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Transduction of Redox Signaling by Electrophile-Protein Reactions

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Abstract

Over the last 50 years, the posttranslational modification (PTM) of proteins has emerged as a central mechanism for cells to regulate metabolism, growth, differentiation, cell-cell interactions, and immune responses. By influencing protein structure and function, PTM leads to a multiplication of proteome diversity. Redox-dependent PTMs, mediated by environmental and endogenously generated reactive species, induce cell signaling responses and can have toxic effects in organisms. PTMs induced by the electrophilic by-products of redox reactions most frequently occur at protein thiols; other nucleophilic amino acids serve as less favorable targets. Advances in mass spectrometry and affinity-chemistry strategies have improved the detection of electrophile-induced protein modifications both in vitro and in vivo and have revealed a high degree of amino acid and protein selectivity of electrophilic PTM. The identification of biological targets of electrophiles has motivated further study of the functional impact of various PTM reactions on specific signaling pathways and how this might affect organisms.

Introduction

Our still-blossoming insight into genetically encoded cellular communication mechanisms stemmed from two seminal advances rooted in the 1950s. When studying steroid hormone function, Mueller and Jensen pioneered the concept that mRNA transcription and translation into protein could be regulated by receptor-ligand interactions (1). The posttranslational modification (PTM) and regulation of proteins by O-phosphorylation, first described by Kennedy, Krebs, and Fischer (2), revealed another exciting aspect of metabolic regulation—that protein function could be modified by secondary chemical reactions that are linked to metabolic and environmental stimuli. Propelled by the discovery of protein O-phosphorylation, additional PTMs of proteins have been discovered that expand the scope of the functional proteome one to two orders of magnitude over that encoded by the genome (3).

Approximately 5% of the genome encodes enzymes that catalyze the PTM of proteins, which dictates proteome diversification and plasticity. In addition to O-phosphorylation, PTMs include acetylation, acylation, adenosine diphosphate–ribosylation, biotinylation, carboxylation, glycosylation, hydroxylation, methylation, ubiquitinylation, SUMOylation, and sulfation, to name a few. These structural modifications of proteins can affect catalytic activity, trafficking, stability, and immunogenicity, thus linking biochemical stimuli with the physiological responses of organisms.

Redox Signaling

Within the ever-expanding universe of PTMs, the regulation of protein structure and function by oxidation-reduction (redox) reactions has emerged as a vibrant area of investigation. Many discoveries in redox biology continue to emerge since McCord and Fridovich's 1969 discovery of biological superoxide generation and the existence of the oxygen radical-scavenging enzyme superoxide dismutase (4). Soon thereafter, it was appreciated that activated inflammatory cells and various forms of metabolic stress (such as ischemia-reperfusion, hyperoxia, and xenobiotic exposure) increased tissue production rates and steady-state concentrations of reactive oxygen species (5–8). Reactive species, first viewed as toxic by-products of inflammatory responses and xenobiotic exposure (9, 10), are now also appreciated as signaling mediators that are produced and inactivated in a regulated manner. Cellular redox reactions and their regulation rely on an extensive array of proteins and small molecules that evolved (i) to generate reactive species in a regulated manner as host defense and signaling intermediates, (ii) to scavenge catalytically or to inactivate reactive species, and (iii) to sense the cellular redox milieu chemically by undergoing oxidation and reduction reactions.

The discovery of nitric oxide ($\cdot\text{NO}$) as a free radical mediator of cell signaling and inflammation expanded the scope of redox-regulated cell signaling. In addition to activating guanylate cyclase through heme-iron coordination, $\cdot\text{NO}$ reacts directly with superoxide ($\text{O}_2^{\cdot-}$) and indirectly with hydrogen peroxide (H_2O_2)–derived species, to yield some of the most reactive molecules found in biology (9, 11, 12). Through the formation of secondary $\cdot\text{NO}$ -derived species, such as peroxynitrite (ONOO^-) and nitrogen dioxide ($\cdot\text{NO}_2$), $\cdot\text{NO}$ thus accelerates the chemical reaction rate and increases the breadth of reactions that transduce redox signaling. In cells and tissues, mediators of redox signaling are a diverse population of small molecules and proteins that serve as molecular sensors of cellular metabolic and stress status. When alterations in the redox state of the target molecules act as sensors, organisms respond by modulating many cellular functions, including gene expression, enzyme catalysis, cytoskeletal function, membrane transport, intermediary metabolism, respiratory chain function, and neurotransmission.

The broad reactivities and short half-lives of redox signaling mediators have engendered productive debate regarding the rates of generation, steady-state concentrations, chemical identities, specific molecular targets, and mechanisms of signal transduction of particular reactive species. Notably, nonbiological mediator concentrations are often required to produce detectable responses in model systems, sometimes promoting primary and secondary reactions that would be more limited or absent in vivo. Finally, if labile PTMs are

induced by redox mediators, the chemical characterization and quantification of these modifications becomes a challenge. Overall, unappreciated reactions and the analytical challenges inherent in studying short-lived redox intermediates have both churned controversy and motivated investigators to advance knowledge in this area at a rapid pace.

Herein, we focus attention on one class of reactive species that stem from redox reactions, electrophiles. These molecules attract electrons, and by virtue of this property, electrophiles transduce cell signaling by mediating reversible structural and functional changes in susceptible protein targets. We also offer perspective about what constrains the regulated signaling actions of biological electrophiles, as opposed to initiating toxicological outcomes.

Cysteine Thiol—A Primary Target of Redox Reactions

Cysteine is the most nucleophilic amino acid of proteins and readily undergoes Michael addition with electrophiles (13, 14). Simply put, Michael addition is the reaction of a Michael donor (nucleophiles, such as amine or thiol groups) with a Michael acceptor (typically an alkene with an electron-withdrawing substituent, such as a nitro or keto group). Other key biological nucleophiles include the imidazole in the histidine side chain; the amine in the lysine side chain; and the amino-containing substituents of sugars, phospholipids, and DNA. Although less nucleophilic than thiols, these species are also susceptible to electrophile reaction. The thiol of cysteine (Cys-SH) is responsible for its high reactivity with secondary products of oxygen-dependent and NO-dependent reactions and the downstream electrophilic products generated by reaction with biomolecules (15–17). Partially reduced oxygen species (18), NO-derived species (17, 19–21), electrophilic by-products of redox reactions, and glutathione (GSH) can transduce redox signaling by modifying protein cysteine residues to sulfenic (Cys-SO[•]), sulfinic acid (Cys-SO₂[•]), sulfonic acid (Cys-SO₃²⁻), S-nitrosothiol (Cys-S-NO), and disulfide derivatives. Disulfides include cysteinylated proteins (Cys-S-SP), cysteinylated glutathione (Cys-S-SG), and glutathionylated protein (GS-SP), as well as intramolecular disulfides and disulfide cross-links between different proteins (22–24). Because of the diversity of reactive species that are formed during inflammation, metabolic stress, and ·NO signaling, it is not tenable to expect the formation of highly selective thiol-electrophile adduct formation, the generation of a predominant thiol oxidation state, or the formation of predominantly S-nitrosation products. Take, for example, the signaling mediator ·NO, which does not directly react with thiols at an appreciable rate. Rather, ·NO mediates protein S-nitrosation either through transnitrosation from another RS-NO derivative or upon ·NO reaction with a sulfenyl radical (often termed thiyl radical) intermediate after metal-catalyzed thiol oxidation. These reactions are also occurring in a milieu where the generation of O₂^{•-}, other partially reduced oxygen species, and by-products of their reactions are ubiquitous under aerobic conditions. This pro-oxidative environment can thus result in an array of biomolecule reactions mediated by ·NO that are independent of NO stimulation of cyclic guanine monophosphate (cGMP) production after activation of soluble guanylate cyclase. Notably, reactions of ·NO in an aerobic environment will also include the concurrent formation of disulfides, the generation of the further-oxidized thiol derivatives noted above, protein and lipid nitration, and the production of various electrophile adducts to be described herein.

Nucleophiles, such as cysteine, react with electrophiles through a mechanism involving donation of a pair of electrons from the nucleophile to the electrophile (electrophilic attack), resulting in the formation of a covalent bond. When this reaction involves an alkyl group as the electrophile, then this reaction is termed S-alkylation (Fig. 1). The nucleophilic reactivity of the cysteine thiol strongly depends on the surrounding chemical milieu and how this in turn affects a thiol's acid dissociation constant (pK_a). The pK_a of the thiol of the amino acid cysteine in solution is 8.33, with most protein-associated cysteine thiols also displaying pK_a values between 8.2 and 8.5. This translates to relatively low protein thiol reactivity at a typical intracellular pH of 7.2, because only ~10% of thiols are deprotonated to the reactive thiolate anion (Cys-S⁻) species that is much more readily oxidized and alkylated than neutral protonated thiols. The pK_a of the cysteine thiol is decreased when proximal to (i) basic amino acids, such as histidine, lysine, and arginine, that can deprotonate the cysteine and form a hydrogen-bonded ion pair with the cysteine thiolate anion; (ii) the aromatic amino rings of tyrosine or tryptophan that can undergo pi interactions with the SH group; or (iii) metal centers, such as heme-metal complexes, that can interact with the thiol. All of these can facilitate deprotonation to the more reactive thiolate anion, promoting more facile Michael addition or oxidation reactions (25, 26). Therefore, the secondary and tertiary structure of proteins, local pH changes dictated by tissue oxygenation and metabolic state, and the presence of alternative scavenging species will all strongly influence cysteine reaction with electrophiles. Thus, site-specific cysteine deprotonation to the thiolate anion, an event shaped by these anatomic and metabolic factors, can lend target molecule specificity to thiol redox modification. This precept is borne out by a mass spectrometry-based profiling study, where only a limited and reproducible number of highly selective protein sites for electrophilic adduction were identified in a human cell line (897 cysteine residues on 539 proteins). Specific surrounding protein structural motifs influenced the extent of cysteine reactivity with different electrophiles, which indicated that the physical microenvironment of protein cysteine residues undergoing adduction can substantially modulate the reactivity of individual cysteine thiols with electrophiles (27).

Defining a Biological Electrophile

An electrophile ("electron-lover") is attracted to electrons and undergoes chemical reactions by accepting an electron pair in order to chemically bond with a nucleophile. Unsaturated compounds with electron-withdrawing substituents are "soft" Michael acceptors and are susceptible to reaction with a "soft" base (nucleophile), such as a thiolate. "Soft" acids and bases tend to interact covalently, as opposed to "hard" acid and bases, which tend to interact ionically. This means that protein thiols interact with the carbonyl carbon (starred in Fig. 1) when undergoing Michael addition reaction.

Arthur Michael first reported in 1887 that olefins (also known as alkenes, organic molecules with at least one carbon-carbon double bond) conjugated with electron-withdrawing groups are susceptible to attack by nucleophiles. This type of reaction was named after Michael, thus leading to the terms Michael acceptor (the electrophile), Michael donor (the nucleophile), and Michael addition reaction (a type of electrophile-nucleophile reaction). In the context of the electrophilic signaling reactions discussed herein, typical electron-withdrawing groups present on biological Michael acceptors can include (but are not limited

to) aldehyde (-COH); acyl (-COR, where R typically stands for a carbon if the group is a keto); carboxylic acid (-COOH); ester (-COOR, where R typically stands for a carbon or hydrogen); halide (-Cl, -F, -Br, -I); sulfonyl (-SO_n); sulfone (-SO₂R, where R typically stands for a hydrogen or carbon); sulfonic acid (-SO₃H); and nitro (-NO₂) substituents. It can be generalized that alkenes or alkynes (molecules with a triple bond between two carbons, also called acetylenes) that are conjugated to electron-withdrawing groups will display Michael acceptor functionality.

The most prevalent electron-withdrawing group present in both xenobiotic and endogenously produced electrophiles is a carbonyl (or acyl) substituent, with a carbon-oxygen double bond conjugated to an alkene in the electrophiles. Virtually all classes of biomolecules (protein, lipid, carbohydrate, DNA, and RNA) are susceptible to oxidation reactions that can generate the carbonyl (or acyl) functional group. Because of overall abundance and susceptibility to oxidation, the unsaturated fatty acids of membranes and lipoproteins are key cellular sources of electrophilic derivatives after autocatalytic or enzymatically catalyzed peroxidation reactions. Various electrophiles, including fatty acid derivatives, react with the thiol of GSH at second-order reaction rate constants ranging from ~ 1 to 350 M⁻¹s⁻¹ (Table 1). Of note, during oxidative inflammatory conditions, protein carbonylation is primarily attributable to the electrophilic adduction of lipid-derived aldehydes, rather than to the direct oxidation of susceptible amino acids to carbonyl derivatives (28).

Cells and tissues are continuously exposed to electrophiles that stem from exogenous and endogenous sources. Species having electrophilic reactivity are ingested with foodstuffs and are endogenously produced as metabolic by-products after exposure to compounds that undergo oxidation-reduction reactions (for example, phenolic xenobiotics and anticancer drugs) (29). Electrophiles are also present in and can be generated by environmental sources (for example, combustion products, such as those in cigarette smoke) (30). In addition, insects and plants produce various defensive chemically irritating electrophiles that protect the host from other insects or herbivores (31, 32). Electrophiles are also formed in abundance by the oxidative conditions inherent during aerobic metabolism and inflammation (28, 33). A sampling of the chemical structures of biological electrophiles and their electrophilic centers is provided in Fig. 2. Specific examples of biological electrophiles are described individually below.

α,β-Unsaturated aldehydes

α,β-Unsaturated aldehydes come from endogenous and exogenous sources and include the lipid peroxidation by-products 4-hydroxynonenal (4-HNE), 4-oxononenal (4-ONE), and acrolein. The last-named is inhaled with cigarette smoke; is formed endogenously during lipid peroxidation, or protein and amino acid oxidation reactions; and is present in cooked oils and other foodstuffs (34, 35). Elevated plasma concentrations of acrolein are detected in patients with chronic renal failure, and the abundance of proteins adducted by acrolein is increased in tissues obtained from patients with Alzheimer's disease, Parkinson's disease, atherosclerosis, and chronic obstructive lung disease. The potent electrophilic reactivity of acrolein leads to its S-alkylation of several proteins. For example, animal model studies

reveal that acrolein modifies extracellular matrix proteins in the lung, resulting in enhanced macrophage adhesion and activation (36). Proteomic and immunochemical analyses also reveal that acrolein impairs cardiac function by covalently reacting with proteins in the cardiac sarcomeric and cytoskeletal network, as well as with enzymes involved in myocardial energy metabolism (34). Of note, many of these cardiovascular effects of administered acrolein are reversible and can be limited by administering the low-molecular-weight thiol *N*-acetylcysteine to acrolein-treated animal models (34).

4-HNE and 4-ONE are produced endogenously during peroxidation of membrane and lipoprotein polyunsaturated fatty acids (37, 38). Cell and animal model studies reveal that these electrophiles can traverse intracellularly, escaping reaction with cytosolic GSH and GSH transferase defenses to diffuse to the nucleus and to react with both DNA-associated proteins and DNA bases. Generally considered cytotoxic, 4-HNE and 4-ONE act in part by stimulating cell cycle arrest and c-Jun N-terminal kinase (JNK)-dependent apoptotic signaling (39, 40). In addition to forming Michael adducts with cysteine, histidine, and lysine, and a Schiff's base with lysine, 4-ONE also can induce intra- and intermolecular cross-links. For example, α - and β -tubulin are differentially modified by 4-HNE and 4-ONE, with 4-HNE leading to adduction of cysteine residues at physiologically relevant concentrations. 4-ONE induces protein cross-linking upon modification of Lys residues by reductive propylation (41). In phagocytes, the PTM of protein kinase C by 4-HNE inhibits the phagocytic function of these cells (42). The Cys¹²⁰ of human epithelial fatty acid-binding protein (e-FABP) is also a target of 4-HNE reaction. Despite irreversible S-alkylation of this residue, intracellular e-FABP remains functional and continues to bind fatty acids noncovalently; thus, this modification of e-FABP may serve a protective or antioxidant function (43). Finally, the observation that 4-HNE potentially can induce multiple stress response-related gene expression pathways suggests that organisms have evolved mechanisms to cope with low endogenous rates of production of 4-HNE and related α,β -unsaturated aldehydes (39, 44).

ω -6 and ω -3 unsaturated fatty acids

A common structural feature of some electrophilic fatty acid derivatives is a cyclopentenone prostaglandin ring containing an α,β -unsaturated carbonyl group [see 15-deoxy-^{12,14}-prostaglandin J₂ (15-deoxy-PGJ₂) in Fig. 2] or a more linear acyl chain with an α,β -unsaturated carbonyl (4-HNE and acrolein in Fig. 2). Cyclopentenone prostaglandins are formed by enzymatic peroxidation of the ω -6 fatty acid, arachidonic acid (20:4), and include 15-deoxy-PGJ₂, as well as E₁, E₂, G₂, F₂, and F_{1 α} prostaglandin derivatives (45). Arachidonic acid, oxidized by lipoxygenase-dependent catalysis or nonenzymatic oxidative and free radical pathways, also yields α,β -unsaturated carbonyls including 5-, 12-, and 15-oxo-eicosatetraenoic acid and other derivatives (45–47). Longer-chain, more unsaturated ω -3 unsaturated fatty acids [eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6)] also undergo both enzymatic and nonenzymatic oxidation to yield an array of electrophilic cyclopentenone derivatives and other α,β -unsaturated carbonyl products (48). The signaling actions of oxidized ω -3 unsaturated fatty acids can, in part, account for physiologically beneficial actions ascribed to dietary polyunsaturated fatty acids. This is ascribed to the

redox-dependent generation of electrophilic ω -3 unsaturated fatty acid oxidation products that are able to activate the signaling pathways described in the sections below (49–52).

Nitro-fatty acids

Nitro derivatives of unsaturated fatty acids (NO₂-FAs) are generated endogenously upon oxidation of unsaturated fatty acids by NO-derived species, a reaction ultimately mediated by $\cdot$ NO₂ (53). These species are formed both during inflammatory conditions, where inducible nitric oxide synthase activity is elevated, and during oxidative stress by both $\cdot$ NO and nitrite (NO₂[−])–dependent nitration reactions (54–56). The alkenyl nitro configuration of NO₂-FAs is responsible for the electrophilic reactivity of the β -carbon adjacent to the NO₂-bonded carbon (57, 58) (see 9-nitro-octadecenoic acid in Fig. 2). In red cells, NO₂-FAs react with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which inhibits its catalytic activity and induces trafficking of this protein to the plasma membrane. This process is facilitated by the hydrophobic interactions that will occur between the GAPDH–NO₂-FA adduct and membrane lipids (57). NO₂-FAs exhibit broad anti-inflammatory signaling actions through the modulation of transcriptional regulatory mechanisms (53), including serving as agonists of peroxisome proliferator–activated receptors (PPARs) (59–61). NO₂-FAs are by-products of oxidative inflammatory reactions that in turn promote signaling reactions that facilitate adaptive gene expression responses to a stressful environment. PTM reactions induced by NO₂-FAs are reversible in vivo; the NO₂-FAs adducted to protein can transfer between thiols present on both extra- and intracellular proteins, as well as glutathione (55, 57, 62, 63).

Detecting Protein Adduction by Electrophiles

The in vivo detection and quantification of protein adduction by electrophiles initially relied on spectrophotometric and radiolabel-based analyses (64, 65). Although useful as indices of the net conversion of endogenous or exogenous molecules to protein-reactive electrophilic species, these approaches often lacked the sensitivity and precision required to shed light on specific protein or amino acid targets. Even when coupled with electrophoretic separation and immunoaffinity-based protein identification and purification strategies, molecular insight was confounded by specificity and sensitivity limitations (66). These studies of the nature and extent of electrophilic PTM reactions laid a foundation of knowledge and provided inspiration for mass spectrometry (MS)–based advances that can be supplemented by immunoaffinity purification of protein adducts, genetic manipulation of susceptible nucleophilic amino acids, and chemistries that more selectively profile electrophilic species and their adduction sites. With these tools, investigators have identified both critical amino acid targets of electrophiles within a particular protein and specific protein targets within different organelles, cell types, and tissues.

Enzymatic protein hydrolyzates obtained from biological sources are expected to have a relatively low abundance of electrophilic PTM in the absence of acute dietary or toxicological exposure; thus, any adducted species that are endogenously present may be below the limits of detection of tandem MS precursor ion or neutral loss scans. The use of scoring algorithms for spectral analysis (SALSA) of MS/MS spectra has enhanced the sensitivity and specificity of detection of peptide PTM by electrophiles (67). Also useful are

activity-based protein-profiling strategies that permit fractionation of protein mixtures into electrophile-reactive subproteomes. Below, we describe some different “baits” that can be utilized to “fish” for electrophile targets and to define the factors that discriminate electrophile reactivity (Fig. 3).

Click chemistry

This robust reaction strategy capitalizes on the introduction of small and selectively reactive components into a larger structure, so that an irreversible chemical bond is formed with an added target molecule that facilitates target identification and affinity capture. Of great utility in identifying electrophile targets is the process of introducing an azido group into the electrophile and then using a copper (Cu) catalyst to induce covalent linkage with a reporter molecule or an affinity-capture reagent having an alkyne functional group. Overall, this “click” reaction is termed an azide alkyne Huisgen cycloaddition and has the advantage of reducing purification times for electrophile-biomolecule adducts and increasing the sensitivity of adduct detection (68). Modified azido-reactive difluoro cyclooctyne derivatives have been incorporated into living cells, allowing Cu-independent cycloaddition with azido derivatives of biomolecules (for example, lipids and carbohydrates). These difluoro cyclooctyne reagents are useful because they avoid the promotion of secondary and possibly artifactual oxidative reactions that might be mediated by the Cu catalyst that they replace, and they also are amenable to use in live-cell studies (69).

Using a click-chemistry strategy, biomimetic azido and alkynyl derivatives of the fatty acid oxidation product 4-HNE have been prepared and utilized to define protein targets of this electrophilic α,β -unsaturated aldehyde (70). Although both derivatization approaches were informative, the most sensitive and discriminating results were obtained upon affinity capture of azido-HNE-adducted protein. This is accomplished by (i) addition of alkyne-labeled biotin, (ii) enrichment of the “click product” between HNE and biotin with streptavidin beads, (iii) digestion of affinity-purified proteins with trypsin, and (iv) “shotgun proteomic” identification of protein-electrophile adducts by liquid chromatography separation of peptides in concert with electrospray ionization (ESI) liquid chromatography and tandem MS (LC-MS/MS) detection. If adducted peptides are abundant enough, peptide sequencing can also reveal specific amino acids responsible for electrophile adduction. This azido-alkyne click chemistry approach also facilitated comparison of the protein targets of distinctly different carbon electrophiles (a phenylsulfonate ester, linear epoxide, spiral epoxide, and the α,β -unsaturated ketone α -chloroacetamide) (71). Rhodamine azide was used for in-gel fluorescence scanning of protein targets in the soluble fraction of murine organ homogenates or COS-7 cells after resolution by SDS–polyacrylamide gel electrophoresis. More detailed analysis, including the study of engineered cells expressing proteins with specific nucleophilic amino acid mutations, revealed that different electrophile classes displayed unique amino acid specificities after reaction with different proteins. In addition to noncomplexed cysteine residues of proteins, other targets included cysteine thiols participating in metal coordination and the nucleophilic amino acids tyrosine, glutamate, and aspartate (71).

Biotin-electrophile probes

The linkage of biotin to iodoacetamide- and maleimide-based electrophiles has provided a chemical handle for the subsequent harvesting of target proteins from cells after capture by avidin. Alternatively, a more sensitive approach can be used where, after biotin-electrophile reaction, fractionated organelles or whole-cell lysates are subjected to proteolysis and then avidin is used to capture peptides that are conjugated to biotin-electrophile complexes. Once fractionated, peptides and corresponding proteins are identified and sequenced by high-performance liquid chromatography (HPLC)–MS/MS. Addition of biotinylated electrophiles to either intact cells or subcellular fractions (mitochondria, microsomes, nuclei, or cytosol) has provided important new insight into electrophile-sensitive subproteomes (27, 30, 72–74). This strategy has also been extended to the biotin labeling of complex lipids that are then subjected to per-oxyl radical-induced oxidation (75, 76). The parent biotinylated 1-palmitoyl-2-linoleoyl-phosphatidylcholine, upon oxidation, yields a spectrum of electrophilic products that preferentially targeted apolipoprotein A1 when reactions were conducted in human plasma *ex vivo* (76). Electrophile-reactive amino acids identified by this lipid affinity–tagging approach were located in a functionally important region of the lipoprotein. Notably, product profiles revealed that modified peptides predominantly reflected reactions of carboxylated products of lipid oxidation [such as phospholipid-esterified 9,12-dioxo-10(*E*)-dodecenoic acid, 9-hydroxy-12-oxo-10(*E*)-dodecenoic acid, 7-oxoheptanoic acid, 8-oxooctanoic acid, and 9-oxononanoic acid], as opposed to species derived from the ω -terminus of linoleate (such as 4-HNE).

β -Mercaptoethanol exchange

An exchange reaction between an excess of added β -mercaptoethanol (β -ME) and electrophile-adducted biomolecules, when coupled with HPLC-MS/MS, permits sensitive detection and quantification (with standards) of reversibly adducted electrophiles in plasma, organelles, cells, and tissue homogenates (62). Because β -ME is the major ion undergoing neutral loss, the parent electrophile can be further structurally characterized by additional fragmentation. With this strategy, electrophilic NO_2 -FAs and their metabolites, present in both the free form and after adduction to protein and low-molecular-weight targets, have been profiled (55, 63, 77). This β -ME exchange reaction is applicable to fishing for other reversibly adducted electrophiles and has revealed the presence of electrophilic NO_2 -FAs in individual proteins that were resolved by affinity capture and electrophoretic separation. Finally, this approach provided evidence that electrophilic fatty acid derivatives could transfer between individual proteins and from protein to GSH and that electrophilic NO_2 -FAs are protected from secondary decay reactions when adducted to GSH or protein (63, 78).

Electrophiles as Signaling Mediators

Early appreciation of the occurrence and importance of biomolecule reactions with electrophiles came from studies of chemical carcinogenesis and mutations induced by electrophile adduction to DNA bases (79). From this work, an initial viewpoint evolved, namely, that electrophiles mediate noxious reactions by forming adducts with biomolecules. Evolution of a more contemporary view, that electrophile generation and reaction comprises

an important aspect of cell adaptation to environmental and metabolic stimuli, was catalyzed by two related discoveries in redox biology. First, the superfamily of GSH *S*-transferases was described as enzymes that catalytically conjugate GSH with electrophiles (80). This revealed that organisms evolved mechanisms that accelerate the conjugation of xenobiotic-derived and endogenously generated electrophiles and then export these adducts from cells through specific transport mechanisms (81). Second, the landmark discovery was made that a critical denominator of many inhibitors of carcinogenesis was their degree of electrophilic reactivity, which led to the term “chemopreventive agent” (79). Linked with the relative electrophilic reactivity of different compounds was a corresponding relative ability to increase the gene expression of antioxidant response element (ARE)–regulated enzymes, such as GSH *S*-transferases and quinone reductases (82). This insight propelled the subsequent description of multiple oxidant– and electrophile-regulated transcription factors controlling the transcription of genes encoding proteins that defend against the chemical stress caused by reactive species.

The discovery of integrated systems that control, sense, and respond to the abundance of electrophiles reinforces the concept that organisms have developed regulated mechanisms whereby biochemical detente can be established with electrophiles stemming from the diet, xenobiotics, or oxidative inflammatory conditions. This new understanding of endogenous signaling mechanisms that capitalize on electrophilic reactivity also presents new vistas for drug discovery and the treatment of cell growth disorders, metabolic diseases, and inflammatory disorders. The following are key biological targets of electrophiles that result in downstream signaling events, with additional information detailed in Table 2.

Nuclear factor κ B (NF- κ B)

NF- κ B is a cytosolic protein heterodimer composed of combinations of the p50, p52, cRel, RelB, and p65 subunits that acts as a rapid-response transcription factor. In unstimulated cells, NF- κ B is present in the cytoplasm in an inactive form complexed with inhibitor of κ B α (I κ B). After exposure to inflammatory-derived reactive species (or alternatively, different viral and bacterial antigen stimuli), NF- κ B is released from I κ B, translocates to the nucleus, binds to DNA, and induces transcription of various genes involved in inflammatory responses, cell proliferation, and cell survival. Consequently, NF- κ B has been closely linked to inflammatory responses and carcinogenesis (83). The p50 and p65 subunits of NF- κ B are posttranslationally modified by both oxidants and electrophiles at multiple thiols, which results in either an inhibition of nuclear translocation or impaired DNA binding of NF- κ B. For example 15-deoxy-PGJ₂ covalently modifies both subunits, which in turn inhibits DNA binding of NF- κ B (84). NO₂-FAs also form adducts with p65, which inhibits the DNA binding activity of p65 and represses the expression of lipopolysaccharide (LPS)–induced target genes, such as those encoding proinflammatory cytokines (85).

Keap1 and Nrf2

Similar to NF- κ B, Nrf2 (nuclear factor erythroid 2–related factor 2) is a transcription factor that is basally regulated by rapid degradation by the proteasome. Nrf2 proteasomal degradation, in turn, is regulated by interactions with the cytosolic Nrf2-specific Cul3 ubiquitin ligase complex adaptor protein Keap1 (Kelch-like ECH-associated protein 1).

Keap 1 has multiple electrophile-reactive cysteine residues that, when covalently adducted, result in inhibition of Keap1-dependent Nrf2 ubiquitination, leading to enhanced Nrf2 stability, nuclear translocation, and expression of Nrf2 target genes (86). Both this Nrf2 liberation and the synthesis of Nrf2 in the absence of Keap1 capture facilitate Nrf2 translocation to the nucleus. Nrf2 target genes encode antioxidant enzymes, enzymes of GSH biosynthesis, detoxification enzymes, scavenger receptors, chaperone proteins, and others that are regulated by the Nrf2 regulatory element termed the antioxidant response element or electrophile response element (87). Because the Keap1-Nrf2 pathway and particularly the thiols of Keap1 are sensitive to oxidative modification and adduction by electrophiles, this pathway represents an important detoxification and cell protection mechanism. Electrophiles, such as 15-deoxy-PGJ₂, NO₂-FAs, isothiocyanates, and 4-HNE, covalently modify Keap1 by Michael addition and thereby promote Nrf2-regulated gene expression (88–93).

Peroxisome proliferator-activated receptors (PPARs)

PPARs are members of the nuclear lipid receptor superfamily and are ligand-activated transcription factors. PPAR agonists can activate transcription by binding PPARs, altering receptor conformation, and thus promoting the association of coactivator proteins with PPAR either before or after binding to target gene promoters. Alternatively, ligand binding can also induce dissociation of corepressor proteins. In aggregate, PPAR-coreulatory protein interactions define the organization of the transcriptional apparatus and promote an open chromatin structure by facilitating PTMs, such as histone acetylation. PPARs also heterodimerize with the retinoid X receptor, and all PPAR-containing transcriptional complexes bind to PPAR response elements in the 5' untranslated region of target genes (94). Endogenous ligands of PPAR γ , a PPAR isoform that plays a pivotal role in cell differentiation and metabolism, include electrophilic keto-, nitro-, and cyclopentenone-fatty acid derivatives (95, 96). These electrophilic fatty acid derivatives can undergo Michael addition with the Cys²⁸⁵ residue in the human PPAR γ ligand-binding pocket and thus display nanomolar affinities (60, 97, 98). The covalent adduction of electrophilic ligands with lipid sensors, such as the PPAR γ receptor, eliminates traditional equilibrium kinetics as a predictor of PPAR γ activation at different ligand concentrations. Also, the patterns of coregulator protein association with PPAR γ and the genes that are expressed, compared with those stimulated by synthetic and noncovalently binding ligands (such as thiazolidinediones), are different for electrophilic ligands (60).

Transient receptor potential (TRP) channels

TRP channels are a family of multimeric cation channels. Some TRP channels are located in nociceptive neurons, where they serve to sense peripheral stimuli mediated by temperature extremes and diverse chemical factors, many of which share electrophilic reactivity. For example, TRPA1 and TRPV1 channel activation results in acute pain sensation and neurogenic inflammation. Click chemistry revealed that the electrophiles cinnamaldehyde and mustard oil activated TRPA1 through irreversible S-alkylation of reactive cysteines. In addition, electrophiles, such as acrolein, 4-HNE, and iodoacetamide, activated TRPA1, all with micromolar median effective concentrations (99). Allyl isothiocyanate—responsible for the pungent taste of mustard, wasabi, and horseradish—also activates the TRPA1 channel

through covalent PTM of reactive cysteines (100). Of note, the covalent modification of TRPA1 by allyl isothiocyanate is reversible, and the three cysteines susceptible for electrophile adduction and responsible for changes in receptor function by this compound have been identified (100). Although only displaying 13% sequence identity and 22% conservation overall, TRPA1 and TRPV1 are both activated by plant-derived electrophiles, including capsaicin, allyl derivative, and other onion or garlic extracts, through covalent modification of cysteine residues. Only one cysteine seems to be responsible for the activation of TRPV1 by these electrophiles (101). Although NO has also been reported to activate TRPC and TRPV channels through thiol S-nitrosation, high concentrations of NO sources were utilized, creating the distinct possibility that alternative oxidation products and secondary electrophile reactions could be mediating changes in channel function (102). The overall observation that an ion channel activity can be regulated by PTM of functionally important reactive cysteines, in addition to “traditional” noncovalent agonist binding to the active site (ligand-gated ion channels) or activation or inactivation by changes in voltage (voltage-gated ion channels), has broad-reaching implications. For example, this insight can reveal new mechanisms and drug strategies whereby pain and inflammatory signaling may be therapeutically modified.

Heat shock factor (HSF)

HSFs are redox and electrophile-activated transcription factors that regulate the expression of the family of “heat shock response” proteins (HSPs). The HSPs are highly conserved from bacteria to humans and serve as protein chaperones by regulating protein folding, trafficking, and degradation. The expression of genes encoding HSPs is stimulated in response to changes in temperature, the formation of reactive species, and other inflammatory conditions (103). Transcriptional activation is induced by DNA binding of homotrimeric HSFs that interact with promoter sequences termed heat shock elements (HSEs). Through covalent HSF thiol modification, electrophiles, such as 15-deoxy-PGJ₂ (104) and 4-HNE (39), activate HSF and stimulate expression of genes encoding HSPs. Enhanced cellular protein thiol oxidation, as well as thiol adduction by electrophilic species, also stimulates HSF DNA binding and transcriptional activation (105, 106). The discovery of small-molecule HSF activators that may include electrophilic species is a promising drug discovery area for treating inflammatory and degenerative diseases.

Protein tyrosine phosphatases (PTPs)

PTPs, responsible for the dephosphorylation of phosphotyrosine residues, are key regulators of receptor tyrosine kinase- and nonreceptor tyrosine kinase-mediated signaling pathways. PTPs are highly conserved and have, in their signature motif, a catalytic cysteine thiolate and several other reactive cysteine residues that, when modified, substantially affect enzyme activity (107). Acrolein and naphthoquinones, representative electrophiles, irreversibly inhibit PTP1B by covalently binding the active-site reactive cysteine. Most electrophiles display one to two orders of magnitude greater second-order rate constants for the inhibition of PTPs than does H₂O₂. This insight provides useful perspective, as H₂O₂ is a redox mediator that is typically viewed as a key biological inhibitor of PTPs (108) (109).

Mitogen-activated protein kinases (MAPKs)

When activated by growth factors and stress mediators (including cytokines, irradiation, heat shock, reactive species, and electrophilic lipid derivatives), MAPKs transduce cell signals through the phosphorylation of serine and threonine residues on various target proteins. MAPKs principally regulate cell differentiation, proliferation, and survival. Electrophiles, such as isothiocyanate derivatives and 4-HNE, posttranslationally modify MAPKs to influence their downstream signaling actions (110, 111).

Electrophile modulation of signaling mediator activity—pitfalls

Substantial gaps of knowledge still exist regarding the impact of electrophilic species on key signaling pathways, including those noted above. In many instances, specific target residues in modified proteins are not known, a reaction that is likely to vary depending on the physical characteristics of the electrophile. Also, observations collected from in vitro studies may not accurately reflect in vivo responses, because target molecules subjected to lower concentrations and more diverse populations of electrophilic mediators for longer periods of time may respond differently. Better understanding of the pharmacophore for electrophiles—the aggregate of steric and electronic features that facilitate optimal supramolecular interactions of electrophiles with their biological targets—can propel future drug discovery efforts by elucidating how greater specificity can be obtained for better directing target molecule reactions mediated by a particular electrophile structure. For example, an electrophilic PPAR γ ligand having both Cys²⁸⁵ reactivity and strong electrostatic and hydrophobic interactions with other residues in the ligand-binding pocket may show greater promise in treating metabolic and inflammatory disorders than the currently prescribed class of thiazolidinediones. Finally, the nature and extent of many electrophile-signaling mediator interactions in vivo, under more clinically or toxicologically relevant electrophile exposure conditions, frequently has not been evaluated.

Clinical Implications of Electrophile-Stimulated Cell Signaling

Chemopreventive and anti-inflammatory actions induced by administration of (i) synthetic homologs of endogenously produced electrophilic metabolites or (ii) purified electrophilic natural products may be of possible clinical benefit. One might predict therapeutic value upon modulation of the above pathways. In the context of central nervous system disorders, administration of the plant-derived electrophile curcumin is neuroprotective in murine models of ischemia-reperfusion injury and Alzheimer's disease (112, 113). Similarly, administration of the plant-derived electrophile carnosic acid to mice stimulated Nrf2-dependent gene expression, increased the abundance of GSH in the brain, and protected the brain from middle cerebral artery occlusion and reperfusion injury (114, 115). Through a mechanism dependent on Nrf2, topical administration of a broccoli sprout extract containing the electrophile sulforaphane prevented ultraviolet (UV)–induced carcinogenesis in mice (116). Isothiocyanate- and sulforaphane-based chemopreventive strategies have also shown therapeutic promise—for example, in an experiment in which these were administered to a population at risk for aflatoxin-induced hepatocellular carcinoma (117). Individuals treated with a broccoli sprout tea, which contains both the sulforaphane precursor glucosinolate and various isothiocyanates, increased the abundance of Nrf2-regulated cytoprotective enzymes

and excretion of aflatoxin-mercapturic acid conjugates (117–121). Thus, electrophile-based strategies are yielding promising preclinical and early phase clinical study results in the treatment of inflammatory and cancer-related problems.

Regulation and Reversibility of Protein Modification by Electrophiles

Although electrophiles can exert physiologically beneficial effects through PTM and the activation or inactivation of key signaling mediators, the reversibility of most electrophile-protein reactions has not been adequately addressed in the context of protein turnover, transfer of protein-electrophile adducts to different nucleophilic centers on the same or different macromolecule, and cellular export. The potential for unique signaling actions emanating from different classes of electrophiles—sometimes previously viewed as harmful chemical irritants and damaging inflammatory by-products—also warrants more extensive evaluation. It appears that the specific sites and extents of protein adduction, the reversibility of adduct formation after exposure to particular electrophiles, and the outcome variables that are selected for measurement can influence interpretation of electrophile actions in cell and animal models or clinical studies.

Signal selectivity and the existence of mechanisms contributing to the regulation of electrophilic adduction are essential prerequisites if one is to consider electrophiles as a class of cell signaling mediators. The accessibility and preferential reactivity of specific cysteine residues with electrophilic species are two factors that appear to confer a degree of specificity to electrophile signaling. This is confirmed by the observation that mutant proteins lacking a specific reactive cysteine [for example, p65 (122), TRPV1 (101), and Keap1 (123, 124)] show modifications in electrophilic PTM and, in concert with this, subsequent changes in protein function. Besides accessibility and selective amino acid reactivity, subcellular compartmentalization and the formation of more complex protein–macromolecule interactions are additional factors that can affect electrophilic protein modification in cells. This concept, in the context of redox signaling, has been addressed with S-nitrosation and tyrosine nitration reactions (26, 125–127). For these PTMs, surrounding amino acid motifs, protein compartmentalization, and organization of proteins in complexes can either inhibit or facilitate the interaction between molecular targets and reactive species that typically have short half-lives and limited diffusion distances.

GSH biosynthesis, its rereduction following oxidation to a disulfide, and its catalyzed transfer to proteins all contribute to the regulation of electrophilic PTM. GSH is the most abundant cellular reductant and, with its oxidized form, serves as an intra-cellular redox buffer system (15). In addition to its role as a redox buffer, GSH can react with oxidized protein thiols to yield mixed disulfides, thus limiting irreversible oxidation reactions (25, 128). GSH can also undergo an exchange reaction with a disulfide. Disulfide exchange reactions are kinetically slow; however, they are accelerated intracellularly by enzymes, such as glutaredoxins, thioredoxin, and sulfiredoxin. Despite its low reactivity, explained by the relatively high thiol pK_a of 9.2, GSH is still an important mediator of redox-based PTMs because of its abundance in cells and its rapid enzymatic regeneration (25).

Some electrophiles permanently and irreversibly modify a target protein and others induce more short-lived and reversible adduction of the target protein (30). From this work, it has been appreciated that reversibly reactive electrophiles display less or no cytotoxic effects at low concentrations. In addition to specificity of reaction, the reversibility of electrophilic PTM supports that these reactions may function as a signaling event that is sensitive to cellular metabolic and redox status. The reversible electrophilic adduction of cysteine can include inter- or intramolecular electrophile exchange between different thiols, with ultimate transfer to GSH and export of the electrophile from cells through specific multidrug resistance protein transport mechanisms (Fig. 4). The clinical promise of electrophile-based therapeutic strategies has motivated additional investigation of factors influencing when redox-mediated PTM reactions cross the threshold from physiological signaling to pathologic reactions. The fact that some redox-based modifications are irreversible and lead to a permanent loss of protein function also supports that some redox-dependent modifications do not exclusively play a role in cell physiology (129, 130).

Conclusions and Future Directions

In recent years, redox-dependent and, in particular, electrophile-mediated posttranslational protein modifications have emerged as cell signaling mechanisms that link cell function with inflammatory and metabolic status. Multiple transcription factors have prominent amino acids that are both electrophile reactive and functionally important and so enhance a capability for stress-related adaptive signaling reactions. These highly conserved signaling pathways allow organisms to respond to electrophilic species that are present in the diet, generated as a consequence of toxin exposure, and produced by oxidant and free radical mediators of inflammation and metabolic stress. The availability of more incisive chemical and bioanalytical strategies for identifying and quantifying both the mediators and the molecular targets of electrophile signaling is rapidly propelling new discoveries in this area. Recent advances have shown that there exist subproteomes that are targets of reversible adduction by particular electrophiles. Thus, fertile waters exist for spawning important discoveries related to the consequences of proteomic versus genomic reactions of electrophiles and whether these events have therapeutic or toxicological implications.

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Fig. 1. PTM of a protein by an electrophile. The electrophile attacks the nucleophilic thiolate anion of a reactive cysteine residue through Michael addition. Asterisk denotes the electrophilic carbon.

Fig. 2. Chemical structures of selected biological and environmentally induced electrophiles. Asterisk denotes the electrophilic carbon. The target of electrophiles, a protein with the reactive thiol (nucleophile), is shown in the center.

Fig. 3.

Three different chemical approaches for detecting specific protein-electrophile reactions.

(A) To identify the amino acid target of an electrophile (E) of interest, the azide-coupled electrophile is reacted with a protein and an alkyne-coupled tag (for example, an alkyne adduct of biotin or rhodamine) is added. The azide and the alkyne react irreversibly to form a stable triazole product. The protein-electrophile-triazole tag complex is isolated by affinity purification (for example, with streptavidin beads). After proteolysis with trypsin, labeled peptides are detected by MS or fluorescence-based strategies. (B) After adding a biotinylated electrophile to a protein, the complex is either trypsinized and reacted with avidin or treated with avidin and then trypsinized (the second approach is less sensitive). The covalently modified site on the protein is determined by mass spectrometry. (C) After

electrophile reaction with a target protein or tissue, followed by sample solubilization, an excess of β -ME is added to detect and quantify the protein-adducted electrophile. Reversibly bound electrophiles undergo transfer from the protein to β -ME. The E- β -ME complex is detected by mass spectrometry, and the concentration is determined by comparison with an internal standard. The protein to which the electrophile is attached is glyceraldehyde-3-phosphate dehydrogenase (PDB accession number 1rm5).

Fig. 4. Intracellular transfer of electrophiles. After reversibly binding to a reactive cysteine, the electrophile can be either transferred to a reactive cysteine of another protein or transferred to GSH and exported out of the cell. Most reversibly adducted electrophiles are transferred to GSH and ultimately exported. Irreversible electrophile adduction is typically cleared by protein degradation.

Table 1

Rate constants of electrophile reaction with GSH.

Electrophile	Second-order rate constant ($M^{-1}s^{-1}$)	Selected references
Acrolein	121	(131)
4-HNE	1.3	(132)
4-ONE	145	(132)
Isothiocyanate	2.5	(133)
15-Deoxy- 12,14 -prostaglandin J_2	0.7	(58)
Nitro-octadecenoic acid	183	(58)
Nitro-octadecadienoic acid	355	(58)

Table 2

Biological targets of electrophiles and the reactive amino acid residue(s). BMCC, 1-biotinamido-4-butane; IAB, *N*-iodoacetyl-*N*-biotinylhexylenediamine; NEPP, neurite electrophilic outgrowth-promoting prostaglandin.

Target	Electrophile	Reactive amino acid residue	Selected references
NF-κB (human)	15-Deoxy-PGJ ₂	Cys ⁶¹	(84, 122, 134)
	Nitroalkene Acrolein	Unknown	(85)
	Isothiocyanate	Cys ⁶¹ , Arg ³⁰⁷	(135, 136)
		Unknown	(137)
IκB (human)	4-HNE	Unknown	(137–141)
Keap1 (human)	Isothiocyanate	Unknown	(142)
	4-HNE	Unknown	(143)
	15-Deoxy-PGJ ₂	Cys ²⁷³ , Cys ²⁸⁸	(92, 123, 124, 144)
	Nitroalkene	Unknown	(93)
	IAB, BMCC	Cys ²⁸⁸	(145)
	NEPP	Cys ¹⁵¹	(146)
PPAR (human)	15-Deoxy-PGJ ₂	Cys ²⁸⁵	(45)
	Nitroalkenes	Cys ²⁸⁵	(60)
	4-HNE	His ⁴¹³	(97)
	Onion or garlic extract	Cys ¹⁵⁷	(101)
TRPV1	Aldehydes or acrolein Isothiocyanate	Cys ⁴¹⁵ , Cys ⁴²² , Cys ⁶²²	(99)
TRPA1 (murine)		Cys ⁶¹⁹ , Cys ⁶³⁹ , Cys ⁶⁶³	(100)
HSF (human)	15-Deoxy-PGJ ₂	Unknown	(104, 147)
	4-HNE	Unknown	(39)
	Acrolein	Unknown	(148)
PTP (human)	Acrolein	Cys ²¹⁵	(108)
MAPK (human)	Isothiocyanate	Unknown	(110)
	4-HNE	Unknown	(111)