Regulation of stress signaling pathways by protein lipoxidation

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1. Introduction

Post-translational regulation of signaling proteins via oxidation-reduction (redox) status of certain amino acids, cysteines in particular, has expanded our understanding of the role of reactive oxygen species mediating cellular signaling events in cells [1]. Oxidation of thiols residues within proteins to yield multiple oxidative states or disulfide formation by hydrogen peroxide (H$_2$O$_2$) has been widely studied, but the concept has now expanded to include thiol reactions with reactive electrophilic species formed e.g. via enzymatic and non-enzymatic oxidative reactions with unsaturated fatty acids [2,3]. In this graphical review, we provide a brief overview of the key adaptive pathways responsive to stress elicited by lipoxidation products (Fig. 1), the thiol targets of lipid-derived electrophiles (LDEs) identified in signaling proteins, as well as introduce novel methods to identify additional targets for lipid-derived electrophiles. Specifically, this review will focus on KEAP1-NRF2 and HSF1 pathways and highlights the protein targets modified by lipoxidation affecting these pathways.

2. Stress signaling by lipoxidation

Oxidation of polyunsaturated fatty acids generate different electrophilic species that can covalently modify nucleophilic residues within redox sensitive protein sensors in a process called lipoxidation. This triggers adaptive signaling pathways that ultimately lead to increased resistance to stress. In this graphical review, we will provide an overview of pathways affected by protein lipoxidation and the key signaling proteins being altered, focusing on the KEAP1-NRF2 and heat shock response pathways. We review the mechanisms by which lipid peroxidation products can serve as second messengers and evoke cellular responses via covalent modification of key sensors of altered cellular environment, ultimately leading to adaptation to stress.

Abbreviations: ABPP, Affinity based protein profiling; aLA, Alkyne-labeled linoleic acid; ARE, Antioxidant response element; BTB, Broad complex, Tram-track, and Bric-a-Brac; CTAD, C-terminal transactivation domain; CTD, C-terminal domain; CUL3, (Cullin-3)-ubiquitin E3 ligase complex; DBD, DNA binding domain; DMSO, Dimethyl sulfoxide; GCLC, Glutamate cysteine ligase catalytic; GCLM, Glutamate-Cysteine Ligase Modifier Subunit; G-REX, Genome-wide variant of T-REX; GST, Glutathione S-transferase; HSE, Heat shock element; HSF1, Heat shock factor 1; HSP, Heat shock protein; HSR, Heat shock response; HSPA8, Heat shock protein family A (Hsp70) member 8; HSPD1/E1, Heat shock protein family D/E (Hsp60/Hsp10) member 1; HSP40, 40-kDa heat shock proteins from HSP40 family; HSP70, 70-kDa heat shock proteins from HSP70 family; HSP90, 90-kDa heat shock proteins from HSP90 family; H$_2$O$_2$, Hydrogen peroxide; IA, Iodoacetamide alkylene; IER5, Immediate early response 5; IVR, Intervening region; KEAP1, Kelch-like ECH-associated protein 1; LC-MS, Liquid chromatography-mass spectrometry; LDE, Lipid-derived electrophiles; LNO$_2$, N-10-nitrosoperoxidolase, 12-nitro-octadec-9, 12-diNO$_2$ acid; LOX12/15, 12/15-Lipoxygenase; LZ1–3, Leucine zipper 1–3 domains; LZ4, Leucine zipper domain; MD, Middle domain; MS, Mass spectrometry; MRP, Multidrug-resistance-associated protein; Neh, Nrf2-ECH homology; NO$_2$-CLA, nitro-conjugated zipper domain; MD, Middledomain; NTR, N-terminal region; NQO1, NAD(P)H: quione oxidoreductase 1; OA-NO$_2$, Nitro-oleic acid; oxPAPC, Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphorylcholine; PARP1, Poly(ADP-ribose) polymerase 1; PARP13, Poly(ADP-ribose) polymerase 13; PEIPC, 1-palmitoyl-2-(5, 6)-epoxy isoprostane E2)-sn-glycerol-3-phosphocholine; PGA$_2$, Prostaglandin A$_2$; POLK, DNA polymerase kappa; PRDX1, Peroxiredoxin 1; PRDX13, Poly(ADP-ribose) Polymerase 13; RAC, Reactive oxygen species; ROS, Reactive oxygen species; SILAC, Stable isotope labeling with amino acids in cell culture; SH, sulphydryl group; sMAF, Small musculoaponeurotic fibrosarcoma oncogene homolog protein family; SQSTM1, Autophagosome cargo protein Sequestosome 1; TEV, Tobacco etch virus; T-REX, Targetable reactive electrophiles and oxidants; TXNR1D1, Thioredoxin reductase 1; Ub, ubiquitin; ZAK, Animal-specific MAP kinase found in stress responses; 4-HNE, 4-hydroxy-2-nonenal; 15d-PGJ$_2$, 15-deoxy-Δ2,14-prostaglandin J$_2$.
Fig. 1. Activation of the KEAP1-NRF2 and HSF1 pathways by electrophilic lipoxidation products. To survive, cells have developed an intricate set of stress signaling pathways that are activated by endogenous or exogenous signals [63]. Central to these defenses are the KEAP1-NRF2 and HSF1 pathways that together regulate hundreds of genes via binding to antioxidant response and heat shock elements, respectively [64,65]. A) The main signaling proteins of KEAP1-NRF2 pathway are the transcription factor NRF2 and its negative regulator protein KEAP1, which is a cullin-3 (CUL3)-RING ubiquitin ligase adaptor/scaffold protein enabling rapid proteasomal degradation of NRF2 during unstressed conditions. NRF2 is bound by the BTB domains of the KEAP1 dimer via DLG and ETGE motifs residing in NRF2 Neh2 domain. During the activation by e.g. lipid-derived electrophiles, the proteasomal degradation machinery is disrupted and de novo synthesized NRF2 is free to enter the nucleus to heterodimerize with the members of the musculoaponeurotic fibrosarcoma oncogene homolog protein family (sMAF) and drive the expression of cytoprotective genes [66]. SH; Sulfhydryl group of free cysteine residue, Ub; Ubiquitin. B) The HSF1 pathway consists of inactive HSF1 monomer that is bound by heat shock proteins from HSP90α [67] and HSP70 [68] families. HSF1 is negatively regulated by HSPs [69]. During stress, the interaction is disrupted and HSF1 trimerizes and enters the nucleus to regulate the heat shock response genes [70]. While the two pathways are largely distinct, they converge at the level of shared stimulus (e.g. electrophilic lipid peroxidation products) and mode of action (i.e. modification of redox-active cysteines that are regarded as molecular “switches”) [63].

Fig. 2. Cysteine residues are critical mediators of the antioxidant and heat shock responses. A) In the KEAP1-NRF2 pathway, the KEAP1 protein is responsible for sensing oxidant/electrophile stress. Human KEAP1 has 27 cysteines in total, enriched in the IVR-domain [17,18]. Cysteine modification leads to conformational changes in KEAP1, resulting in disruption of KEAP1-mediated ubiquitination of NRF2 [71]. B) There are seven cysteine residues in human NRF2, implicated to take part in oxidant/electrophile sensing [72]. However, no studies have addressed their impact on LDE-mediated NRF2 activation. C) Human HSF1 contains five cysteines, two of which (C35 and C135) have been shown to form a dimer upon heat stress or by H2O2 [73,74]. However, no studies to date have identified LDE targets in HSF1. D) Molecular chaperone family of HSP70s are one of the more abundant HSPs that regulate HSF1 [75,76]. HSP70s have five cysteines. E) Human HSP90α family of HSPs contain seven cysteines that are reactive towards heat shock and oxidative stress [73,77].
electrophilic lipoxidation products. These electrophilic products derived from lipids can interact with nucleophilic protein residues covalently through Michael addition reaction, leading to conformational changes in proteins that can affect their function [4]. Some target proteins that can be modified by the interaction with electrophilic lipids are transcription factors, proteins involved in cell defense, enzymes (such as glutathione S-transferase, GST) and regulators of signaling pathways (such as RAS) [4,5]. Importantly, electrophilic lipids exert biphasic effects, low concentrations being able to elicit adaptive cell signaling pathways that are cytoprotective and anti-inflammatory, while higher concentrations can aggravate inflammation and cause cell death. These effects depend not only on the concentration but also the cell type being affected, as well as the chemical nature of the lipid species [2,6,7].

Several stress-activated pathways are key targets for regulation by lipoxidation. These include the antioxidant response pathway governed by the KEAP1-NRF2-system [8] and the heat shock response via HSF transcription factors [9], that are in the focus of this review. However, protein lipoxidation can also affect stress kinase pathways, as kinases, phosphatases and/or their regulators can be targeted by lipoxidation [10]. Affected pathways include the c-Jun N-terminal kinase (JNK) pathway, as well as the structurally similar p38 pathway, which belong to the mitogen-activated protein kinase cascades. These pathways regulate e.g. differentiation, motility and apoptosis [2,11], and can be modulated by LDEs such as 4-HNE and cyclopentenone prostaglandins [10,12].

3. Activation of the KEAP1-NRF2 signaling pathway by lipid-derived electrophiles

The KEAP1-NRF2 pathway (Fig. 1A), is the key pathway mediating the transcriptional response to oxidative and electrophilic stress [13]. Under unstressed conditions, the transcription factor NRF2 (NF-E2-related factor 2) is tethered by KEAP1 (Kelch-like ECH-associated protein 1), an adaptor protein within the CUL3 (cullin-3)-ubiquitin E3 ligase E3 ligase complex, resulting in proteosomal degradation of NRF2 via the 26S proteasome [8,14,15]. NRF2 binds to KEAP1 via two different and highly conserved motifs present in Neh2 domain: a weak affinity, DLG motif and a high affinity, ETGE motif (Fig. 1 A). Upon exposure to stimuli, ubiquitination is disrupted and newly synthetized NRF2 translocates to the nucleus, where it binds to the antioxidant response element (ARE) within the regulatory regions of NRF2 dependent genes driving their expression [13,16].

KEAP1 has four functional domains: Bric-a-Brac, tram-track, broad complex (BTB) domain, the intervening region (IVR), the Kelch domain and the C-terminal region. [13] The human KEAP1 protein contains altogether 27 cysteine residues, of which three have unambiguously shown to have functional importance (Fig. 2A) [17,18]. These are Cys151 in BTB domain and Cys273 and Cys288 in the IVR region [13,19].

LDEs are produced endogenously by both enzymatic and non-enzymatic reactions from unsaturated fatty acids [2,20]. Given their electrophilic character, they are able to activate NRF2 in a KEAP1-dependent manner. With respect to the mechanism of activation, arachidonic acid-derived cyclopentenone prostaglandins and isoprostanes (prostaglandin A2, PGA₂; 15-deoxy-Δ12,14-prostaglandin J2, 15d-PGJ₂ and structurally similar cyclopentenone isoprostanes produced by nonenzymatic oxidation), lipid-derived aldehydes, especially 4-hydroxynonenal (4-HNE), and nitroalkenes (nitro-conjugated linoleic acid, NO₂-CLA) have been studied in some detail. There are discrepant findings of KEAP1 and NRF2 thiols that are modified by LDEs (Fig. 2A,B and Table 1). Particularly in the case of 15d-PGJ₂, PGA₂ and OA-NO₂, it is clear that C151 in KEAP1 is not the primary target unlike with C151-prefering activators such as cyclic cyanoenones, some of which are currently in clinical stages of drug development [21]. In addition to free oxidized lipid species, oxidized phospholipids can activate NRF2 [22–24]. 1-palmitoyl-2-(5,6-epoxy isoprotease E2)-sn-glycerol-3-phosphocholine (PEIPC), the major active component of oxidized 1-palmitoyl-2-arachidionyln-s-Glycerol-3-phosphorylcholine (oxPAPC), has been shown to activate NRF2 in a thiol-dependent manner, but the mechanism of action and thiol targets are currently unknown [22,23].

4. Lipoxidation targets of the HSF1 pathway

Heat shock response (HSR) is an evolutionarily conserved pathway that has evolved to provide protection to eukaryotic cells against heat, oxidative and other forms of proteotoxic stress [9,25]. HSR includes a sequence of events in which heat shock proteins (HSPs), acting as molecular chaperones and heat shock factors 1 and 2 (HSF1 and 2), transcription factors mediating transcriptional responses coordinate to maintain protein homeostasis in affected cells [26]. Though there are different HSF family members (HSF1–4 in mammals), HSF1 is the factor primarily orchestrating protein homeostasis [27,28]. HSR (Fig. 1B) is a

| DOMAIN       | CY5 | OA-NO₂ | PGA₂ | 15d-PGJ₂ | 4-HNE | Ref. |
|--------------|-----|--------|------|---------|-------|------|
| KEAP1        | 38  | ☐      | ☐    | ☐       | ☐     | [109]|
| N-terminal   | 12  | ☐      | ☐    | ☐       | ☐     |      |
|             | 13  | ☐      | ☐    | ☐       | ☐     |      |
|             | 23  | ☐      | ☐    | ☐       | ☐     |      |
| BTB          | 77  | ☐      | ☐    | ☐       | ☐     | [19][81]|
|              | 171 | ☐      | ☐    | ☐       | ☐     |      |
|              | 196 | ☐      | ☐    | ☐       | ☐     | [109]|
|              | 226 | ☐      | ☐    | ☐       | ☐     |      |
|              | 241 | ☐      | ☐    | ☐       | ☐     |      |
| Interneaving | 207 | ☐      | ☐    | ☐       | ☐     | [109]|
| region       | 288 | ☐      | ☐    | ☐       | ☐     | [82][109]|
|              | 289 | ☐      | ☐    | ☐       | ☐     | [19][82][109]|
|              | 297 | ☐      | ☐    | ☐       | ☐     | [82]|
| Kelch        | 498 | ☐      | ☐    | ☐       | ☐     | [82][109]|
|              | 513 | ☐      | ☐    | ☐       | ☐     |      |
|              | 518 | ☐      | ☐    | ☐       | ☐     |      |
|              | 583 | ☐      | ☐    | ☐       | ☐     |      |
|              | 613 | ☐      | ☐    | ☐       | ☐     |      |
|              | 614 | ☐      | ☐    | ☐       | ☐     |      |
|              | 697 | ☐      | ☐    | ☐       | ☐     |      |
| C-terminal   | 622 | ☐      | ☐    | ☐       | ☐     |      |
|              | 624 | ☐      | ☐    | ☐       | ☐     |      |
| HSP90α       | 374 | ☐      | ☐    | ☐       | ☐     |      |
| MD           | 420 | ☐      | ☐    | ☐       | ☐     |      |
|              | 529 | ☐      | ☐    | ☐       | ☐     | [41]|
| ATPase domain| 607 | ☐      | ☐    | ☐       | ☐     |      |
| Lid          | 574 | ☐      | ☐    | ☐       | ☐     | [40]|
|              | 603 | ☐      | ☐    | ☐       | ☐     |      |
for specific autophagy [97]. Upon activation of the heat shock response, HSF1 forms first an active trimer and activating genes having a HSE regulatory element, such as immediate early response 5 (IERS) [97] and multiple heat shock proteins (chaperones) that have various different functions. These genes include heat shock protein family A (HSP70) member 8 (HSPA8) [98], Heat shock protein family D/E (HSP60/HSP10) member 1 (HSPD1/E1) [99], 40-kDa heat shock proteins from HSP40 family (HSP40) [100]. HSF1 can also provide the means to repair already damaged DNA by encoding DNA polymerase kappa (POLK) [101] and through a complex with Poly(ADP-ribose) polymerase 1 and 13 (PARP1 and PARP13) [102–104]. KEAP1-NRF2 and HSF1 pathways co-activate and/or regulate heme oxygenase 1 (HMOX1). Both pathways are thus complementary and overlapping in their functions [105–108].

transcriptional response, where HSF1 binds to DNA to regulate transcription of hundreds of genes including a number of HSPs [29]. Under basal conditions, HSF1 is bound to an inhibitory complex consisting of HSP40, HSP70 and HSP90 existing only as latent and transcriptionally inactive monomer in the cytoplasm [30,31]. During HSR, HSF1 is released from the complex and it forms transcriptionally active trimer by binding to two other HSF family members via hydrophobic repeats in leucine zipper (LZ1–3 and LZ4) domains [32]. After trimerization, the complex translocates to the nucleus and utilizing specialized DNA binding domains the trimer binds to heat shock elements (HSE) that lie within enhancer regions of genes for specific autophagy [97]. Upon activation of the heat shock response, HSF1 forms first an active trimer and activating genes having a HSE regulatory element, such as immediate early response 5 (IERS) [97] and multiple heat shock proteins (chaperones) that have various different functions. These genes include heat shock protein family A (HSP70) member 8 (HSPA8) [98], Heat shock protein family D/E (HSP60/HSP10) member 1 (HSPD1/E1) [99], 40-kDa heat shock proteins from HSP40 family (HSP40) [100]. HSF1 can also provide the means to repair already damaged DNA by encoding DNA polymerase kappa (POLK) [101] and through a complex with Poly(ADP-ribose) polymerase 1 and 13 (PARP1 and PARP13) [102–104]. KEAP1-NRF2 and HSF1 pathways co-activate and/or regulate heme oxygenase 1 (HMOX1). Both pathways are thus complementary and overlapping in their functions [105–108].

It has long been known that LDEs can trigger HSR in cells. One of the earliest reports found that cyclopentenone prostaglandins PGA2 and 15d-PGJ2 increase the expression of inducible HSP70, the key marker of HSR in K562 erythroleukemia cells [36] and later on, other LDEs such as 4-HNE and OA-NO2 have been shown to evoke HSR in an HSF1 dependent manner [34,35]. Though the exact mechanism by which HSF1 is activated by LDEs is not well understood at the moment, it is widely believed that LDEs target cysteines within HSPs resulting in HSF1 release and activation [2,38]. Other mechanisms contributing to the modulation of this pathway by LDEs, include the lipoxidation of histone deacetylases which affects the expression of HSF70 [39]. Cysteine residues, C572 and C267 respectively, of rat HSP90 and HSP70 are modified by 4-HNE (Figs. 2D,E and 3) [40,41]. HSF1 has also been shown to contain redox-sensitive cysteines, as a disulfide bridge can be formed between C35 and C267 respectively, of rat HSP90 and HSP70 are modified by 4-HNE cyclopentenone prostaglandins, 15d-PGJ2 and or PGA2 [44–46]. HSF1 has also been shown to contain redox-sensitive cysteines, as a disulfide bridge can be formed between C35 and C267 respectively, of rat HSP90 and HSP70 are modified by 4-HNE cyclopentenone prostaglandins, 15d-PGJ2 and or PGA2 [44–46].
modified proteins can be then coupled with high resolution mass spectrometry (MS) for identification of protein targets [48]. For a more quantitative analysis of protein targets, Wang et al. have developed an affinity based protein profiling (ABPP) method using isotope-labeling for quantifying the reactivity of 4-HNE and 15d-PGJ2 in the human proteome [53]. In this approach, human breast cancer cells were first treated with LDEs or DMSO (control), followed by alkylnlated iodoacetamide (IA) probe, and conjugated by copper-catalyzed azide-alkyne cycloaddition ("click") chemistry to light and heavy protease–cleavable biotin tags. After enrichment with streptavidin, sequential on-bead protease digestion is done to yield probe-labeled peptides for MS analysis. For a more quantitative analysis of protein targets, Wanget al. have developed an affinity based protein profiling (ABPP) method using isotope-labeling for quantifying the reactivity of 4-HNE and 15d-PGJ2 in the human proteome [53]. In this approach, human breast cancer cells were first treated with LDEs or DMSO (control), followed by alkylnlated iodoacetamide (IA) probe, and conjugated by copper-catalyzed azide-alkyne cycloaddition ("click") chemistry to light and heavy protease–cleavable biotin tags. After enrichment with streptavidin, sequential on-bead protease digestion is done to yield probes for MS analysis. For a more quantitative analysis of protein targets, Wanget al. have developed an affinity based protein profiling (ABPP) method using isotope-labeling for quantifying the reactivity of 4-HNE and 15d-PGJ2 in the human proteome [53]. In this approach, human breast cancer cells were first treated with LDEs or DMSO (control), followed by alkylnlated iodoacetamide (IA) probe, and conjugated by copper-catalyzed azide-alkyne cycloaddition ("click") chemistry to light and heavy protease–cleavable biotin tags. After enrichment with streptavidin, sequential on-bead protease digestion is done to yield probes for MS analysis. For a more quantitative analysis of protein targets, Wanget al. have developed an affinity based protein profiling (ABPP) method using isotope-labeling for quantifying the reactivity of 4-HNE and 15d-PGJ2 in the human proteome [53]. In this approach, human breast cancer cells were first treated with LDEs or DMSO (control), followed by alkylnlated iodoacetamide (IA) probe, and conjugated by copper-catalyzed azide-alkyne cycloaddition ("click") chemistry to light and heavy protease–cleavable biotin tags. After enrichment with streptavidin, sequential on-bead protease digestion is done to yield probes for MS analysis.
Historically, the established methods to detect LDE modifications are based on bolus treatment, which may represent poorly the actual lipidoxidation events occurring within cells, but in return can identify many RES sensors [54]. These methods use excess amounts of LDEs and can thus identify protein targets not encountered when more physiological levels of electrophiles are present [55]. Recently, novel methods with more sophisticated dosing and detection procedures have been introduced to reduce the inherent bias due to bolus dosing and to provide new means to quantitatively detect endogenous modifications [54,56–58].

To avoid bolus treatment with a high concentration of LDEs, a method in which the LDE in question is delivered inside the cells as a photocaged precursor (Fig. 4B and C) and then liberated in situ to allow local delivery of LDE within cells has been developed [59]. The method can be coupled to proteomics to identify novel thiol targets in an unbiased manner [60] or to a specific signaling protein of interest [61]. Intriguingly, the method appears to produce modifications that have very little overlap with those evoked by external bolus addition. For instance, 4-HNE modified C513 and C518 within KEAP1, neither of which have been identified as sensitive cysteines in previous studies nor to have functional importance [3].

Also, methods to identify the modifications by endogenously produced lipid electrophiles need to be developed in order to address the (patho)physiological role of these in cellular processes. Beavers et al. combined stable isotope labeling with amino acids in cell culture, click chemistry, and ABPP techniques to explore addition of lipid electrophiles endogenously generated during macrophage activation (Fig. 4D) [57]. In this study, mitochondria was identified as both the source and target of LDEs in activated macrophages, indicating the role of mitochondrial protein modifications in inflammatory diseases [57]. Similar approach has been used to identify targets of 12/15-Lipoxygenase (LOX-12/15) derived LDEs in macrophages [62]. It is enticing to speculate that LDEs could mediate also other stress responses and therefore it is necessary to expand the selection of stressors and cell types further.

6. Conclusions

It is now clear that reactive lipid electrophiles elicit signaling functions that are specific and biologically relevant, adding to the repertoire of post-translational modifications affecting cellular functions. Especially well studied are the KEAP1-NRF2 and HSR pathways, which have cytoprotective, anti-inflammatory and proteostatic functions. Especially well studied are the KEAP1-NRF2 and HSR pathways, which have cytoprotective, anti-inflammatory and proteostatic functions.

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