Published in final edited form as:

Biochim Biophys Acta. 2014 February ; 1840(2): . doi:10.1016/j.bbagen.2013.09.003.

Detection of electrophile-sensitive proteins

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Abstract

Background—Redox signaling is an important emerging mechanism of cellular function. Dysfunctional redox signaling is increasingly implicated in numerous pathologies, including atherosclerosis, diabetes, and cancer. The molecular messengers in this type of signaling are reactive species which can mediate the post-translational modification of specific groups of proteins, thereby effecting functional changes in the modified proteins. Electrophilic compounds comprise one class of reactive species which can participate in redox signaling. Electrophiles modulate cell function via formation of covalent adducts with proteins, particularly cysteine residues.

Scope of Review—This review will discuss the commonly used methods of detection for electrophile-sensitive proteins, and will highlight the importance of identifying these proteins for studying redox signaling and developing novel therapeutics.

Major Conclusions—There are several methods which can be used to detect electrophile-sensitive proteins. These include the use of tagged model electrophiles, as well as derivatization of endogenous electrophile-protein adducts.

General Significance—In order to understand the mechanisms by which electrophiles mediate redox signaling, it is necessary to identify electrophile-sensitive proteins and quantitatively assess adduct formation. Strengths and limitations of these methods will be discussed.

Keywords

electrophile; proteomics; oxidative post-translational modification; redox signaling; thiol; reactive species

Introduction

Oxidative protein modification is a mechanism of redox signaling which expands the complexity of protein regulation, function, and/or structure available outside of the genetic code. Oxidative modifications are important for normal cell function, as well as response to stress, initiation of programmed cell death, and other critical cell functions [1, 2]. A number of different reactive species can cause oxidative modifications, including hydrogen

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peroxide, oxidized lipids, and electrophiles. Of interest in this review are electrophiles, which are electron-loving and can form covalent adducts with electron-rich nucleophiles, such as the side chains of cysteine, lysine, histidine residues as well as DNA bases [3, 4]. The formation of electrophiles can occur endogenously, for example, through enzymatic and non-enzymatic lipid peroxidation pathways [5], and nitration of nucleic acids [6] and lipids [7]. In addition, exposure to exogenous electrophiles can occur through diet, inhalation, and dermal contact [8–10]. The overall cellular effect of an electrophile will depend on its reactivity, reversibility, accessibility in the cell, the dose and time of exposure, and the available proteins that are targeted [1, 11]. Since electrophiles modify multiple proteins simultaneously, these electrophile-sensitive proteins have collectively been termed the “electrophile-responsive proteome” [2, 5, 12]. Studies by several groups have identified a number of these protein targets [13–17].

Electrophiles have been shown to have biological and pathological roles, and are increasingly being investigated for their potential therapeutic properties [18–28]. Consequently, the detection of electrophile-sensitive proteins and the determination of the sites of modification have become instrumental in understanding the mechanisms of action for these electrophilic compounds [16]. Indeed, there are important chemical properties of both the electrophiles and the adducted nucleophilic species which must be considered. For example, not all electrophiles are the same. Although the term “electrophile” means electron loving, there are different types of electrophiles which have different reactivities with different nucleophiles. In order to understand this, it is helpful to consider the reactions between electrophiles and nucleophiles as analogous to hard and soft acids and bases [29]. Briefly, soft electrophiles (analogous to acids) are more readily reactive with soft nucleophiles (analogous to bases). Conversely, hard electrophiles (e.g. carbonium ions) are more readily reactive with hard nucleophiles (DNA bases) due to the shape and energy of the frontier molecular orbitals [30–33]. In contrast, soft electrophiles (which are the focus of this review), are more readily reactive with soft nucleophiles (e.g. thiol groups of cysteine, primary/second amino groups of lysine/histidine). Based on this theory and from experimental observations, the primary biological effects of soft electrophiles are mediated via the direct adduction of the electrophile to cysteinyl thiols within the cell [30, 34]. It is important to note that the deprotonated form of the thiol group is nucleophilic, but not the protonated form, and this can be determined by the acid dissociation constant (pKa). Therefore, knowing the pKa of a target protein’s cysteinyl thiol is important in predicting the interactions with electrophiles [35]. Interestingly, this means that the local environment within the structure of a protein can affect the state of the nucleophilic residues in proteins. It is possible that genetic mutations affecting the local environment of a cysteine may alter the pKa of a cysteine. Changing the thiol pKa or the local pH can promote or inhibit adduct formation for a given cysteine residue. The average thiol pKa of cysteine is 8.4, however, the pKa of electrophile-sensitive cysteines is often lower than this average. For example, the pKa of the active site thiol of cysteine 32 of thioredoxin-1 is 6.8 [36, 37] and this thiol is sensitive to oxidation [38]. Therefore, proteins containing low pKa thiols in the active site have been shown to be sensitive electrophile targets [39] since these thiol groups are more likely to be in the deprotonated, nucleophilic form [36, 40]. Indeed, low pKa thiols have been characterized in proteins such as methionine sulfoxide reductase A [41], peroxiredoxin [42], tubulin [43], and thioredoxin [36]. Many other protein cysteines have been studied and are found to be modified by electrophiles such as: H-Ras [44, 45], soluble epoxide hydrolase [46], thioredoxin [36, 38], and Keap1 [47]. It is thought that these proteins have low thiol pKa and possibly other structural features that cause specific residues to be sensitive sites of electrophile adduct formation. It also is possible for electrophiles to form adducts with GSH either through direct reaction, or through enzymatic conjugation via glutathione S-transferase (GST) [48]. It should be noted that the thiol pKa of GSH is relatively high, at 8.8. Even though the pKa being high would indicate that it is less likely to form direct

adducts at physiological pH, the concentration and enzymatic control of glutathione adduct formation by GST can catalyze the formation of these adducts [49, 50]. Other important considerations in determining the extent of protein adduct formation include factors such as lipophilicity of the electrophile, differential expression of target proteins, and the concentration and time of electrophile exposure.

Another important experimental consideration is the reversibility of electrophile-protein adducts which is dependent on the mechanism of adduct formation. In cases where the reaction involves a leaving group (e.g. in S_N2 reactions), reversibility does not readily occur. However, Michael addition reactions can be reversible depending on many factors, including the stability of the original compounds, temperature, and the presence of competing reactants [51, 52]. It is worth noting that for many electrophiles, protein adducts are stable for a number of hours to days [51, 53]. Other considerations in adduct stability include enzymatic adduct removal and/or protein turnover.

Advancements have recently been made in understanding the effects of protein microenvironment on susceptibility to electrophile modification, and in some cases it is possible to predict modifications based on amino acid sequence and known binding motifs targeted by specific electrophiles [54]. Recently, Labenski *et al.* showed that lysine-rich regions of proteins promote adduct formation with electrophilic quinones [55]. As more is discovered about specific electrophile-responsive proteomes, additional electrophile binding motifs may be discovered. Though this review will primarily focus on cysteinyl thiol modifications, many of the concepts will also be applicable to other nucleophilic residues (e.g., nucleophilic amine of lysine and histidine). A special emphasis will be placed on current methodologies to detect adducts, including model electrophiles, tags, and derivatization techniques. Overall, an understanding of these methods will facilitate the identification of critical electrophile-sensitive proteins, which in turn will be essential in ultimately determining the mechanisms by which electrophiles mediate redox signaling.

Two overall approaches have been applied to search for electrophile-sensitive proteins in discovery-based experimental formats. One involves using model electrophiles to scan for possible protein targets, and the other involves detection of endogenously-formed electrophile-protein adducts. There are advantages and limitations for each of these approaches. Regardless, it is often helpful to employ high-resolution protein separation methods, or to decrease sample complexity by enrichment of adducted proteins. Other considerations include selection of appropriate tags and detection systems, and targeting proteins within specific organelles, which will be discussed in the following sections.

An overview of model electrophiles

Model electrophiles include either synthetic or natural electrophiles of interest which can be administered exogenously and tracked. These compounds can be pre-labeled with detection tags, and have been used in variety of biological model systems. Methods using model electrophiles include indirect detection of modified proteins by labeling free thiols (Table 1, top scheme), and direct detection of modified protein using a tagged electrophile (Table 1, bottom scheme).

Detection of potential protein targets of electrophiles

Monitoring the overall status of protein thiols can give insight into the extent of protein thiol modification. Model electrophiles, such as iodoacetamide (IAM), N-ethylmaleimide (NEM), 1-biotinamido-4-(4'-[maleimidoethylcyclohexane]-carboxamido)butane (BMCC), and N-iodoacetyl-N-biotinyloxyhexylenediamine (IAB) are well-characterized and have been used to identify electrophile-sensitive proteins, and to monitor the status and availability of protein

thiols [15, 56] (see Table 1, top). These reagents are cell permeable and commercially available with a variety of detection tags. Importantly, even though all of these reagents are thiol-reactive, they utilize distinct reaction mechanisms [56]. For example, NEM forms adducts by Michael addition, which involves the formation of an adduct equal to the exact mass of the electrophile. On the other hand, IAM forms adducts by nucleophilic substitution (S_N2) which liberates the leaving group, iodide, from the electrophile. Reaction rates and conditions have been described previously, but it is noteworthy that the reaction of NEM with thiols occurs at a reaction rate which is at least twice as high as other agents [56]. Although the target of both electrophiles is the thiolate anion, the rapid reaction rate of NEM results in the modification of more thiols, when compared with IAM [56]. There are other model electrophiles for the detection of free thiols which may also be useful, for review see [57]. These types of reagents have been useful in identifying the potential protein targets which are sensitive to modification by electrophiles [58].

Detection of specific electrophile-responsive proteins

In addition to IAM and NEM, electrophiles of interest can be tagged and used to identify proteins or groups of proteins which are responsive to a specific electrophile (see Table 1, bottom). This approach has been used successfully to determine the protein targets of 4-HNE, 15d-PGJ₂, and nitroalkenes, among others [13, 59–61]. An advantage to using a tagged model electrophile is that the protein adducts are directly detectable. In the case of reversible Michael adducts, it may further be desirable to stabilize adducts using sodium borohydride (NaBH₄) [62]. In addition, some tags enable affinity precipitation which can be used to enrich for low abundance proteins. However, the addition of a large tag to an electrophile may affect properties such as solubility, pharmacodynamics, and/or subcellular localization. Additionally, some tags may interfere with the binding of the electrophile to certain target proteins. For instance, adding a tag to 15d-PGJ₂ removes the terminal carboxyl group of the electrophile which is critical for the binding to the peroxisome proliferator-activated receptor γ (PPAR- γ); however, the tag does not affect interaction with other known targets such as Keap1 [47] or thioredoxin [17].

There are some concerns that the concentrations of model electrophiles needed to achieve biological responses exceed the concentrations found *in vivo*. Oh *et al.*, discussed the reasons for these discrepancies, and determined that the biological effects of electrophiles are dependent upon the amount of electrophile exposed to a biological system, and that these adducts can accumulate over time [11]. Therefore, it is possible to reproduce a biological response of a single high-dose bolus electrophile exposure, with smaller, sequential exposures of low doses of electrophile over a longer period of time to allow adducts to accumulate [11].

Detection of endogenous electrophile-protein adducts

In addition to using model electrophiles, it is possible to detect electrophile adducts which are formed endogenously. There are examples of antibodies which have been developed to detect electrophile-protein adducts [63–65]. However, the small size of the electrophile adduct relative to the antibody-binding domain, as well as antibody bias due to residues flanking the adduct have limited these reagents in evaluating electrophile sensitive proteins. Nevertheless, antibodies which recognize a particular electrophile-protein adduct can be used in specific applications, as long as these issues do not interfere with the interpretation of the results.

Another method to detect endogenous electrophile-protein adducts relies on the fact that some electrophile-protein adducts are reversible [51]. For example, as mentioned previously, electrophiles which form adducts via Michael addition may be reversible, particularly in the

presence of high temperature and high concentrations of nucleophiles, such as β -mercaptoethanol. This reversibility can be exploited in order to identify or quantify an endogenous electrophile. One such method has been developed in order to release endogenous electrophile adducts from proteins after gel-based protein resolution. In this method, the electrophile-protein adduct is reversed and the electrophile is reacted with excess β -mercaptoethanol, and these adducts are then be quantitatively retrieved and further analyzed (Table 2, top scheme) [66]. A limitation of this method is that the site of protein modification is not preserved. It is also possible to stabilize adducts to minimize reversal for some adducts. For example, Michael adducts containing a carbonyl group adjacent to the electrophilic carbon can be reduced using sodium borohydride (NaBH_4), thereby preventing protein adduct reversal [62].

Other methods are commonly used which derivatize functional groups added during adduct formation with proteins (Table 2, bottom scheme). A number of electrophiles of biological interest, such as electrophilic A- and J- series prostanoids and alkenals, contain α - β unsaturated ketones or aldehydes. The β -carbon of these species is electrophilic and adducts to the nucleophilic side chain of a protein, thereby introducing a carbonyl group to the protein. The carbonyls can then be derivatized using hydrazine or hydrazide reagents containing a traceable tag. For example, dinitrophenylhydrazine (DNPH) is used to derivatize carbonyls and can be detected by anti-DNP antibodies. This method has been widely used in a number of experimental formats such as ELISA, Western blotting and spectrophotometric assays [67–69]. It is also possible to add tags such as biotin and fluorophores using available biotin-hydrazide [70] or BODIPY-hydrazide [71]. Advantages of using biotin or fluorophore tags are that they do not rely on antibody detection, and thus do not suffer from antibody limitations or contaminating immunoglobulins in downstream applications. A significant disadvantage is that detection of carbonyls is not specific to the electrophile of interest. There are a number of sources of protein carbonyls, including formation on protein side chains during severe oxidative stress, which may further complicate the interpretation of results [72]. Also, some electrophiles do not contain carbonyls, underscoring the fact that the types of adducts which can be detected using this approach are limited. Furthermore, the specificity of derivatization reagents should be considered when applying this approach. For example, it has been shown that DNPH can cross-react with cysteine sulfenic acids, and thus is not entirely specific for carbonyls [73].

Another strategy to track endogenous electrophile adducts is to tag the precursor of endogenously produced electrophiles. Higdon *et al.*, have used this approach with the non-electrophilic lipid arachidonic acid. In this study, cells were loaded with labeled arachidonic acid, then subjected to lipid peroxidation, thereby producing a variety of lipid electrophiles [74].

An overview of tags

Biotin tags

A number of methods and protocols have been developed based on biotin and its interaction with the protein avidin [75–78]. The interaction between biotin and avidin is the strongest known non-covalent interaction in biology (K_d approx. 10^{-15} M), due to a very rapid on-rate and extremely slow off-rate [79]. The result is that the avidin-biotin complex is unusually stable, and is resistant to extreme changes in temperature and pH. Advantages include versatility, in that avidin can be coupled to enzyme-linked or fluorescent tags, or immobilized to a resin for affinity precipitation. A spacer region between the biotin moiety and the protein also allows biotin to bind to avidin without bias from the surrounding amino acids. This is a common problem encountered with antibodies directed against protein modifications, which are often affected by flanking residues [80].

The strength of the interaction between biotin and avidin can also be a significant limitation. For example, harsh conditions (e.g. 8M guanidine•HCl, pH 1.5 or boiling in SDS-containing sample loading buffers) are required to break the avidin-biotin interaction. Thus, it is virtually impossible to strip and reprobe a blot after avidin-based detection in a manner which will not interfere with other proteins bound to the membrane. Another limitation of using biotin as a tag is due to endogenous biotin containing proteins which utilize biotin as a cofactor in normal carboxylation reactions. Furthermore, this family of carboxylase enzymes is found within the mitochondrion. These endogenous proteins can interfere with the detection of biotin, particularly in mitochondrial samples, often giving the most intense signal in the blot.

Looking for electrophile modifications in a complex mixture of proteins is challenging, and can be analogous to looking for a “needle in a haystack.” Due to the fact that modifications often occur on proteins of low natural abundance, it is often necessary to decrease sample complexity prior to analysis. In some cases, specific organelles or cellular compartments which are known to be targeted by electrophiles can be enriched using tissue or cell sub-fractionation techniques. In addition, the enrichment of biotin can be accomplished by affinity precipitation. Methods for affinity precipitation of biotin-tagged proteins utilize avidin or other avidin-like proteins, such as streptavidin or neutravidin conjugated to resins. Briefly, samples containing biotin-labeled proteins are affinity precipitated using avidin conjugated resin, and then are eluted for detection [76]. While elution conditions may be suitable for subsequent analysis by gel electrophoresis using denaturing detergents, they are not suitable for many downstream applications, such as enzymatic assays requiring the preservation of protein structure and function, or mass spectrometry. Interestingly, the affinity of avidin with biotin can be decreased to approach that of an antibody-antigen interaction. This can be accomplished by the nitration of tyrosine in the binding site of avidin so that biotin may be removed by less harsh buffers. For example, tetranitromethane (TNM) is used to nitrate a tyrosine residue in the biotin-binding site of avidin, which in turn increases the off-rate of biotin [81]. This allows the elution of biotin-tagged proteins using milder conditions of low pH, without the use of chaotropic agents or denaturing detergents.

Fluorophore tags

In addition to biotin, fluorescent tags can also be used to detect electrophile-sensitive proteins [56]. Fluorescent labeling of thiol-reactive compounds, followed by chromatographic or electrophoretic separation, is a sensitive method for the detection of thiols [82]. For example, fluorescent derivatives of the thiol-reactive compounds, NEM or IAM, are used extensively for thiol detection [57, 83, 84]. The visible light probes vary and include fluorophores such as BODIPY, Alexa Fluor, fluorescein, Oregon Green, tetramethylrhodamine and Texas Red. These all are relatively photostable, and have high fluorescence quantum yields. Blue fluorescent dyes such as aminomethylcoumarin can also be used to tag electrophiles, and these tend to be smaller molecules since the size of the tag is usually proportional to the absorption wavelength [85]. However, these dyes have lower quantum yield than the visible light and infrared probes, resulting in lower signal-to-noise ratios.

Advantages of using fluorescent probes include the ability to image protein adducts directly, enabling the visualization of electrophile-sensitive proteins within polyacrylamide gel, eliminating the need for Western blotting. Furthermore using protein picking workstation, fluorescent bands or dots may be excised from gels for downstream applications such as mass spectrometry. For quantification of adducts, fluorescence detection is superior to Western blotting, because sample handling is decreased, and dynamic range for quantification is increased [56]. Fluorophore-tagged electrophiles have also been used to

track the localization of electrophiles in live cells imaging using confocal microscopy. This technique has been utilized with a BODIPY FL-tagged 15d-PGJ₂ (BD-15d-PGJ₂) [61].

Some limitations to fluorescence-based tags include that they are light-sensitive, and special care must be taken to avoid light during preparation and processing [86, 87]. It is also necessary to utilize specialized detection systems such as fluorescence gel-based imagers and fluorescence microscopes [56, 78]. In addition, the fluorescence signal can be decreased or quenched by other reagents such as bromophenol blue. It is important to note that some rhodamine iodoacetamide and maleimide derivatives, including thiol-reactive Texas Red dyes are difficult to prepare in pure form. Therefore, commercial preparations often have different mixed-isomer products; furthermore, these individual isomers preferentially react with certain cytoskeletal proteins, leading to complications in the interpretation of labeling results [88–91].

Importantly, unless the sample complexity has been decreased by affinity methods or high resolution protein separation techniques, it must be considered that other untagged proteins may be present within a sample (e.g. a protein of similar apparent molecular weight co-migrating with an adducted, tagged protein of interest).

Clickable tags

Recently, a system was described to facilitate the identification and purification of molecules of interest, utilizing relatively small modular units which are joined together via “click chemistry” (Figure 1). These reactions were originally developed by Barry Sharpless in 2001 [92]. As opposed to biotin or fluorophore-based tagged electrophiles, which are larger and may affect localization or other properties of the electrophile, click tags are much smaller and are less likely to adversely impact the biological properties or localization of the electrophile. As depicted simply in Figure 1, a clickable electrophile analog is engineered to have a chemically derivatizable functional group distal to the electrophilic portion of the molecule. The functional group added to the electrophile is usually an alkyne or azide group. Once the electrophile has reacted with a protein, the alkyne or azide is reacted *in vitro* with a tag containing a reciprocal functional group (i.e. azide or alkyne, respectively) containing an identifiable tag. The alkyne and azide groups react to form a stable triazole which serves to “click” the tag to the electrophile. Generally, an alkyne group on the electrophile is more desirable since alkynes are very stable and not generally found endogenously, resulting in less non-specific products after derivatization. As an example, 4-HNE adducts have been detected using click chemistry, where an alkyne group was added to the end of the alkyl chain of 4-HNE. Protein adducts were then detected after derivatization with an azide-containing biotin tag [59].

There are a number of variations on the methods for click reactions, including copper-dependent and independent reactions [93, 94]. However, the fact that the derivatization step occurs during *in vitro* sample processing and after biological adducts have been formed, allows the use of different tags and clicking schemes according to the desired downstream application. In addition, there are variations on click chemistry involving the use of cleavable linkers. In one study, a biotinylated tag was used with an alkyne-containing cleavable linker. The biotin moiety was used to purify the protein(s) of interest. The linker was then cleaved with low pH, revealing the alkyne which was then available to click using an isotope or fluorophore-labeled tag [95]. Other forms of cleavable linkers include photolysable and tobacco etch virus (TEV) protease recognition site-containing linkers have been developed which are compatible with mass spectrometry and other methods [96, 97].

There are a few weaknesses with using click chemistry which include the acquisition of clickable electrophiles. Because this technique is relatively new, there are few clickable

electrophiles which are commercially available, often necessitating the synthesis of a clickable electrophile. In addition, many electrophile-sensitive proteins of interest are low abundance proteins, and may be lost in the background or undetectable. For these reasons, the conditions of the click reaction must be optimized, including protein amounts, reducing equivalents, catalyst, and temperature, which will help to ensure that the reaction is maximally complete and minimize inter-experimental variation. Furthermore, there is an assumption that the clickable electrophile will indeed have the same reactivity as the parent electrophile. However, it is important to compare the clickable and non-clickable electrophiles to ensure that they elicit similar biological endpoints.

Isotope tags

The use of isotope tags for the detection of electrophile sensitive protein seems less intuitive than the other tagging methods, and yet, these tags provide a powerful tool for quantitation of proteins modified by electrophiles and kinetics of the modification to specific amino acid sites (Figure 2). These experiments require the covalent addition of a heavy and light labeling agent in which one or more atoms are replaced with a corresponding stable isotope, such as ^2H , ^{13}C , or ^{15}N to make multiple experimental conditions identifiable and to quantify adduct formation over time. Isotope tags have been applied successfully in conjunction with mass spectrometry for the analysis of electrophile adduction by two chemically different model electrophiles on two Cys residues of Glutathione-S-Transferase P1-1 [98]. In this study, simultaneous isotope labeling of the N-terminus of the digested peptides by phenyl isocyanate was performed under both time and dose responses of the two electrophiles. Using a time course of *in vitro* incubations with these model electrophiles and phenyl isocyanate, a kinetic analysis of electrophilic protein adduction was obtained. While these studies demonstrate the selectivity and reactivity of electrophiles with protein targets *in vitro*, larger scale studies are needed to determine the selectivity of electrophile adduct formation in complex biological samples.

In fact, isotopes can be used in combination with biotin tagged lipids to decrease sample complexity, since these reagents are readily available [99]. Consequentially, the labeled electrophile is biologically indistinguishable from the prototype, and most of the structural and chemical properties are retained, which is a significant advantage over other tagging methods. Since isotope labels have been used extensively as internal standards in mass spectrometry, the methodology for quantitation can be applied to electrophile-protein adducts. This has been demonstrated for the electrophile 4-HNE [100]. Disadvantages of these methods are that isotope-labeled compounds require synthesis and commercially available isotope-labeled compounds can be costly. Also, as of yet this method requires that the modification has been previously identified prior to kinetic analysis. This method requires extensive technical expertise in both mass spectrometry and proteomics. Nevertheless, this is a promising approach which will likely lead to an increased understanding of electrophile-protein interactions, particularly with regard to kinetics.

Approaches to selectively modify sub-proteomes

Electrophiles modify signaling proteins in many cellular compartments. Recently there has been an interest in targeting sub-proteomes with electrophiles in order to elicit cell death, or to fine-tune the biological effects of an electrophile. It has been reported that certain electrophiles accumulate within certain organelles, such as the mitochondria [61]. Our laboratory has also added a chemical moiety, triphenylphosphonium (TPP) to an electrophile in order to increase localization to the mitochondrion [101]. TPP is a delocalized, lipophilic cation which can be linked to an electrophile to selectively accumulate the electrophile within the mitochondrial matrix 200–500 fold, according to the mitochondrial membrane potential [102]. An electrophilic analog,

iodobutyltriphenylphosphonium (IBTP) is considered to be only moderately reactive and reacts with protein thiols by S_N2 chemistry in manner similar to IAM. These adducts are detectable using an antibody raised against the TPP moiety. Thus, IBTP treatment results in the post-translational modification of cysteinyl thiol groups belonging to a discreet sub-proteome located primarily in the mitochondrion, particularly enzymes in the citric acid cycle such as aconitase, isocitrate dehydrogenase and dihydrolipoamide S-succinyltransferase the E2 component of α -ketoglutarate dehydrogenase complex [103, 104]. Recently, our laboratory has investigated mito-15d-PGJ₂ and IBTP, mitochondria-targeted electrophiles, in order to determine the effects on cellular function and survival [101, 105]. Interestingly, mito-15d-PGJ₂ caused increased defects in mitochondrial bioenergetics and triggered apoptosis in breast cancer cells, compared with the non-mitochondrially-targeted analogue. Moreover, mito-15d-PGJ₂ was less efficient at up-regulating Keap1-dependent expression of HO-1 and GSH than 15d-PGJ₂. While it is clear that mito-15d-PGJ₂ localizes to the mitochondrion and adducts with a smaller number of proteins than 15d-PGJ₂, the proteins which mediate these effects have yet to be determined. Thus, it is possible to design a variety of electrophiles which can selectively target the mitochondrion.

At this point, this strategy has only been used to design or select electrophiles which target the mitochondrion, by using lipophilic, cationic molecules or carriers. However, in the future it may be possible to target other subcellular locations such as the, nucleus, or endoplasmic reticulum based on specific transport mechanisms or other unique properties of the organelle.

Applications and importance of detecting electrophile-sensitive proteins

Electrophile-sensitive proteins are involved in a number of physiological and pathological cellular processes. Modification of the electrophile-sensitive protein, kelch-like ECH-associated protein (Keap1) is involved in cytoprotection [106]. The covalent adduction of Keap1 by electrophiles leads to stabilization of the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) and the subsequent induction of antioxidant response element-regulated genes [107]. Our recent study showed that the cytoprotection offered by 15d-PGJ₂ through the activation of Keap1/Nrf2 requires the participation of electrophile-sensitive proteins within the mitochondrion [105]. Electrophile-sensitive proteins also play a role in tissue toxicity. This was demonstrated using a proteomic approach in ethanol-induced hepatotoxicity model [108]. In this study, the electrophile-sensitive protein aldehyde dehydrogenase (ALDH) was shown to be inactivated during chronic ethanol consumption, and this was correlated with a decrease in free thiols in the enzyme. Because ALDH is involved in the detoxification of acetaldehyde generated during ethanol metabolism [109], its modification and loss of activity may contribute to the hepatic injury associated with chronic alcohol consumption [110]. Other proteins which have been reported to be electrophile-sensitive include: I κ B kinase β subunit [111], NF- κ B p50 subunit [112, 113], serine/threonine kinase [114], adapter-related protein complex-1 [115], H-ras [44], thioredoxin [17], and PPAR- γ [116].

Several pathologies such as diabetes, atherosclerosis, cancer, Alzheimer's, and Parkinson's disease have also been associated with increased covalent protein modifications by reactive electrophiles. Damage induced by electrophilic xenobiotics along with endogenous electrophiles and oxidants have been used to model these pathologies [117]. For instance, the electrophilic aldehyde metabolites of glucose, glyoxal and methylglyoxal, have been shown to be associated with diabetes and associated complications including nephropathies, neuropathies, retinopathies and cardiovascular disorders [118]. Another notable example is the functional inactivation of integrin due to modification of the binding sites which result in

endothelial cell shedding, anoikis, increased generation of free radicals, and increased modification of mitochondrial proteins [118]. In relation to cardiovascular disease, 4-HNE has been shown to covalently modify protein components of LDL, thereby activating macrophages and contributing to the vascular inflammation which occurs in atherosclerotic lesions [119]. Moreover, it has been suggested that 4-HNE contributes to cardiac hypertrophy by inhibiting the mitochondrial energy regulating enzyme NADP⁺-dehydrogenase through the formation of HNE-protein adducts [120].

Electrophile-sensitive proteins may also be an important target for therapeutic development. In this context, it has been previously shown that 15d-PGJ₂ at low concentrations (<1μM), does not cause cytotoxicity, but rather attenuates migration of mouse mammary adenocarcinoma cells, suggesting a potential anti-metastatic activity for 15d-PGJ₂ [121]. Electrophiles have anti-inflammatory activity resulting from the modification of proteins in the NF-κB pathway [112, 113]. In addition, electrophilic PPARγ agonists are being investigated for the treatment of type II diabetes and cardiovascular disease [122].

Another recent area of study involves the use of dietary electrophiles, such as curcumin from turmeric and sulforaphane from broccoli. These compounds exhibit beneficial health effects. For example, curcumin is able to mediate anti-inflammatory effects by alkylating two cysteine residues at the catalytic site of thioredoxin reductase, this in turn regulates the activity of NF-κB [19, 123, 124]. Unlike hormones and growth factors, electrophiles have the potential to alter gene expression and inflammation by covalently binding to electrophile-sensitive proteins. Thus, the study of dietary electrophiles and the interaction with electrophile-sensitive proteins will likely provide new information regarding the bioactivity of certain foods and spices and elucidate the correlation between the diet and disease.

Conclusions

Electrophiles are increasingly becoming recognized as mediators of redox cell signaling. Currently, the understanding of how electrophiles mediate redox signaling is limited by the available methods to identify electrophile-sensitive proteins and quantitatively assess adduct formation. This review contains an overview of methods available for electrophile tagging, detection of electrophile/protein adducts, and examples of electrophile signaling in biology. The applications of these methods include the discovery of new protein targets for electrophiles, and a deeper understanding of how site-specific modification of proteins by electrophiles affects protein function. The discovery of new protein targets is important for developing a broad perspective of electrophile signaling, and in understanding how multiple protein adducts may induce a coordinated biological response and/or contribute to overall regulation of cell function. In contrast, these methods can also be used to more deeply study individual protein regulation in order to explore how specific electrophile adducts can regulate protein function. Studies of this type are warranted and important for furthering the understanding of the electrophile sensitive proteome.

Acknowledgments

The authors would like to acknowledge the following sources of support: HL096638 (to A.L.), pilot funding from UAB College of Arts and Sciences (to A.L.), UAB Comprehensive Cancer Center (to A.L.), and the UAB-UCSD O'Brien Core Center Core C-Bioanalytical (DK079337) and NIH Training Grant T32 HL007918 (S.B.W.).

Non-standard abbreviations

15d-PGJ₂

15-deoxy-Δ^{12,14}-prostaglandin J₂

4-HNE	4-hydroxynonenal
bt-15d-PGJ₂	biotin 15-deoxy- $\Delta^{12,14}$ -prostaglandin J ₂
BD-15d-PGJ₂	BODIPY FL-15-deoxy- $\Delta^{12,14}$ -prostaglandin J ₂
DNA	deoxyribonucleic acid
DNPH	dinitrophenylhydrazine
Ep	electrophile
ELISA	enzyme-linked immunosorbent assay
GSH	glutathione
GST	glutathione S-transferase
IAM	iodoacetamide
Keap1	Kelch-like enoyl-CoA hydratase-associated protein 1
mito-15d-PGJ₂	mitochondrially targeted 15-deoxy- $\Delta^{12,14}$ -prostaglandin J ₂
NAC	N-acetylcysteine
NEM	N-ethylmaleimide
Nrf2	nuclear factor-erythroid 2-related factor 2

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Highlights

- We describe approaches for studying electrophile modification of proteins.
- Tags used for detection are discussed, including strengths and limitations of each.
- The therapeutic importance of electrophile sensitive proteins is explored.

Figure Key:

Electrophile with click tag

Biotin with compatible click tag

Stable heterocyclic adduct

Figure 1. Example of click chemistry-based detection of electrophile adducts

The schematic shows the reaction between clickable analogues of an electrophile and a biotin tag. First, the electrophile analogue forms an adduct with a reactive thiolate on the protein, thereby introducing the click tag. A biotin analog is added in a manner which allows the reaction of the two click units to form a stable, heterocyclic ring.

Figure 2. Isotope based method for adduction kinetics

This method is useful to monitor electrophile adduct formation kinetics in cases where the adduct sites on the protein are known. First, as shown above the dotted line, the protein is reacted with an electrophile of interest (red dot) under conditions resulting in maximal adduct formation. The protein is then digested and reacted with a compound which adds an isotope tag (in this case, a heavy isotope) to the N-terminus of all peptides. Below the dotted line electrophile adduction is performed over a time course. The proteins are digested as above, but instead reacted with a compound which adds a distinct isotope tag (in this case, a light isotope). The heavy isotope-containing sample is combined with the light isotope-containing samples in equimolar amounts and the adducts for each time point are measured by mass spectrometry. The relative ratio of heavy to light isotope can then be determined. In the example mass spectra (right side of panel), the relative intensity of light isotope label approaches the intensity of the heavy isotope label as the time course progresses [98].

Table 1

Detection methods using model electrophiles.

Type of detection method	Application	Example	
Indirect, general thiol detection (a subtractive method)	Detection and quantification of a decrease in thiols in response to electrophiles (Ep) or oxidants. Usually, a thiol modifying agent such as NEM or IAM which has been tagged, (shown as) is used. It is an indirect, subtractive method because the experimental group is compared back to the control group to determine the change in available thiols. This method assumes that the thiol tagging reaction is complete. [15,56]
Direct, tagged electrophile	Detection and quantification of protein thiols in response to a tagged electrophile (Ep-) . Depending on the tag, the electrophile can be detected (e.g., by SDS-PAGE, western blot analysis, or mass spectrometry). This requires an Ep which can be conjugated to a tag. This method is direct, because increased signal is directly proportional to the number of electrophile adducts formed. [15, 59–61]

Table 2

Detection methods for endogenous electrophiles.

Type of detection method	Application	Example
Electrophile Capture	Quantification and identification of endogenously produced electrophiles. This method requires a reducing agent ( ) which will trap the electrophile after protein adduct reversal. [65]	<p>Administration Of Reducing Agent and Reversal of Adduct</p>
Electrophile-Adduct Derivatization	Detection, quantification of adducts, and identification of adducted proteins. This method uses a tagged agent ( ) to derivatize a functional group which is added during Ep adduct formation. Can also be used to decrease sample complexity in downstream applications. [66–70]	<p>Derivatize with Tag</p>