are modified in vitro and in vivo by a number of intermediates, including nitrosating agents and 4-HNE (57, 72). 4-HNE forms Michael adducts with tubulin isoforms and disrupts microtubule assembly in neuroblastoma cells, blocking neurite outgrowth

Table 2
Common protein targets of reactive endogenous intermediates

Protein target	Residue modified	Modifying agents	Biological consequences	Ref.
Enzymes				
Caspases	Catalytic cysteine	$HO^{\bullet}$, nitrosating agents	Glutathionylation, inactivation, reduced death by apoptosis, necrosis (?)	80–83, 95, 96
Protein tyrosine phosphatases	Catalytic cysteine, active-site tyrosine (?)	$HO^{\bullet}$, O_2^-, nitrosating agents,ONOOH	Glutathionylation, inactivation, accumulation of phosphotyrosine, prolonged activation of MAPKs (?)	84–90, 97–100
IκB kinase	Active-site cysteine (?)	4-HNE, $HO^{\bullet}$	Inactivation of NF-κB signaling	101, 102
Tyrosine hydroxylase	Active-site tyrosine(s)	ONOOH	Inhibition of dopamine synthesis, possible role in progression of Parkinson disease	77–79
Mn-superoxide dismutase	Active-site tyrosine	ONOOH	Prevention of O_2^- detoxification, potential role in transplant rejection	74–76
H-ras (p21)	Cysteine near GTP-binding site	NO, $HO^{\bullet}$	Destabilization of GDP binding, activation of signaling downstream of Ras	103–107
GAPDH	Catalytic cysteine (?)	4-HNE, $HO^{\bullet}$, O_2^-, nitrosating agents, ONOOH	Glutathionylation, inactivation, modification by NADH	57, 108–115
Structural and membrane proteins				
Tubulin	Cysteine residue(s)	4-HNE, nitrosating agents, ONOOH	Disruption of microtubule networks	57, 72, 116
Neurofilament heavy subunit	Lysine and cysteine residues	4-HNE, nitrosating agents	Unknown	57, 73
N-methyl-D-aspartate receptor channel	Extracellular cysteine residue	Nitrosating agents	Inactivation, reduced Ca^{2+} influx	117–120
Transcription factors				
NF-κB	Cysteine in DNA-binding domain	$HO^{\bullet}$, nitrosating agents	Glutathionylation, inhibition of NF-κB transcriptional activity	121–126
AP-1	Cysteine in DNA-binding domain	Various oxidants, NO	Glutathionylation, inhibition of DNA binding	127–130
OxyR	Cysteine residues	$HO^{\bullet}$, nitrosating agents	Glutathionylation, transcriptional activation of OxyR-responsive genes, clearance of oxidants and other reactive species	91, 92, 131–134

(72). Additionally, neurofilament heavy chains have been identified as targets of nitrosating agents and 4-HNE (57, 73). The mechanistic features and the effect(s) seen with modification of neurofilaments by these and other agents remain to be elucidated.

Damage to metabolic and detoxification enzymes

Disruption of housekeeping enzymatic activities can occur in response to various endogenously produced electrophiles and radicals and may serve to potentiate stress responses. For instance, Mn-superoxide dismutase has been shown to be inactivated in rejected kidney transplants (74). Accumulation of 3-nitrotyrosine, an adduct of $ONO_2CO_2^-$, is observed on Mn-superoxide dismutase in the same patients (74), presumably on a single active-site tyrosine (75, 76). Upon inactivation of this key detoxifying enzyme, superoxide and $ONOO^-$ can accumulate and potentially trigger more protein and DNA damage.

Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of L-dopamine, an essential chemical for the proper function of dopaminergic neurons. L-dopamine levels are substantially reduced in Parkinson disease patients. It has recently been shown that tyrosine hydroxylase isolated from the brains of mice that are models for Parkinson disease contains 3-nitrotyrosine adducts and exhibits reduced enzymatic activity (77). Upon in vitro modification of tyrosine hydroxylase with ONOOH, several active-site tyrosine residues are damaged (78, 79). These data support the hypothesis that tyrosine hydroxylase is a critical target for oxidative inactivation in Parkinson disease.

Damage to proteins involved in cell signaling and gene expression

Numerous studies have revealed the importance of reactive species in altering cell signaling pathways that are critical for cell growth, differentiation, and/or survival. Targets of reactive intermediates include apoptotic caspases, protein tyrosine phosphatases, certain kinases, and the transcription factors AP-1, NF-κB, and OxyR. Of these, perhaps the most extensive work has been performed on modification of protein tyrosine phosphatases, caspases, and OxyR.

Caspases, the cysteine protease executioners of the apoptotic response, can be modified by oxygen radicals and various nitrosating agents. These enzymes catalyze the proteolysis of proteins necessary for cell survival, using a nucleophilic cysteine residue in the active site to stimulate cleavage of target proteins at consensus aspartate residues. Several studies have shown that caspase-3, upon modification by radicals and nitrosating agents, is inactivated (80–82); presumably the inactivating modification of caspase-3 occurs on its catalytic cysteine residues (80–82). It has recently been demonstrated that *S*-nitrosated caspase-3 molecules are predominantly localized to the mitochondria (83), suggesting that this regulatory modification may prevent activation of caspase-3 in specific cellular organelles. The modification of caspase-3 by various agents represents a potential method for regulating its activity, thereby preventing apoptosis.

A large family of protein tyrosine phosphatases exists to modulate signaling by growth factors and other agonists. All members of the protein tyrosine phosphatase family function via a common catalytic mechanism that utilizes a single cysteine residue; these active-site cysteines have pK_a's of approximately 5.5. Because of their low pK_a's, the catalytic cysteines are susceptible to inactivation by oxygen radicals, ONOOH, and nitrosating agents, being converted to a sulfenic acid, a sulfonic acid, *S*-nitrosocysteine, or a glutathionylated cystine (84–90). In cells subjected to oxidative stress, inactivation of protein tyrosine phosphatases is associated with an accumulation of phosphotyrosine and the potential for prolonged activation of specific stress-responsive and/or mitogenic signaling pathways (87).

One prototypical transcription factor that is regulated by oxidation and *S*-nitrosation is the bacterial protein OxyR. This homotetrameric protein undergoes a conformational change and becomes activated when critical cysteine residues are nitrosated or oxidized (91). Subsequently, OxyR activates transcription of several bacterial genes that protect against and/or repair oxidative damage. Various modifications of OxyR (i.e., *S*-nitrosation, sulfenic acid formation, and glutathionylation) influence its DNA-binding and transcriptional activation properties differently, suggesting that these modifications are responsible for varied biological responses (92). Knowledge gained from the study of OxyR may be useful in studying the effects of oxidants on oxidant-sensitive eukaryotic transcription factors, such as AP-1 and NF-κB.

Proteomic approaches for identifying protein targets of reactive species

Proteome-wide screening for modified proteins promises to be an exciting area of research in the immediate future. High-throughput methods have recently been developed for examining global protein *S*-nitrosation, glutathionylation, and tyrosine nitration (57, 93, 94). Although most current methods use the power of mass spectrometry for identification of proteins, the approaches taken to enrich for and isolate modified proteins differ. For example, an elegant approach to the isolation of *S*-nitrosated proteins was recently reported (57). Free thiol groups were reacted with methyl methanethiosulfonate, and then *S*-NO groups were reduced to free thiols by treatment with ascorbate. Subsequently, using streptavidin agarose beads, the newly released protein thiols were converted to biotin conjugates to enrich for proteins. Peptides from isolated proteins were identified using matrix-assisted laser desorption ionization mass spectrometry. Approximately 15–20 proteins were identified as targets of nitrosating agents. The methodology was validated by confirmation that many targets of exogenous nitrosating agents are in vivo targets, as revealed by the lack of labeling in brain lysates from mice deficient in neuronal NO synthase.

In another method, glutathionylated proteins were identified by incubation of cells with radiolabeled cysteine in the absence of protein synthesis, exposure of T

lymphocytes to agents that create an oxidative intracellular environment, isolation and detection of radiolabeled (i.e., glutathionylated) proteins, and identification of radiolabeled peptides by mass spectrometry (93). This study reveals that numerous proteins are targets for redox-dependent modification. Several of the enzymes identified were inactivated in in vitro assays, implying that glutathionylation of proteins upon alteration of cellular redox status is a potential mechanism for regulating the activity of many targets.

A similar methodology has been developed for identifying tyrosine damage by $ONO_2CO_2^-$ following exposure of cells or animals to inflammatory stimuli (94). This approach uses detection of proteins containing 3-nitrotyrosine residues by Western blotting following two-dimensional electrophoresis. Proteins from A549 cells, rat liver, or rat lung were partially transferred to membranes, and nitrated proteins were identified by Western blotting. The spots of the gel comigrating with those staining positive by Western blot were extracted and identified by mass spectrometry. Approximately 40 proteins were identified in this study as targets of $ONO_2CO_2^-$, although the biological consequences of nitration in most cases remain to be determined.

Concluding thoughts

Research in the past twenty years has heightened our awareness of the importance of endogenous metabolic processes in cellular dysfunction, mutagenesis, and death. Much of this work has been descriptive, because the tools needed to ask discrete molecular questions were not available. However, there has been a dramatic and recent transformation in our ability to conduct qualitative and quantitative analysis of nucleic acid and protein modification. Thus, it is reasonable to assume that the next few years will witness a more precise definition of the events that lead from chemical modification to altered cellular function and/or heritable genetic change. This application of the tools of chemistry to biology will provide a strong experimental basis for the development of new diagnostic, therapeutic, and preventive strategies.

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