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Review Article

Protein lipoxidation: Detection strategies and challenges

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ABSTRACT

Enzymatic and non-enzymatic lipid metabolism can give rise to reactive species that may covalently modify cellular or plasma proteins through a process known as lipoxidation. Under basal conditions, protein lipoxidation can contribute to normal cell homeostasis and participate in signaling or adaptive mechanisms, as exemplified by lipoxidation of Ras proteins or of the cytoskeletal protein vimentin, both of which behave as sensors of electrophilic species. Nevertheless, increased lipoxidation under pathological conditions may lead to deleterious effects on protein structure or aggregation. This can result in impaired degradation and accumulation of abnormally folded proteins contributing to pathophysiology, as may occur in neurodegenerative diseases. Identification of the protein targets of lipoxidation and its functional consequences under pathophysiological situations can unveil the modification patterns associated with the various outcomes, as well as preventive strategies or potential therapeutic targets. Given the wide structural variability of lipid moieties involved in lipoxidation, highly sensitive and specific methods for its detection are required. Derivatization of reactive carbonyl species is instrumental in the detection of adducts retaining carbonyl groups. In addition, use of tagged derivatives of electrophilic lipids enables enrichment of lipoxidized proteins or peptides. Ultimate confirmation of lipoxidation requires high resolution mass spectrometry approaches to unequivocally identify the adduct and the targeted residue. Moreover, rigorous validation of the targets identified and assessment of the functional consequences of these modifications are essential. Here we present an update on methods to approach the complex field of lipoxidation along with validation strategies and functional assays illustrated with well-studied lipoxidation targets.

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Abbreviations: ACR, acrolein; AGE, advanced glycation end product(s); ALE, advanced lipoxidation end product(s); CID, collisional induced dissociation; CHH, 7-(diethylamino)coumarin-3-carbohydrazide; cyPG, cyclopentenone prostaglandin(s); DNPH, 2,4-dinitrophenylhydrazine; ESI, electrospray ionization; GO, glyoxal; 4-HHE, 4-hydroxyhexanal; HNE, 4-hydroxynonenal; HEL, Nε-hexanoyl-lysine; MALDI, matrix assisted laser desorption/ionization; MDA, malondialdehyde; MS, mass spectrometry; NLS, neutral loss scans; ONE, 4-oxononenal; 15d-PG₂, 15-deoxy-Δ^{12,14}-prostaglandin J₂; RCS, reactive carbonyl species.

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1. Introduction

Redox balance is emerging as an important physiological and pathophysiological regulator of cellular behavior and outcome. The functions and activities of a growing number of proteins have been found to be altered by the oxidation state of the protein, especially at redox-sensitive cysteines. Proteins can also be modified by covalent reactions with oxidized sugars, often referred to as advanced glycation end products (AGE), or with oxidized products of lipids (advanced lipoxidation end products, or ALE) [1]. These reactions can occur mainly on the nucleophilic residues cysteine, histidine, arginine and lysine, although reactions with glutamine and asparagine have also been reported; this leads to the formation of a wide variety of adducts, as described previously [1–4]. While, originally, such modifications were considered solely as detrimental to protein function, more recently there have been some reports of protein lipoxidation increasing the activity or altering the nature of the activity of specific proteins, and therefore lipoxidation is starting to be considered alongside cysteine thiol-sulfenate-disulfide switches as an additional mechanism of protein regulation [3,5,6]. The aim of this article is to provide a succinct update on the importance of lipoxidation *in vivo* and progress in the methods employed for its study.

2. Types of oxidized lipids that generate adducts

Phospholipid peroxidation occurs following radical attack, usually on polyunsaturated fatty acyl chains, and generates many different products including full-chain length oxidized fatty acids or phospholipids, chain-shortened oxidized phospholipids and small fragmentation products from the chain scission reactions. These reactions are now quite well understood and have been described in detail in several recent reviews [7–9], showing that the structure of the parent lipid and the site of radical damage determine the products. There are also enzymatic pathways for producing oxidized fatty acids and phospholipids, starting with cytochrome P450 enzymes, lipoxygenases and cyclooxygenases; products of the latter are further metabolized by a variety of prostaglandin synthases [10]. Many of the products generated by both enzymatic and non-enzymatic pathways are reactive and electrophilic owing to the presence of carbonyl groups (aldehydes or ketones) or α , β -unsaturated moieties, and can be categorized into five principal groups: alkanals (and hydroxyalkanals), 2-alkenals, 4-hydroxy-2-alkenals, keto-alkenals, and alkanedial (dialdehydes) [3]. The most reactive and commonly studied are malondialdehyde (MDA), acrolein (ACR), 4-hydroxyhexanal (4-HHE) and 4-hydroxynonenal (HNE), which also reflects the fact that these products are produced at higher levels than many other products [7] (please see Fig. 1 for the structures of some electrophilic lipids involved in protein lipoxidation). In addition, compounds with more complex structures, such as oxidized phospholipids, arachidonic acid metabolites and nitrated fatty acids are emerging as important lipid mediators in pathophysiological situations, in some cases associated with the onset and/or the resolution of inflammation. The type of adducts formed depends on the reactivity of the oxidized lipid species. Compounds containing aldehydes or ketones can react with amines (e.g. on lysine) to form

Schiff base adducts by loss of water, whereas those containing an α , β -unsaturated moiety form Michael adducts by a nucleophilic addition reaction of the protein sidechain at the β -carbon. Furthermore, some electrophilic lipids have been described to contain epoxide moieties, which also react with nucleophiles giving rise to different structures. It is interesting to note that some bi-functional lipid oxidation products, such as dialdehydes or hydroxyalkenals, do react with proteins and still present free carbonyls, which can be exploited in some detection procedures, as discussed below. Nevertheless, in many cases, the carbonyl group is involved in the reaction and is not available for detection. In addition, bi-functional electrophilic lipids can induce protein cross-linking, as has been shown for HNE, isoketals and cyclopentenone prostaglandins (cyPG) with dienone structure, and this may have important consequences on protein fate [11–13].

3. Pathophysiological relevance of lipoxidation adducts

Evidence for occurrence of lipoxidation products *in vivo* has expanded greatly in the last 10 years, as more sensitive and specific methodology has been developed, and now there are many examples of lipoxidized proteins in both healthy and diseased tissues. Much of the work has focused on HNE, but there are also many examples of adducts formed by other short chain electrophilic products, whereas studies of lipoxidation by long chain and esterified products are rarer.

As discussed below, generation of reactive species is increased in pathological conditions, and, in parallel, levels of protein lipoxidation increase in several diseases, favoring progress in the detection of adducts and identification of the modified proteins. A

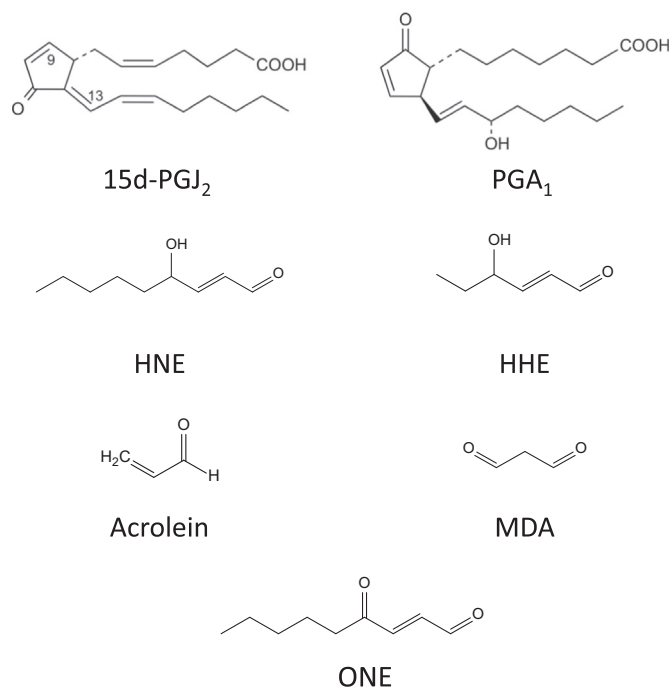


Fig. 1. Structure of some of the electrophilic lipids involved in protein lipoxidation.

condition in which protein lipoxidation may have a particularly high relevance is ageing. ALE-adducted proteins have been found to accumulate during ageing and certain age-related diseases (see [14] for review). Both enzymatic and non-enzymatic lipid metabolism can contribute to the generation of increased levels of reactive lipid species in ageing [2]. On one hand, even physiological ageing is associated with a sub-clinical systemic inflammatory state, in which pro-inflammatory cytokines may elicit the enzymatic formation of inflammatory lipid mediators such as prostaglandins. On the other hand, the oxidative state can contribute to the non-enzymatic oxidation of unsaturated lipids. Nevertheless, the potential for oxidized lipid products to cause lipoxidation will depend on a balance of the rate of formation of the product, its reactivity, and the rate of detoxification by glutathione peroxidases [15], glutathione S-transferases [16], or aldoketoreductases [17]. Importantly, protein lipoxidation in ageing and age-related diseases can result in protein dysfunction contributing to the accumulation of damaged proteins. This occurs because the modified proteins can be involved in mitochondrial function, clearance of damaged proteins or in the antioxidant cellular defense; thus, their impairment can give rise to a vicious circle of protein lipoxidation and accumulation.

Therefore, an important issue regarding the pathophysiological relevance of protein lipoxidation is the nature of the adducts formed. Various proteomic studies indicate that protein lipoxidation occurs at restricted sets of proteins within the cellular proteome [18,19]. Interestingly, there seem to be certain “hot spots” among lipoxidized proteins. Regarding plasma proteins, albumin appears to be the protein most sensitive to lipoxidation, not only because of its abundance but of the high reactivity and accessibility of some nucleophilic sites, such as Cys34 and Lys199 [20]. In the cellular environment, particular pathways or protein classes have been detected as lipoxidation targets in several experimental or clinical settings. Among them, mitochondrial, protein homeostasis-related, such as the chaperones Hsp70 and Hsp90 [21], key regulators of the antioxidant response such as Keap 1 [22], and cytoskeletal proteins emerge as targets for oxidation and/or lipoxidation in oxidative stress, cellular senescence and ageing or age-related diseases [14]. Not only actin, but also tubulin and vimentin are frequently identified cytoskeletal lipoxidation targets [18,23–27]. Within proteins, some nucleophilic residues act as “hot spots” towards the electrophilic agents. Usually, the residues that undergo covalent adduction by reactive carbonyl species (RCS) are cysteine, histidine and amino acids bearing a reactive amino group, such as lysine and arginine. In general, the order of reactivity of nucleophilic amino acids towards electrophilic compounds is Cys>H is > Lys. However, factors including solvent accessibility and nucleophilicity regulate the reactivity of such hot spots. In addition, the microenvironment around the residue greatly affects the susceptibility to RCS adduction, as in the case of Cys34 and Cys374 in albumin and actin, respectively. The high reactivity of these particular residues is due to their significant accessible surface together with the remarkable acidity of the S–H bond, as indicated both by the polarity of S–H bond and the stability of the corresponding sulfur anion [20,23].

From a biological perspective, the selectivity of protein lipoxidation elicits great interest. According to some hypothesis, selectivity could be related to specific biological pathways whereas others consider that it reflects defense mechanisms. The modification of Keap 1 results in the activation of transcription factor Nrf-2 and induction of antioxidant response element-dependent genes [22]. Actin scavenges reactive electrophilic aldehydes, up to C9, such as ACR and HNE, through its highly reactive Cys374 residue, thus contributing to detoxification mechanisms without undergoing significant polymerization impairment [28]. Similarly, albumin, through its reactive nucleophilic sites, acts as a protein

carbonyl scavenger in the extracellular milieu, which contains only low levels of glutathione (1.5–4 μ M in human plasma with respect to 1–10 mM in cells) [20]. Hence, actin and albumin can act as carbonyl scavengers, protecting more vulnerable cellular and circulating proteins whose covalent adduction would lead to damage. From an analytical point of view, several limitations should be taken into account when defining the selectivity of protein lipoxidation. Currently, most *in vitro* studies attempting identification of lipoxidation targets in cells or tissues use non-physiological excess of RCS leading to high noise levels due to off-target effects. To overcome this lack of biochemical specificity, some analytical platforms have been developed that reduce the off-target effects by minimizing the amounts of exogenous RCS [22,29]. In addition, factors such as protein abundance and the number of studies addressing protein lipoxidation of given targets or performed in particular experimental or pathological conditions, can introduce a bias in these results. For instance, neurodegenerative and cardiovascular diseases have attracted a great deal of attention, and the number of studies addressing protein lipoxidation in these pathological conditions may determine the nature of some of the targets most frequently identified. Extensive work has been carried out on HNE-modified proteins in neurodegenerative diseases [30] and it was recently reported that lysines on neurofilament heavy and medium subunits are major sites of intramolecular cross-linking by HNE [11]. Strong evidence has been accumulating over many years for lipoxidation adducts of aldehydes in atherosclerotic plaques [31–33], while oxidized phosphatidylcholine adducts of ApoB-100, extensively studied in atherosclerosis [34,35], appear to be good biomarkers and predictive of disease [36]. More recently, it has been suggested that HNE-adducts on myocardial alcohol dehydrogenase 2 might contribute to cardiomyocyte hypertrophy in a mouse model of disease [37]. Lipoxidation has also been observed in several other inflammatory diseases. For example, HNE-modified catalase has been reported in erythrocytes of patients with systemic lupus erythematosus [38], while Hsp90 modified by HNE was found in chronic alcoholic liver disease [39]. A more recent study identified increased levels of HNE adducts of AMPK α in a murine model of alcoholic liver disease that might contribute to changes in lipid metabolism, and analysis of recombinant AMPK α *in vitro* demonstrated that several cysteines were susceptible to formation of Michael adducts with HNE [40]. Recent work on chronic periodontitis in humans found increased prevalence HNE-His adducts both in serum and gingival crevicular fluid, which was exacerbated in patients who also had type 2 diabetes [41].

The importance of lipoxidation has been broadly discussed in the context of disease in several recent reviews. Pamplona [2] reviewed several products of lipoxidation (N_ϵ -hexanoyl-lysine or HEL, HNE-lysine, carboxymethyl lysine, glyoxal-lysine dimer) and the proteins bearing these adducts, and discussed evidence for their accumulation in longevity. The role of α , β -unsaturated aldehydes and their adducts in the pathophysiology of vascular diseases (including diabetes and atherosclerosis), Alzheimer's disease and chronic obstructive pulmonary disorder has been reviewed by Lee and Park [42]. There are recent focused reviews on the occurrence of ACR–protein adducts [32] and the role of HNE–protein adducts [43] in human diseases. Another area of growing interest is the role of ALE end products in age-related ocular diseases and diabetic retinopathy, where evidence for carboxymethyl lysine, N^ϵ (3-formyl-3,4-dehydropiperidino)lysine and other adducts has been collected, as reviewed recently [44,45].

While it can be seen that there is abundant evidence for the occurrence of lipoxidation and ALE in a range of different diseases, and it is clear that many have value as biomarkers of ageing, damage and inflammation, it is more challenging to demonstrate a physiological role of the modifications. Lipoxidation can be

detected in healthy tissue; for example, healthy human plasma has been found to contain HNE adducts on Apolipoprotein B-100 [46] and HNE-adducts were detected in healthy human erythrocyte membranes [47], while protein adducts of HNE, 4-oxononenal (ONE), HHE, ACR and 3-hydroxyacrolein have all been identified in healthy rat heart mitochondria [48]. In many cases, protein modification by reactive electrophilic species has been found to inhibit protein and enzyme function, in some cases leading to beneficial effects. In addition, the possibility that moderate levels of electrophilic species participate in adaptive or preconditioning responses needs to be considered. In these cases, electrophilic species would trigger cell defense mechanisms, such as expression of phase II enzymes or antioxidant defenses, thus protecting cells from further damage. The cyPG 15-deoxy- Δ 12,14-prostaglandin J₂ (15d-PGJ₂) has been found to inhibit soluble epoxide hydrolase by forming adducts with a catalytic cysteine (Cys521), but this contributes to the coronary vasodilation observed in hypoxia in mice, and can be considered a compensatory effect [49]. Furthermore, a role of 15d-PGJ₂ in the resolution of inflammation in relation to its electrophilic nature has been set forth in various experimental systems [50,51]. A role of electrophilic lipids in cell signaling is exemplified by the fact that the central signaling protein H-Ras is a target for modification and activation by 15d-PGJ₂ and other electrophilic prostanoids, which bind to critical cysteine residues in a structure-dependent fashion leading to modulation of downstream cascades [52,53]. Thus strong support for regulation of physiological processes by lipoxidation is emerging, but more research in the area is required. This in turn depends on the application of robust and specific methods for identifying and quantifying proteins modified by these electrophilic lipid species, which are discussed in the following sections. In addition, lipidomic approaches that shed light on the reactive lipid species generated both under basal and pathological conditions, as well as on their reactivity, will greatly help to understand of the consequences of lipoxidation, and even predict the modification of certain targets. Currently, controversy exists about the levels of some of the species generated and whether they are sufficient to promote changes in protein activity through lipoxidation. Some of the difficulties arise from the high reactivity of these species, which makes it challenging to detect/quantify free and total levels. In addition, analysis of whole cell or tissue extracts can overlook potential compartmentalization of these species, which may be generated at, or preferentially act in, specific subcellular environments.

4. Detection of reactive oxidized lipid species, particularly aldehydes, as precursors of lipoxidation

The electrophilic lipids that have received most attention are those containing carbonyl groups. Analysis of free reactive carbonyl species (RCS) and of electrophilic lipid species in general, especially in biological matrices is very challenging due to their intrinsic physicochemical properties (i.e. high reactivity towards the matrix and low molecular weights), low amounts in biological samples, high hydrophilicity not suitable for their retention on common reversed phase LC columns, and the fact that most of the detectors are unsuitable for direct detection. In addition, most of these reactive species do not have a chromophore or their absorbance is very limited, and they do not possess ionizable functional groups, which makes their ionization yield in electrospray ionization (ESI) very poor and hampers their detection by mass spectrometry (MS). Due to these limitations, most of the analytical approaches for profiling RCS in biological matrices are based on derivatization approaches. The derivatizing agents of RCS are characterized by a functional group that rapidly reacts with the

carbonyl group, such as hydralazine, and by a moiety required for their detection. Usually the derivatizing agent is chosen on the basis of the available detector used. Table 1 summarizes the most commonly used derivatizing agents for RCS.

As explained below, some of these derivatization methods can be applied to the detection of bifunctional electrophilic lipids bound to proteins, when the adducted lipid still possesses a free carbonyl group. The first popular methods for RCS analysis were based on 2,4-dinitrophenylhydrazine (DNPH) as a derivatizing agent and the corresponding reaction products, the hydrazones, were detected by UV detection for an overall analysis of RCS or by a UV detector coupled to HPLC when each individual aldehyde needed to be identified [54,55]. This approach was widely used for the analysis of free RCS *in vitro*, but was inadequate for *in vivo* conditions due to the lack of sensitivity and selectivity. Later, some methods based on LC-MS and GC-MS analysis of 2,4-dinitrophenylhydrazone derivatives of free aldehydes and ketones were proposed and they were mostly applied to volatile airborne carbonyls or carbonyls contained in food [56]. Use of DNPH as a derivatizing agent is currently limited due to disadvantages such as limited solubility in aqueous solvents, explosiveness, and tendency to deposit in the MS ion-source [57].

In line with these limitations, in recent years some innovative analytical approaches have been reported for RCS profiling and for their quantitative analysis in biological matrices including serum, urine and cells. The proposed methods so far reported can be roughly divided into two groups: (1) untargeted methods able to profile unknown RCS; (2) targeted methods characterized by high sensitivity for an absolute quantitative analysis of a known RCS.

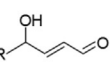
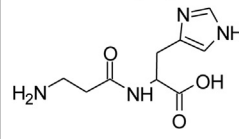
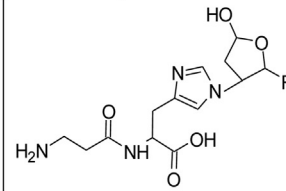
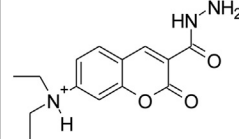
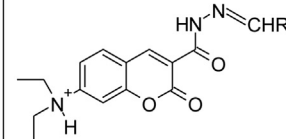
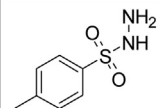
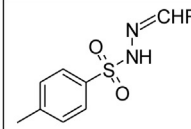
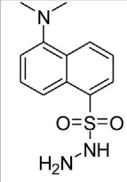
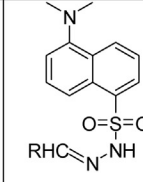
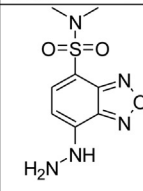
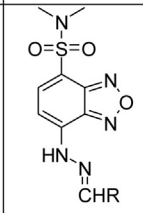
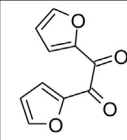
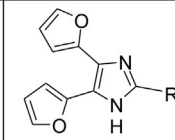
5. Untargeted methods for RCS profiling

In recent years, some oxo-lipidomic approaches have been proposed with the aim of (i) obtaining a comprehensive measurement of the oxidized lipids and of the relative break-down products present in a biological matrix and (ii) profiling the individual analyte responses in various pathophysiological conditions.

One of the first oxo-lipidomic approaches to identify 4-hydroxyalkenal species in lipid extracts used carnosine as a derivatizing agent followed by a direct infusion on a nano ESI source connected to a triple quadrupole [58]. Carnosine rapidly reacts with unsaturated aldehydes and the Michael adducts formed are ionizable and hence, easily detectable using an ESI source. Moreover, collisional induced dissociation (CID) of carnosine adducts leads to diagnostic neutral losses that were used to specifically identify unknown adducts, whereas the structures of 4-hydroxyalkenal species were readily deduced from the detected molecular weight and the knowledge of the existence of naturally occurring polyunsaturated fatty acid structures. Four hydroxyalkenal species were identified in the lipid extracts of various mouse tissue samples, namely 4-hydroxyhexenal, 4-hydroxynoninal, 4-hydroxyundecenal, and 4-hydroxydodecatrienal. Moreover, by using deuterated standards, a quantitative measurement was also performed.

More comprehensive oxo-lipidomic approaches have taken advantage of the derivatization of RCS with 7-(diethylamino)coumarin-3-carbohydrazide (CHH) followed by ESI-MS analysis in positive ion mode [59]. Identification of CHH-derivatized compounds and elucidation of the cleavage sites and the oxidative modifications were carried out by specific neutral losses or fragment ions. This method allowed the identification of 69 and 122 products of *in vitro* oxidation of free fatty acids and phosphatidylcholine vesicles, respectively, thus illustrating the complexity of the reactive species generated. More recently this method has

Table 1
Compounds used in the derivatization of RCS.

Target	Derivatizing agent	Conditions	Reaction product	Detector	Ref.
 4-hydroxyalkenals	 carnosine	H ₂ O, 37°C, 24 hours		MS	[58]
Reactive carbonyl species	 CHH	50% aqueous methanol, 37°C, 1 hr		MS	[59]
Reactive carbonyl species	 TSH	H ₂ O and CH ₃ CN mixture, acid (HCl) conditions		MS	[57]
Reactive carbonyl species	 DH	CH ₃ CN, <i>p</i> -toluenesulfonic acid, 4 hr, room temperature		MS	[61]
Reactive carbonyl species	 DBD-H	20 min, 25°C, 10% trifluoroacetic acid		Fluorescence and chemiluminescence	[64]
Reactive aldehydes	 2,2' furil	Ammonium acetate, 90°C, 30 min		Fluorescence	[63]

CHH: 7-(diethylamino)coumarin-3-carbohydrazide

TSH: *p*-toluenesulfonylhydrazine

DH: dansyl hydrazine

DBD-H: 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole

been applied to the characterization of lipid extracts from rat primary cardiomyocytes treated with the peroxyxynitrite donor SIN-1 [60]. Another derivatization method for comprehensive analysis of oxidized lipids uses *p*-toluenesulfonylhydrazine-(TSH) [57], which has a reactivity similar to DNPH but improved solubility and high volatility, and is suitable for the global derivatization of aldehydes and ketones coupled to a detection based on SWATH

(Sequential Window Acquisition of all Theoretical Fragment-Ion Spectra) at high mass resolution. The approach was applied to the comprehensive quantification of known compounds as well as the identification of uncharacterized compounds in biological samples based on the detection of signature fragment ions originated from the derivatization reagent sub-structure. Similarly, another LC-ESI-MS approach uses dansyl hydrazine as a derivatizing agent and

a MS analyzer operating in SRM mode, monitoring the ion at m/z 236.1 generated by the CID of the derivatized aldehydes whose $[M+H]^+$ is scanned from m/z 275–949 [61]. A derivatization-free approach based on UHPLC–HRMS has also been proposed as a tool for the identification of aldehydes (2-alkenals, 4-hydroxy-2-alkenals, and 4-hydroxy-2,6-alkadienals). This method was suitable for detecting aldehydes from 6 to 16 carbons, and signals were found for $[M+NH_4]^+$ and $[M+H]^+$ adducts in positive-ion mode ESI and for $[M+HCOO]^-$ adducts in negative-ion mode [62].

6. Targeted methods

Several methods have been reported for the quantitative analysis of free RCS in biological fluids, including capillary GC–MS, micellar electrokinetic chromatography, LC–MS/MS and various derivatizing procedures. Methods targeting a wide spectrum of RCS, including lipid- and glucose-derived RCS are desirable since, in pathological conditions, there is cross-talk between modification of proteins by the two classes of reactive species.

A method based on derivatization with 2,2'-furyl followed by HPLC coupled to fluorescence detection has been validated for the quantitative analysis of MDA, ACR, glyoxal (GO) and HNE in sera of humans, with detection limits ranging from 0.03 to 0.11 nmol/ml [63]. MDA was found to be the most abundant species in healthy volunteers, with a serum concentration greater than 10 nmol/ml, followed by the other aldehydes at concentration around 1 nmol/ml. Interestingly, this method detected significantly higher concentrations of ACR, MDA, and HNE in sera from diabetic and rheumatoid arthritis patients as compared to healthy controls. Carbonyl groups can also be derivatized with 4-(N,N-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole to generate peroxyoxalate chemiluminescence [64]. This method has been applied for the analysis of methylglyoxal, ACR, crotonaldehyde and trans-2-hexenal with results ranging from 4.4 to 6.5 nM.

In spite of the comprehensive oxo-lipidomic methods developed over the last years, several limitations and unanswered questions on RCS analysis in biological matrices still remain. On one hand, the stability of RCS can be widely different, with α , β -unsaturated aldehydes being less stable than saturated aldehydes. This can affect the recovery yield and the accuracy of the method. Hence the recovery of RCS from biological samples should be carefully standardized and made reproducible by suitable sample preparation with addition of the derivatizing agent during sample collection and spiking of deuterated internal standards. On the other hand, it is important to consider the source of RCS detected by derivatization methods, and specifically, what proportion of RCS is in a free or protein-bound form, and what proportion can arise from reversible protein adducts such as Schiff bases or Michael adducts. Furthermore target analysis based on derivatization misses Schiff base protein–lipid adducts.

Notwithstanding these unanswered questions, owing to advances in analytical strategies there is now a better understanding of RCS. In particular, we now know that there are many more RCS besides the well-known RCS species such as HNE, HHE, ACR, GO and methylglyoxal, which have been extensively studied. Indeed, comprehensive lipidomic approaches have identified more than 400 different RCS species in rodent serum [61]. This, together with the knowledge about other lipidic reactive species, such as nitrated fatty acids, which do not possess carbonyl moieties [65], highlights the structural variety of reactive species that can modify proteins. Taking into account that the functional consequences of the modification may depend on the structure of the adducted moiety, a vast number of functionally diverse protein species may arise through lipoxidation. Moreover, significant advances have been made in quantitative detection of RCS in biological samples.

From a quantitative point of view it would be important to determine the amount, not only of free RCS but also of protein-bound species. This information would be very valuable for studies in model systems to ascertain the role of lipoxidation using concentrations in the range of those occurring *in vivo*. A combination of strategies will therefore be needed for understanding the roles of lipoxidation in pathophysiology.

7. Analysis of protein lipoxidation through MS-based methods

7.1. Label-free MS methods

Mass spectrometry (MS)-based analytical approaches are one of the most popular methodologies to study biological systems and are used extensively in the study of protein–lipid adducts. MS technology offers unmatched performance in identification and quantification of these compounds, providing precise, sensitive and high-throughput analysis. It allows detailed structural information to be obtained at a molecular level, providing structural characterization of protein–lipid adducts *in vitro* and *in vivo*. Although the ultimate goal is to characterize the adducts formed in real pathophysiological conditions, an initial characterization of potentially important adducts can be obtained from the analysis of peptide–lipid or protein–lipid adducts prepared *in vitro* under controlled reaction conditions using pure preparations of the candidate peptides/protein and electrophilic lipids. The high reactivity of the electrophilic lipids allows the formation of adducts with the amino ($-NH_2$) or sulfhydryl ($-SH$) groups almost spontaneously. The new peptide–lipid adducts can be easily detected by MS analysis with matrix assisted laser desorption/ionization (MALDI) or electrospray (ESI), often coupled to liquid chromatography (LC–MS), in positive ion mode. In the case of protein–lipid adducts, characterization by MS can follow the top-down or bottom-up proteomic strategies. Bottom-up approaches are by far the most popular, involving enzymatic hydrolysis of the protein–lipid adducts, usually using trypsin, followed by analysis of tryptic peptides and peptide–lipid adducts by MS (MALDI-MS or LC–MS) and MS/MS [66].

Peptide–lipid adducts are identified in MS as protonated molecules $[M+H]^+$ or multiple charged ions $[M+nH]^{n+}$ and modified peptides are typically recognized in the MS spectrum based on the mass shift against the unmodified peptide. In the case of Michael adducts, the mass shift caused by the modification is equal to the molecular weight of the RCS, while for the Schiff base it is equal to the molecular weight of the RCS minus 18 amu, owing to loss of H_2O during adduct formation. For example, regarding peptide adducts with MDA, the mass deviation is +72 amu for Michael adducts and +54 for Schiff adducts in the case of single charged ions, or +36 amu for Michael adducts and 27 for Schiff adducts in the case of double charged ions, as observed in the study of MDA adduction of beta-lactoglobulin by LC–MS [67,68]. Tandem mass spectrometry (MS/MS) of peptide–lipid adduct ions is usually performed to confirm the nature of the oxidative modification and to pinpoint its location in the peptide backbone. MS/MS commonly utilizes collision-induced dissociation (CID), and details about the site of modification and type of adducts in peptides are inferred by the deviation of the typical y or b -type fragment ions or by the presence of modified immonium ions [69]. CID-tandem mass spectra also show typical loss of the carbonyl moiety, leading to the formation of the non-modified b and y -ions, which might prevent the identification of the position of the adduct. It is possible to take advantage of this characteristic loss of the electrophilic lipid and of the modified immonium ions. These typical fragmentation pathways can be used to define specific target reporter ion-based MS approaches, namely neutral loss

scans (NLS) or precursor ions scan (PIS). NLS have been widely used for the detection of HNE–peptide adducts. MS³ neutral loss scanning of 156 amu (singly charged), 78 amu (doubly charged) or 52 amu (triply charged) approaches were used to locate HNE–peptide Michael adducts [70,71], as reported for example for HNE–cytochrome C oxidase adducts [71] or HNE with beta-spectrin in human blood samples [72]. More recently, dissociation techniques that do not induce fragmentation of labile bonds, such as electron capture dissociation (ECD) and electron transfer dissociation (ETD) have been used [4,73]. MS/MS spectra obtained under these conditions can be more informative, showing abundant c- and z-type fragment ions of modified peptides, since the carbonyl–peptide bond is more stable, preventing the loss of the carbonyl moiety. ETD is also preferred for the analysis of bigger peptides and intact proteins and thus for top-down approaches.

Peptide and protein adducts with small electrophilic lipids, like the α , β -unsaturated aldehydes HNE, MDA and ACR are by far the most studied [3,66,74], and protein adducts involving these lipids have been detected and characterized both *in vitro* and in many pathophysiological situations [30,32]. Nevertheless, for other lipid classes, the evidence is scarcer. There are numerous reports of the formation of protein adducts of cyPG in several experimental models of pathophysiological situations through various techniques, either involving endogenously generated or exogenously added cyPG. Most studies combine the detection of modified proteins through the use of metabolic labeling (see below) with MS characterization of the candidate cyPG–peptide adducts in the positive mode, using purified protein or peptides incubated *in vitro* with cyPG (for review see 3,19). Nevertheless, at present, the MS detection of prostaglandin–protein adducts formed endogenously is yet to be established. Peptide/protein modifications by electrophilic lipids esterified to phospholipids have rarely been considered [3]. Recently, several groups have identified adducts of RCS derived from phosphatidylcholine with His, Cys and Lys in synthetic peptides of ApoA and ApoB-100 [75,76]. This is a field of research that should be explored further since adducts of proteins with oxidized phospholipids have been detected in HDL and in human platelets enriched with electrophilic truncated oxidized phosphatidylcholine (KODA-phosphatidylcholine) [77].

Thus, the number of protein–lipid adducts formed under controlled chemical conditions is relatively low, and their spectra are relatively easy to analyze. Data obtained through this approach allows the identification of the type of adducts that can be formed for each electrophilic lipid. Additionally, this approach has the advantage of decoding the reactivity of each specific electrophilic lipid species and specific fragmentation patterns of lipoxidation adducts under tandem MS conditions can be identified, which can be very useful for target analysis in biological samples. However, use of more complex samples from cells, biological fluids and tissues requires more complicated, specific and targeted approaches to identify and characterize the protein adducts. These can take advantage of enrichment procedures, chemical labeling and bottom-up proteomic strategies combining enzymatic digestion, chromatographic methods and analysis by MALDI-MS and/or LC-MS and MS/MS. Enrichment and derivatization methods, followed by MS analysis, are commonly used to improve sensitivity and selectivity, which aids in the structural identification and may be performed before or after protein digestion. In this regard, it should be noted that aldehydes that react with lysines and arginines pose additional difficulties since these modifications may interfere with trypsin digestion, resulting in long peptides that are difficult to sequence. Also, false positives of HNE adducts can be obtained since they would add the same mass as arginine. Stabilization of lipid adducts with NaBH₄ prior to enzymatic digestion and MS analysis is usually performed [78]. Targeted analysis based on NLS scans, of either underivatized or derivatized samples is

becoming a popular analytical strategy to search for specific protein modifications arising from a particular lipoxidation product. Recently published papers illustrate how combined MS approaches (MALDI-TOF and LC-ESI-MS/MS) can contribute to uncover protein lipid adducts in biological samples. HNE adducts of metalloproteinase rhMMP-13 were identified by bottom-up approaches using HR-LC-MS/MS and target analysis by multiple-reaction monitoring (MRM) based on specific neutral loss of HNE *in vitro* and in chondrocytes from osteoarthritic patients [79]. Results from proteomic approaches, using MALDI-TOF/TOF combined with SDS-PAGE and enzymatic digestion, showed that liver fatty acid-binding protein (L-FABP) is a target for modification by HNE [80]. Several nuclear proteins including actin, chromodomain-helicase-DNA-binding protein 4, heterogeneous nuclear ribonucleoprotein L(hnRNPs), and neuroblastoma differentiation-associated protein AHNK, were found to be modified by HNE, HHE, and ONE, among others, using SDS-PAGE and LC-MS in a model of liver steatosis [81].

7.2. Label-based MS methods

The most commonly used measurement of protein oxidation is probably the formation of protein carbonyl groups (often referred to as protein carbonylation); this is because robust, simple and economical methods are available for detection of carbonyl groups on proteins [82]. While bi-functional lipid oxidation products, such as dialdehydes or hydroxyalkenals, do react with proteins to yield free carbonyls, a substantial number of other products do not. Moreover, protein carbonyls can also be formed by direct oxidation of the side chains of lysine, proline, arginine and other residues, for example through oxidative deamination. Thus while many studies report on the occurrence of protein carbonyls in disease, this cannot be considered as evidence of lipoxidation unless specific antibodies against lipid oxidation products have been used, or the modifications are analyzed by mass spectrometry, as described in later sections.

To improve sensitivity and selectivity in the detection of carbonylated proteins, some analytical approaches take advantage of chemical labeling of carbonylated lipid–protein adducts prior to MS detection [83]. These derivatization procedures, as discussed in previous sections, usually exploit the high reactivity of the free carbonyl groups of the Michael lipoxidation adducts, which are very prone to react with free amine containing molecules of the derivatizing reagent. Lipid–protein Schiff adducts cannot be derivatized by this approach, unless the electrophilic lipid is a di-aldehyde. Derivatization with DNPH has been used for more than a decade, since DNPH labeled carbonylated peptides showed favorable ionization, providing a sensitive detection method. Nevertheless, this method presents some disadvantages, outlined above for the analysis of free RCS together with a lack of selectivity because DNPH also reacts with sulfenic acid [84]. 7-(Diethylamino) coumarin-3-carbohydrazide has also been used for derivatization of lipid-bound carbonyls [85], although other chemical labels can also be used [86]. One of the most common chemical labeling procedures employs biotin-based hydrazide-functionalized reagents. This approach allows enrichment of biotin-linked adducts by avidin chromatography [4], thus reducing the amount of unmodified peptides in the MS analysis. Two routes can be considered: enrichment of biotinylated proteins followed by enzymatic digestion and MS, or initial digestion of protein and further separation of biotinylated peptides by affinity chromatography [66]. MS based approaches combined with enrichment procedures using biotin are increasingly being used to recognize site-specific modifications of proteins in complex proteomes from biological samples. Recently, Aluise et al. [87] identified a peptidylprolyl cis/trans isomerase A1 (Pin1) adduct with HNE linked to the catalytic cysteine (Cys113) using click chemistry conjugation of biotin to

Pin1 and LC-MS/MS analysis. *In vitro* covalent modification of the mitochondria protein NAD-dependent deacetylase sirtuin-3 (zrSIRT3) by HNE at Cys280 was recognized using biotin hydrazide (BH) treatment and avidin pull-down and further analysis by LC-MS/MS and MALDI-TOF/TOF [88].

As mentioned above, until now most published work has focused on HNE-protein lipid adducts, but many more electrophilic lipid species are generated during lipid oxidation. Further investigation and experimentation in this field will be needed in order to understand the impact of lipoxidation in health and disease. Future research should therefore concentrate on new methods for *in vivo* detection of carbonylated proteins and other lipid adducts.

8. Detection of protein lipoxidation through non-MS approaches

Metabolic labeling methods are based on the incubation of biological samples, mostly cells in culture, with labeled analogs of the oxidized or electrophilic species or their precursors. As stated above, some of these methods can be used both in MS and non-MS approaches. Incubation of cells in the presence of radioactive precursors of oxidized lipid species, in particular with radioactive arachidonic acid, which is a precursor of electrophilic prostaglandins and of other reactive lipids, has allowed the detection of incorporation of radioactivity into proteins [89]. These methods have the advantage that the structure of the resulting lipoxidized species is not affected; thus, metabolism and interactions are preserved. As a drawback, this labeling method requires handling of radioactive material and does not allow imaging or enrichment procedures.

Biotinylated derivatives of electrophilic lipid species or their parent compounds have been widely used for the study of protein lipoxidation. Biotinylated analogs of cyPG or their precursors are among the most widely used probes. The fact that some cyPG showed beneficial effects in experimental models of inflammation and tissue injury spurred research in the identification of the proteins modified by these mediators, seeking novel therapeutic targets, even before their real pathophysiological importance had been elucidated. Indeed, the study of protein modification by biotinylated cyPG has paved the way for studies with other lipid species with similar structure or reactivity, including nitrated fatty acids and isoprostanes. In fact, many of the targets of cyPG identified, including NF- κ B and transient receptor potential (TRP) channels [90,91], have later been confirmed to be modified by the other lipids [65,92]. The progress with cyPG may be due to the fact that these compounds are relatively stable, as are the resulting adducts with proteins, and they are amenable to derivatization through modification of the carboxyl group by various moieties. Therefore, since the first descriptions of biotinylated PGA₂ [93] and 15d-PGJ₂ [90], many biotinylated derivatives of arachidonic acid-derived electrophilic lipids have been commercialized and are readily available. Advantages of these derivatives include the exploitation of the high affinity avidin-biotin interaction for both detection and enrichment of the samples for purification and identification. Among the drawbacks of this approach is the fact that the biotin moiety imposes structural restrictions that may affect interaction with proteins (see below). Thus, although biotinylated analogs mimic many of the effects of their parent compounds, there are important functional differences [50,94]. In addition, none of these metabolic labeling procedures are suitable for use in humans.

Analogously, fluorescent labels have been introduced in electrophilic lipid species for the detection and quantitation of lipoxidized proteins either in gels or in cells [95,96]. In combination

with proteomic techniques, these approaches are also suitable for the identification of potential targets of lipoxidation, although it should be taken into account that, as in the case of biotinylated tags, due to the bulky nature of the fluorescent moieties, validation of the identified targets is required and the biological interaction may be affected.

Click chemistry has also been used to monitor the fate of HNE and oxidized phospholipids [87,97] and presents the advantage of the smaller tagging moiety, which is considered in most instances not to interfere with the metabolism or interactions of the labeled lipids. This type of labeling allows the derivatization of the tag by an alkyne-azide reaction *ex vivo*, that is, in tissue extracts, partially purified samples or permeabilized cells, thus facilitating detection, enrichment, imaging, *etc.* This strategy has been successfully used to detect the HNE-induced cross-linking of peptidylprolyl cis/trans isomerase A1 (Pin1) [87].

As discussed above, lipoxidation is a selective process that does not affect cellular proteins randomly but is directed by structural features of the protein and the reactive lipid species [19,98]. Intracellular redox status and availability of small molecule antioxidants such as glutathione is also an important determinant for this selectivity [98,99]. In addition, lipoxidation could present a selective compartmentalization depending on the site of generation of electrophilic lipid species, or on the distribution of small molecule thiols that could act as decoys or the presence of oxidized lipid detoxifying enzymes such as GSTs [94,100]. Therefore, topography of lipoxidation may also be important in its functional outcome. In this context, some probes have been synthesized that combine derivatization of electrophilic lipids with biotin or fluorescent tags, and organelle-specific tags or targeting moieties for selectively detecting lipoxidation at particular subcellular environments [101].

Importantly, all these label-based methods can be used both in “positive” (or direct) and “negative” (or indirect) approaches to detect protein modification. In the positive approaches, the modified targets are identified. In the negative approaches, modification is induced first with an oxidant or electrophilic lipid and the reduction of the signal with the labeled probe is observed [96,102], or vice versa. These approaches can be used *in vitro* and in cells or tissues, and are useful to study the competition between the tagged and the parent lipid or the potential interplay between lipoxidation and concurrent modifications, either oxidative, or by endogenous or exogenous electrophiles, such as drugs [53,103], particularly those affecting the versatile cysteine residues (see below) [104]. Nevertheless, as considered in the following section, especially when using live cells, these approaches need to be complemented with other methods since the modifications occurring in cells treated with oxidized lipids can be structurally quite varied and involve not only lipoxidation but also diverse oxidations related to oxidative stress generated by the reactive species [100].

The *ex vivo* derivatization of lipoxidized proteins with various reagents has been considered above. In addition to MS procedures, the derivatized moieties can be detected using fluorescence methods, avidin-biotin based approaches or specific antibodies. Nevertheless, some derivatization procedures present limitations. As mentioned above, although lipoxidation can lead to an increase in the amount of carbonyl groups on proteins as a result of the incorporation of some lipid moieties containing these structures, carbonyl groups formed by oxidative modification of certain residues will also be detected by methods based on derivatization of carbonyl groups, such as reaction with DNPH. Moreover, this method will not be specific for lipoxidation, nor will it detect all types of lipoxidation, but only those in which a carbonyl moiety is preserved after lipid addition. In spite of this, the dinitrophenylhydrazone generated after reaction of DNPH with

carbonyl groups can be detected spectrophotometrically or by antibodies against the dinitrophenyl group, which has contributed to the wide use of this method. Similar detection can be achieved with biotin hydrazide derivatization, which reacts with carbonyl groups but in this case adds a biotin moiety to the modified residue, enabling avidin-based affinity detection or enrichment strategies. The methods for detecting carbonyl formation on proteins have been reviewed recently [105].

Lastly, there are several antibodies that directly detect adducts of lipid peroxidation products with proteins. These include antibodies against various types of HNE adducts [106], which may be selective for cysteine or histidine adducts, and antibodies against other adducts like ACR–protein, MDA–lysine and oxidized phospholipid–protein (please see [3] for review). In addition, antibodies against 15d-PG₂ have been used to detect this PG intracellularly by immunohistochemistry [107], as well as protein adducts involving this lipid through ELISA [49]. Kato et al. [108] reviewed antibody-based methods for detection of a range of unusual modifications of proteins with hydroperoxide-derived products that form amide-type lipid–lysine adducts like HEL. In all these cases, assuming an optimal specificity of the antibody, information on the proteins modified can be obtained, but not information on the site of modification, unless the use of antibodies is combined with other strategies, such as the study of site-specific mutants as described below.

9. Validation strategies

The results of the detection and identification of lipoxidation targets may give a general view of the extent of these modifications in a given sample and may also pinpoint targets of potential pathophysiological or therapeutic interest. Lipoxidation is usually not random but occurs at precise residues within proteins [100]. The affected residues are often strong nucleophiles, and may be located in environments that favor the docking or interaction of the reactive lipid species [109,110]. Frequently, the modified residues are involved in catalysis or interaction with other proteins. Therefore, validation of the modification and assessment of its functional consequences is of special importance in delineating its contribution to the overall effect of electrophilic lipids.

For validation of lipoxidation, usually a combination of strategies is required. In the most favorable scenario, a protein would be identified from a sample of a pathophysiologically relevant model, in which the modification by endogenously generated species would be detected and the sites of addition directly spotted by MS approaches. In this case, functional assessment of the modification can be undertaken. However, in many cases, protein modification is achieved by the use of exogenously added lipid electrophiles, either unmodified or tagged. Above all in this situation, validation strategies are required. In Fig. 2 we present a summary of some of the currently used methods for validation of targets of lipoxidation. If the electrophile was tagged, it is important to confirm the results using the parent compound, since any tag can confer steric

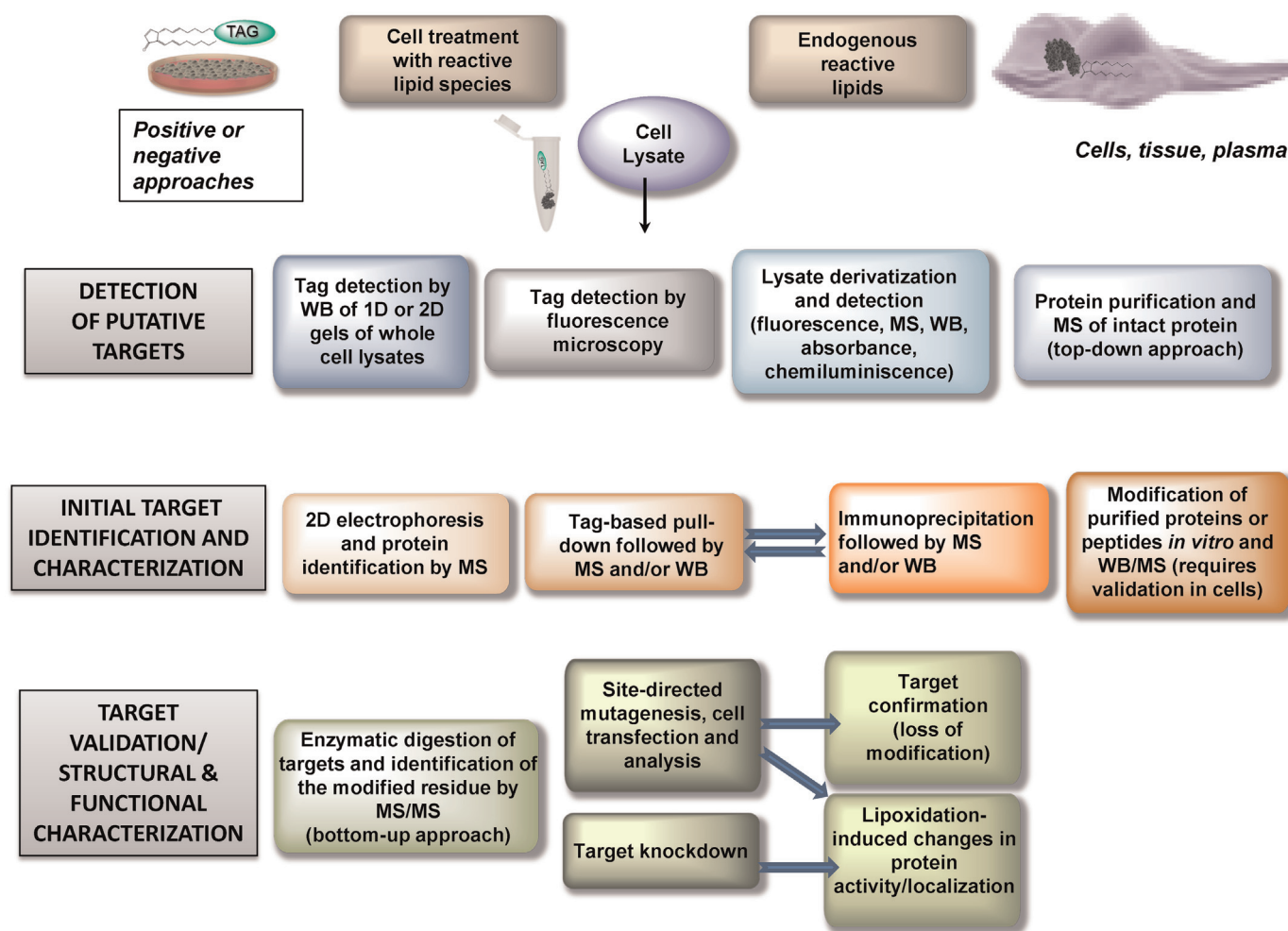


Fig. 2. Summary of procedures useful for the identification, characterization and functional assessment of protein lipoxidation. The rows represent increasing complexity and depth of information from top to bottom. At each level of complexity there are several alternative but complementary approaches that can be used. WB, western blot.

hindrance to the interaction either with the targets of interest or with other structures, leading to indirect effects [94]. Nevertheless, the differential behavior of tagged and untagged electrophilic lipids can be exploited to infer functional or structural consequences of the modification [50]. Immunoprecipitation of the modified protein or other affinity-based purification procedures such as those based on avidin–biotin interaction have proved extremely useful for assessing protein lipoxidation in the case of low abundance proteins or of peptides modified in a low proportion [19,111]. Fig. 3 outlines the identification and characterization approaches that have been used in the case of a thoroughly studied lipoxidation target: the cytoskeletal protein vimentin. In addition, we illustrate several features of the functional characterization of the modification (see below for detailed comments).

From a functional point of view, it is important to take into account that oxidized lipids bind to many targets in the cell proteome and their effects arise from the complex interplay of the diverse modifications. As stated above, induction of oxidative stress may be a source of concomitant modifications that may

coexist or compete with lipoxidation, hence complicating the structural and functional outcomes. This is particularly relevant in the case of modifications of cysteine residues, which, in addition to lipoxidation by various reactive species, may be targeted by oxidative and nitrosative modifications of varied structure, including glutathionylation, nitrosylation, cysteinylation, sulfenylation, etc. Some recent comprehensive reviews have addressed the interplay between lipoxidation and oxidative modifications [100,112]. Therefore, care should be exercised to assign functional roles to lipoxidation of proteins by specific moieties without prior assessment of the overall oxidative state of the protein and its cysteine residues. Moreover, lipoxidation may result in activation or inhibition depending on the target and the structure of the adducted moiety. Modifications of Ras proteins may have different consequences on subcellular localization and activation platforms. Whereas modification by small hydrophobic moieties appears to favor localization at and/or signaling from the Golgi, modification by fatty acids (i.e. palmitoylation) or addition of cyPG or cGMP to the C-terminal cysteines seems to promote localization and/or

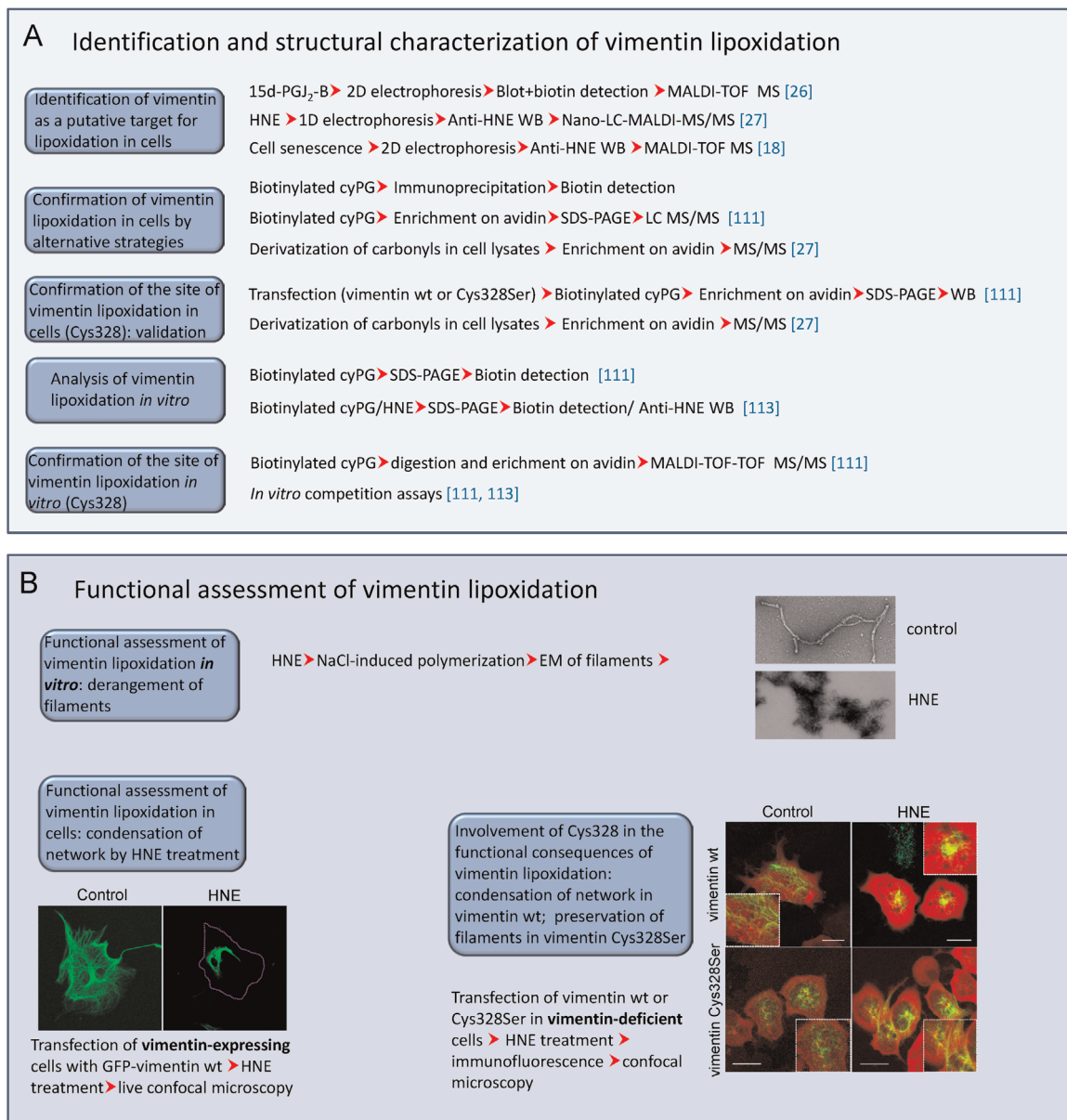


Fig. 3. Methods employed in the study of the lipoxidation of vimentin. (A) Combination of strategies employed in the chemical and structural characterization of vimentin lipoxidation. (B) Several approaches used in the assessment of the consequences of vimentin lipoxidation *in vitro* and in cellular contexts.

signaling at the plasma membrane (reviewed in [100]). Use of labeled electrophilic species in combination with markers specific for various subcellular compartments has been used to obtain information on the presence of a particular target at a defined compartment through fluorescence microscopy. Nevertheless, given the high number of cellular targets of lipoxidation, colocalization studies may offer information on the subcellular localization of adducts but is insufficient for target validation.

The proportion of the target modified is also an important issue which may have diverse implications, if we consider a signal versus background effect. Activation of a small proportion, i.e. 1% of an otherwise inactive protein may have significant effects, whereas inhibition of a higher proportion, i.e. 10% of a fully active protein may not be enough to elicit a detectable outcome. In order to establish a correlation between target modification and functional effects, the use of mutant constructs and/or model systems in which the levels of the target can be modulated are needed. In cases in which lipoxidation occurs at a specific residue within one protein, mutation of that residue can sometimes block the modification and completely rescue the functional alteration. Such is the case with the transcription factor NF- κ B and the intermediate filament protein vimentin [26,90] (see Fig. 3B). While the effect of the mutation can be clearly observed *in vitro* when working with purified proteins, in cellular systems it is usually necessary to work with a model that does not express the wild type form. In the case of vimentin, transfection of cells that do not express the endogenous protein has allowed the response of homogeneous protein constructs/variants to electrophilic lipids to be explored [113]. These studies have shown that mutation of the target residue, in this case Cys328, protects the mutant protein from the morphological derangement induced by electrophilic lipids [113], as illustrated in Fig. 3 for HNE. Thus, this residue behaves as a sensor for this type of stress, either through direct modification by HNE addition or by reactive species generated during HNE treatment. In some cases, mutation of the target residue is deleterious for protein function, making it more difficult to assess the functional consequence of lipoxidation. This may be the case of actin, which is targeted by various electrophiles at Cys374 [23–25], a residue located at the interface between actin monomers in such a way that its mutation may induce altered microfilament patterns *per se* [114]. In some cases, although the function of a particular protein may be spared by mutation of the target residue, the general consequences for the cell could even be worsened if the mutated site was acting as a decoy, thus scavenging the reactive species.

It is also important to consider that lipid electrophiles are key regulators of gene expression, thus, the functional effects, above all in the long run can be the result of protein modification plus altered gene expression, which can amplify or counteract the effect of the electrophiles [50,98].

Ultimately, confirmation of the modification of a given target will be achieved when the adduct is detected on a peptide from the protein, whereas functional confirmation will be achieved by combined strategies, such as those involving mutation of the modified residue or alterations of expression levels.

10. Concluding remarks

Protein lipoxidation is obtaining recognition as a mechanism for regulation of protein function in health and disease. Given the wide structural variability and complexity of these posttranslational modifications, MS-based methods are essential for their characterization. In turn, functional assessment of the modification requires integrated approaches that take into account other potential modifications. Lipoxidation may interact or compete

with other modifications, including oxidative modifications or adduct formation with drugs in therapeutic regimes of patients, thus creating complex patterns, the characterization of which requires highly specific and sensitive methods. *In vitro* studies using either labeled or tag-free oxidized lipids are essential to unveil the potential sites of modification by these compounds, though further work regarding protein modifications in biological samples by endogenously generated species and their functional relevance will be key for a full understanding of the role of these lipids in pathophysiological scenarios.

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