Biochem. J. (2012) **442**, 453–464 (Printed in Great Britain) doi:10.1042/BJ20111752



REVIEW ARTICLE

# Cell signalling by reactive lipid species: new concepts and molecular mechanisms

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The process of lipid peroxidation is widespread in biology and is mediated through both enzymatic and non-enzymatic pathways. A significant proportion of the oxidized lipid products are electrophilic in nature, the RLS (reactive lipid species), and react with cellular nucleophiles such as the amino acids cysteine, lysine and histidine. Cell signalling by electrophiles appears to be limited to the modification of cysteine residues in proteins, whereas non-specific toxic effects involve modification of other nucleophiles. RLS have been found to participate in several physiological pathways including resolution of inflammation, cell death and induction of cellular antioxidants through the modification of specific signalling proteins. The covalent modification of proteins endows some unique features to this signalling mechanism which we have termed the 'covalent advantage'. For example, covalent modification of signalling proteins allows for the accumulation of a signal over time. The activation of cell signalling pathways by electrophiles is hierarchical and depends on a complex interaction of factors such as the intrinsic chemical reactivity of the electrophile, the intracellular domain to which it is exposed and steric factors. This introduces the concept of electrophilic signalling domains in which the production of the lipid electrophile is in close proximity to the thiol-containing signalling protein. In addition, we propose that the role of glutathione and associated enzymes is to insulate the signalling domain from uncontrolled electrophilic stress. The persistence of the signal is in turn regulated by the proteasomal pathway which may itself be subject to redox regulation by RLS. Cell death mediated by RLS is associated with bioenergetic dysfunction, and the damaged proteins are probably removed by the lysosome-autophagy pathway.

Key words: electrophile-responsive proteome, Kelch-like ECH-associated protein 1 (Keap1), lipid peroxidation, nuclear factor-erythroid 2 related factor (Nrf2), protein modification, reactive lipid species (RLS).

## INTRODUCTION

The oxidation of PUFAs (polyunsaturated fatty acids), such as arachidonic acid, generates a broad range of oxidation products which historically have been used as markers of oxidative stress [1,2]. For example, the unique structural attributes of the non-specific oxidation products known as the isoprostanes have allowed for the development of accurate high-throughput assays for their measurement in complex biological systems [3]. Lipid peroxidation products have been detected in the blood, plasma, urine, and tissue samples of humans and animal models using an array of techniques, and, in many cases, their levels are elevated in pathological conditions [4–7]. The application of these analytical techniques has led to the concept that RLS (reactive lipid species) are mediators, not simply by-products, of multiple pathophysiological conditions [8–11]. The cell signalling mediated by RLS has some unique biochemical attributes. Importantly, many lipid peroxidation products are also electrophilic, which allows them to form stable covalent adducts with nucleophilic residues on proteins [12–14]. This is important since it is now well recognized that the thiol groups on cysteine residues act as redox switches controlling cell signalling and metabolism [15–17]. The cysteine thiol group is particularly versatile, and the concept has emerged that different thiol-reactive signalling molecules can selectively modulate protein function [16]. Specific mechanisms that have been shown to modify redox cell signalling include S-nitrosation, S-glutathionylation and Michael addition with biologically active electrophiles [15,18,19]. Other oxidative mechanisms mediated by either hydrogen peroxide or lipid peroxides to form sulfenic or sulfinic acids were initially thought to be markers of oxidative damage. However, a previous study suggested that they may also play a role in cell signalling [20]. Interestingly, although early studies implied that lipid peroxidation always results in damage, a more refined view of this process has evolved and suggests that oxidized lipids can elicit different cellular effects depending on the species present, their concentrations and their reactivity with protein targets [14,21–23].

Oxidized lipids can mediate biological responses through two diverse mechanisms: classic reversible binding and irreversible covalent modification of receptors [15,22,24–26]. Some oxidized lipids are ligands for specific receptors [e.g. PG (prostaglandin) receptors] and mediate biological effects through reversible receptor–ligand interactions [27,28]. This is best understood for the enzymatically produced PGs and LTs (leukotrienes) [29]. In contrast, some lipid peroxidation products modulate cellular activity through irreversible covalent modification of nucleophilic amino acid residues on proteins [15,30]. This concept was initially in conflict with the classical paradigms for cell signalling since to 'turn-off' the signal, the protein must be selectively degraded.

Abbreviations used: BLT, leukotriene B receptor; COX, cyclo-oxygenase; CysLT, cysteinyl leukotriene receptor; 15d-PG$J_2$, 15-deoxyprostaglandin $J_2$; EpRE, electrophilic-response element; GST, glutathione transferase; HNE, 4-hydroxynonenal; HO-1, haem oxygenase-1; HSF, heat-shock factor; Hsp, heat-shock protein; Keap1, Kelch-like ECH-associated protein 1; LOX, lipoxygenase; LT, leukotriene; Nrf2, nuclear factor-erythroid 2-related factor; PG, prostaglandin; PPAR$\gamma$, peroxisome-proliferator-activated receptor $\gamma$; PUFA, polyunsaturated fatty acid; RLS, reactive lipid species; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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However, signalling through the covalent modification of proteins is now accepted for a number of well-defined protein–lipid interactions, and selective degradation is mediated through the proteasome [31,32]. Interestingly, signalling though the covalent modification of proteins changes the relationship between the concentration of the ligand, in this case an oxidized lipid, and the resultant signal [33]. Since irreversible covalent modifications of proteins can accumulate over time and amplify a signal [33], even low levels of oxidized lipids initiate signalling. We have termed this concept 'the covalent advantage' [22].

In the present review, we will discuss: (i) the formation of RLS through both non-enzymatic and enzymatic processes; (ii) oxidized lipid signalling through classic receptor-mediated pathways and by covalent modification of protein targets; and (iii) susceptibility of thiols to modification by RLS. We will then relate these concepts to the ability of oxidized lipids to trigger adaptive and damaging biological effects with a focus on the role of subcellular localization.

## FORMATION OF RLS

Much of the early research into mechanisms of lipid peroxidation was performed by scientists in the food industry. It was well appreciated that off odours and flavours could be attributed to lipid oxidation, and inhibiting this process results in products with a longer shelf life [34]. As the field evolved, it was recognized that lipid peroxidation is endogenous to living organisms and has multiple functions dependent on the site and mechanism of oxidation. For example, as shown in Figure 1(A), enzymatic sources of lipid peroxidation yield important biological mediators of inflammation such as the PGs from COXs (cyclo-oxygenases), and the LTs from LOXs (lipoxygenases) [35,36]. Importantly, both non-enzymatic and enzymatic oxidation of PUFAs results in the formation of RLS that are electrophilic (Figure 1B). A major substrate for lipid peroxidation is arachidonic acid, and its oxidation results in the formation of several products (Figure 1A). Of these lipid peroxidation products (Figure 1A), a subset are electrophilic in nature, and there are both structurally distinct species derived from either non-enzymatic or enzymatic lipid peroxidation (Figure 1B). Examples of electrophilic products of non-enzymatic lipid oxidation include aldehydes such as HNE (4-hydroxynonenal), malondialdehyde and acrolein as well as the J- and A-series isoprostanes. Other RLS include the isoketals, which result from the rearrangement of endoperoxide intermediates of the isoprostane pathway and have the potential to react with both proteins and lipids [37]. The approach to research with RLS has largely focused on defining the reactivity and biological effects of a candidate molecule. For this reason, we know a great deal about the behaviour of HNE, 15d-$PGJ_2$ (15-deoxyprostaglandin $J_2$) and nitroalkanes [21,38–44]. From these studies, two key facts have emerged: (i) the effects of all the RLS are dependent on the amount exposed to the cell with many exhibiting anti-inflammatory or cytoprotective effects over the lower concentration range; and (ii) the biological effects of the RLS vary according to the specific RLS and target cells [33,45]. The implications of these findings are that each RLS reacts with a specific family of proteins which we have called the electrophile-responsive proteome [12,22]. This concept will be explored in more depth throughout the present review.

## NON-ENZYMATIC LIPID PEROXIDATION

PUFAs, such as arachidonic and linoleic acid, are targets for lipid peroxidation. Non-specific lipid peroxidation proceeds through a chain reaction composed of three main steps: initiation, propagation and termination. In enzymatic lipid peroxidation,

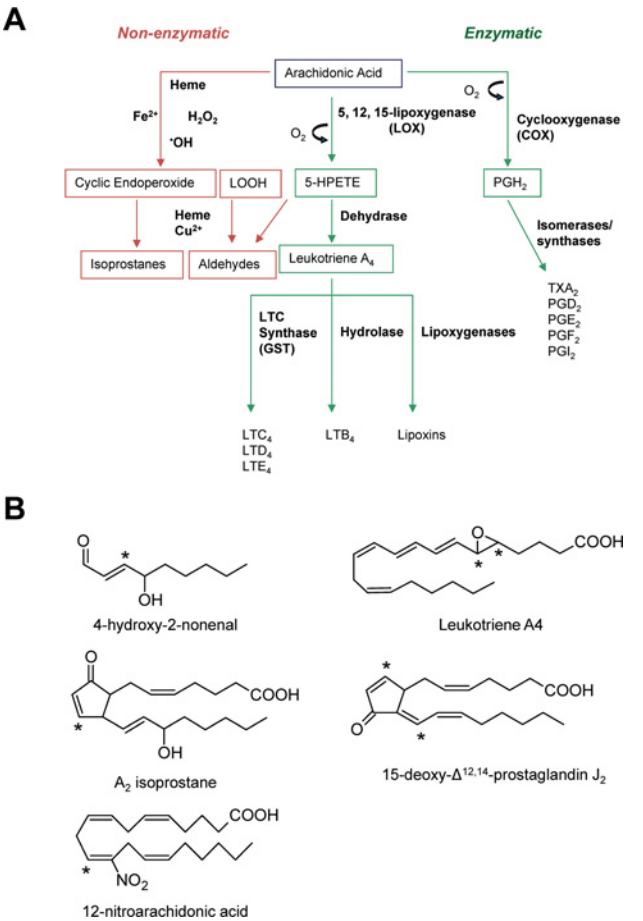


**Figure 1 Formation of lipid electrophiles via non-enzymatic and enzymatic lipid peroxidation**

(**A**) Arachidonic acid can be converted into several products through enzymatic and non-enzymatic lipid peroxidation. Both free-radical-catalysed as well as enzymatically controlled oxidation yields a subset of products that are electrophilic. 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; LOOH, linoleic acid hydroperoxide. (**B**) Examples of RLS produced from arachidonic acid and their structures. For simplicity, stereochemistry is not indicated. $TXA_2$, thromboxane A2; *reactive site.

initiation is controlled and stereospecific and propagation does not occur. The production of specific lipid oxidation signalling molecules is controlled by enzyme pathways and the release of non-enzyme-bound radical intermediates is minimized. Due to their unsaturated double bonds, the allylic hydrogen atoms in PUFAs are readily abstracted by initiating species such as ferryl radical, peroxynitrite ($ONOO^-$), hydroperoxyl radicals ($HO_2^{\bullet}$) and hydroxyl radical ($OH^{\bullet}$). This results in the formation of lipid radicals which react with oxygen if it is available. The products that are formed are diverse and depend on the substrate oxidized (e.g. arachidonic compared with linoleic acid) and the mechanism of oxidation (non-enzymatic or enzymatic). Once lipid peroxidation is initiated, lipid alkoxyl ($LO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$) radicals are capable of abstracting a hydrogen atom from another fatty acid molecule, thus contributing to the propagation of lipid peroxidation [46]. In biological membranes, the presence of proteins can result in transfer of the lipid radicals to protein side chains and adduct formation [47,48]. In this setting, the proteins become active participants in the propagation of the lipid peroxidation reactions. Molecular oxygen ($O_2$) is required for the propagation phase, and, for this reason, lipid peroxidation proceeds at a higher rate when oxygen concentrations are high

[46]. Lipid peroxidation can be terminated by radical–radical reactions with other lipid radical species or with protein radicals. Termination can also occur by radical–radical reaction of a lipid radical species with the nitric oxide radical ($NO^{\bullet}$) [49].

Cardiovascular disease is a pathological condition in which predominantly non-specific lipid peroxidation occurs *in vivo*. For example, in atherosclerotic lesions, the lipid peroxidation products found are mostly those lacking stereospecificity which is a characteristic of the non-enzymatic pathways [50]. However, increases in inflammation do lead to production of low levels of stereospecific enzymatic lipid oxidation products in atherosclerosis [51,52]. Several factors may promote lipid peroxidation through non-enzymatic reactions *in vivo* [53]. For example, the production of ROS (reactive oxygen species) and RNS (reactive nitrogen species) in inflammation may result in damage to iron- or copper-containing proteins and the release of the metal from a protein environment in which radical reactions can be controlled. This can occur with the proteins myoglobin and haemoglobin [54,55]. Haem proteins then play an important role in lipid peroxidation by decomposing lipid hydroperoxides and facilitating the propagation phase [56]. However, unlike free haem, haem proteins can also initiate lipid peroxidation [57]. Interaction of hydrogen peroxide with metmyoglobin or methaemoglobin leads to the formation of an activated haem protein with a porphyrin cation radical ($P^{+}-Fe^{4+}=O$) [58]. This ferryl radical species is the initiator of lipid peroxidation rather than the hydroxyl radical [57]. Because of the ability of hydrogen peroxide to 'activate' these haem proteins to initiating species, myoglobin- and haemoglobin-mediated lipid peroxidation may be important for catalysing lipid peroxidation in biological systems where hydrogen peroxide is elevated [56,59].

Peroxynitrite ($ONOO^{-}$), formed from the rapid reaction of $NO^{\bullet}$ with superoxide has also been shown to promote lipid peroxidation [38,60,61], probably due to the reactivity of decomposition products hydroxyl radical and nitrogen dioxide ($NO_2^{\bullet}$). These radical species are capable of abstracting a hydrogen atom from unsaturated fatty acids and this process is iron-independent [60]. iNOS (inducible nitric oxide synthase) and NADPH oxidases are cellular sources of $NO^{\bullet}$ and superoxide respectively, and their expression is concomitantly increased in several pathologies and can form $ONOO^{-}$ [62,63]. The lipid peroxidation reactions initiated by $ONOO^{-}$ produce isoprostanes, aldehydes and oxysterols, but unique RLS such as nitrated lipids only occur with this mechanism of oxidation [64–66]. The interaction of lipid radicals with RNS such as nitrogen dioxide, or possibly nitrite, results in a family of electrophilic RLS known as the nitroalkenes [38,39,67].

## ENZYMATIC LIPID PEROXIDATION

There are several enzymes that contribute to the controlled peroxidation of PUFAs and the activation of multiple biological pathways [36,68]. One of the best-studied enzymes is COX, which is responsible for the formation of PGs from arachidonic acid (Figure 1). Since COX acts predominantly on free fatty acids, in many cases the production of PGs is dependent upon phospholipase $A_2$ [69]. COX contains two active sites including a COX domain and a peroxidase domain [70]. The COX site is responsible for oxygenating arachidonic acid to form hydroperoxide $PGG_2$. The peroxidase site then reduces $PGG_2$ to the alcohol $PGH_2$, the final product of COX. There are two isoforms of COX in the cell [70]. COX-1 is constitutively expressed in all tissues; however, COX-2 is normally only detected in tissues with active inflammation except kidney and brain where COX-2 is constitutively expressed [71]. The protein expression of COX-2 is regulated by several transcription factors relevant to inflammation including NF-$\kappa$B (nuclear factor $\kappa$B), NF-IL-6 (nuclear factor for interleukin-6 expression) and CREB (cAMP-response-element-binding protein) [72,73]. Once expressed, COX's activity can also be regulated in a transcription-independent manner [74,75]. Several ROS are known to regulate COX-2 activity by regulating the levels of the lipid peroxide tone which is required for activation [75–77]. The major product of both COX-1 and COX-2 is $PGH_2$, which can then be metabolized to other PGs through the action of PGD, PGE, PGF, and PGI synthases [78–83]. $PGA_2$, $PGJ_2$ and 15d-$PGJ_2$ are examples of electrophilic PGs.

The COX enzymes generate several anti-inflammatory electrophilic RLS from arachidonic acid (e.g. cyclopentenones) as well as products of $\omega-3$ fatty acids [e.g. DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid)] [40,84]. The latter products derived from COX-2 have been shown to be important anti-inflammatory mediators [84,85]. Interestingly, a subset of these are electrophilic, termed the EFOXs (electrophilic oxo-derivatives of $\omega-3$ fatty acids) [84]. These enzymatically produced RLS may be important for the protection afforded by $\omega-3$ supplementation.

Another important source of enzymatic lipid peroxidation products is through the action of LOXs. LTs and lipoxins are the products of this pathway and have been extensively studied in the field of immunology [29,86,87]. There are three LOX isoforms, with 5-, 12- and 15-LOX expressed in leucocytes, platelets and endothelial cells respectively [29,88]. The active site of LOX contains a non-haem iron which is critical to the enzyme's activity [89,90]. As with COX, LOX activity is also modulated by ROS through regulation of the enzyme's peroxide tone [91,92]. Among the LOXs, 5-LOX is the most well-studied in the context of cardiovascular disease [68]. It was originally found to contribute to asthma and was targeted with inhibitors developed to minimize airway inflammation [86]. It is now well-established that 5-LOX products also contribute to other inflammatory processes including the development of coronary artery disease [51,93]. As shown in Figure 1, following generation of $LTA_4$ from LOX, the product $LTB_4$ is formed by hydration, whereas the cysteinyl LTs, $LTC_4$, $LTD_4$ and $LTE_4$, are produced by a specialized GST (glutathione transferase) enzyme, $LTC_4$ synthase [94,95]. Aside from the known receptor-mediated effects of the LTs, one LT is known to be capable of receptor-independent effects through covalent modification. Because $LTA_4$ is uniquely electrophilic owing to its epoxide group, it is capable of adducting to nucleophilic amino acids as well as DNA bases [96,97]. The nucleophilic attack of 5-LOX by $LTA_4$ leads to the covalent modification and inactivation of the enzyme [98].

## REVERSIBLE RECEPTOR-MEDIATED SIGNALLING BY OXIDIZED LIPIDS

It is well appreciated that oxidized lipids including the PGs and LTs can act through reversible binding to cellular receptors [27,99] (Figure 2). The signalling responses vary depending on the spectrum of oxidized lipids formed, their concentration and which receptors are bound by the ligand. As shown in Figure 2(A), the signals arising from reversible receptor–ligand interactions are dependent on the concentration of specific lipid peroxidation products formed and are typically transient and saturable. Thus it will not be an effective agonist unless a product is present at a concentration close to the binding affinity of the receptor. In many cases, the biological lifetimes of the PGs are also extremely short, and this results in a transient signal.

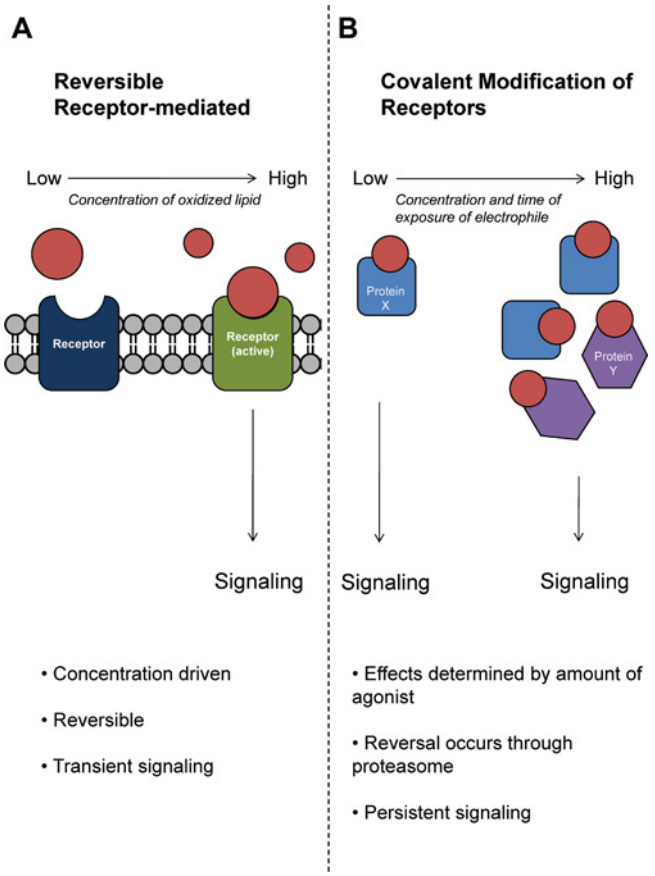


**Figure 2 Classical receptor-mediated signalling compared with signalling mediated through covalent modification**

(**A**) Classical receptor-mediated signalling only occurs when a ligand is present at concentrations which exceed the affinity constant ($K_m$). This binding is reversible and quickly dissipates when ligand concentrations dip below the needed threshold. (**B**) Signalling via covalent modification can occur at low concentrations as well as high concentrations of the ligand. Low concentrations of electrophile accumulate over time, resulting in persistent signalling. Higher concentrations of electrophile may result in modification of more diverse protein targets and thus change the cellular response.

Many of the COX products act through G-protein-coupled receptors, including the EP receptors (EP1, EP2, EP3, and EP4 in humans) for $PGE_2$, the DP receptor and the CRTH2 (chemoattractant receptor homologous molecule expressed on T helper type 2 cells) for $PGD_2$ and its metabolites, the FP receptor for $PGF_2$, and the IP receptor for $PGI_2$ [100]. In contrast, electrophilic PGs can also participate in cell signalling through covalent modification of receptors [22,33], a signalling mechanism that will be discussed further below [23,101]. As with the PGs, LTS formed through LOX act through specific receptors [102]. BLT (LTB receptor) 1 and BLT2 mediate the pro-inflammatory effects of $LTB_4$ [103]. The cysteinyl LTs ($LTC_4$, $LTD_4$ and $LTE_4$) act through CysLT (cysteinyl LT receptor) 1 and CysLT2 [104]. CysLT1 is highly expressed in bronchial smooth muscle, and agonist binding induces smooth muscle contraction [105]. CysLT1 is also expressed in the spleen and platelets [105,106]. CysLT2 is expressed in the heart, adrenal gland, placenta, peripheral leucocytes, spleen, lymph nodes and central nervous system [107].

Other than through specific G-protein-coupled receptors, several lipid peroxidation products have been suggested to act through PPAR$\gamma$ (peroxisome-proliferator-activated receptor $\gamma$). This receptor is interesting since it appears that it can function through both reversible and irreversible binding to the receptor. For example, the reactive PGs (e.g. 15d-$PGJ_2$), electrophilic fatty acids including the nitrated lipids such as nitro-arachidonic acid and nitro-oleic acid have also been shown to bind covalently to this receptor [39]. Binding to PPAR$\gamma$ is thought to be important for the anti-inflammatory effects of a number of RLS [39,41,108]. Upon binding to PPAR$\gamma$, genes involved in metabolism, cellular differentiation and inflammation are up-regulated. PPAR$\gamma$ activation is anti-inflammatory and the protein is expressed on several cell types within the vasculature including endothelial cells, monocytes, macrophages and smooth muscle cells [109].

## ACCUMULATION OF CELL SIGNALLING EFFECTS BY LOW CONCENTRATIONS OF RLS: THE COVALENT ADVANTAGE

Can the formation of a covalent bond between a receptor and a RLS (Figure 2B) elicit a biological response *in vivo*? This is a particularly important issue to address because the 'free' levels of RLS in biological systems are often reported to be in the nanomolar range and yet *in vitro* micromolar ranges are typically needed to elicit cell signalling [11,40,42,43,55,56,110]. For example, PPAR$\gamma$ activation may be the basis of signalling for some electrophilic lipids [39], but it has been suggested that they are present at concentrations insufficient to bind to and activate this receptor [111–113]. However, these RLS can bind covalently to a cysteine residue on the ligand-binding domain of the receptor [114,115]. In addition, quantitative estimation of RLS is confounded by the reactivity of the $\alpha,\beta$-unsaturated ketone group, since substantial amounts of the lipid will be bound to proteins.

The fact that RLS can form covalent bonds with proteins may allow the accumulation of a signal over time. This is governed by several factors. The rate of activation will depend on the concentration of the activating electrophile and the receptor target. However, the net activation will depend on the amount of activating electrophile to which the receptor target is exposed to over time (Figure 2B). For example, the activation of a signalling pathway achieved by 10 pmol of electrophile will be half that achieved by 20 pmol in the cells that are exposed to the same volume. Using a cell culture model system and covalent adduct formation of 15d-$PGJ_2$ with Keap1 (Kelch-like ECH-associated protein 1) as a model electrophile receptor, we have demonstrated experimentally that the covalent modification of Keap1 by 15d-$PGJ_2$ accumulates over time [33]. Thus a low flux of an electrophilic RLS leads to full activation of a receptor even when the concentration is low (Figure 2B). In part specificity can then be attributed to the generation of a low flux of lipid electrophile, which favours reactions with cysteine residues and steric factors controlling the availability of the nucleophilic amino acid residue. Reversibility of the signal is then controlled by integration of the signalling pathway with the proteasome as will be discussed in a later section.

Thus the key feature of the covalent advantage signalling paradigm is that cysteine modification occurs in a specific manner. As such there are several factors which regulate susceptibility to thiol modification. Although cysteine is present in most proteins, only a small percentage of cysteine residues are susceptible to modification [116] as will be discussed in the next section.

## POST-TRANSLATIONAL MODIFICATION OF PROTEINS BY RLS: PROTEIN DAMAGE COMPARED WITH CELL SIGNALLING

Protein adducts with RLS can occur by reaction with nucleophilic centres such as those shown in Figure 3(A) [117]. The RLS shown in this Figure have been extensively studied and include electrophilic lipid oxidation products such as acrolein and HNE

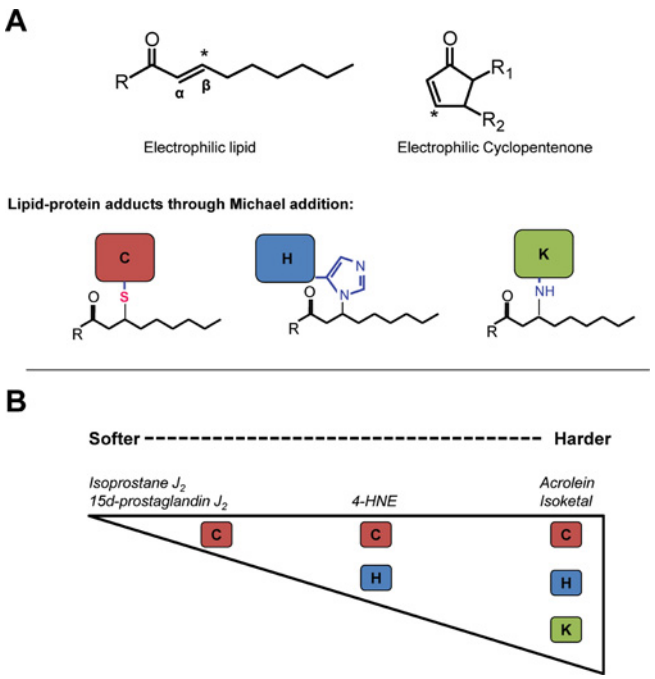


**Figure 3 RLS differ in their reactivity with specific amino acids**

(**A**) Basic structure of an electrophilic lipid and cylopentenone. The $\beta$-carbon of the $\alpha,\beta$-unsaturated carbonyl is electrophilic, making the compound reactive with the nucleophilic amino acids cysteine, lysine and histidine. (**B**) The specificity and reactivity of lipid electrophiles differ depending on relative hardness. Although soft electrophiles such as the cyclopentenones 15d-$PGJ_2$ and isoprostane $J_2$ are largely reactive with cysteine residues, harder electrophiles including the isoketals show less specificity and react with many other nucleophilic targets.

as well as electrophilic cyclopentenone PGs and isoprostanes. Acrolein and HNE possess an aldehyde functional group and an $\alpha,\beta$-unsaturated carbonyl functional group which allow for reaction by both Schiff's base formation and Michael addition respectively. The cyclopentenone structure reacts through Michael addition only. Michael addition occurs by reaction of the electrophile with the nucleophilic amino acids cysteine, lysine and histidine (Figure 3A).

Exposure of electrophiles to biological systems modifies a subset of proteins (termed the electrophile-responsive proteome) which, in concert, orchestrate the biological response. These proteomes are determined by a number of inter-related properties of both the electrophile and protein target. The chemical reactivity of pathologically relevant electrophiles and nucleophiles has been investigated in some depth [118]. As shown in Figure 3(B), an important property determining which nucleophilic amino acids are modified by an electrophile are governed by the hard/soft acid–base principle [118–120]. 'Hard' electrophiles include a number of mutagenic compounds and often react with the 'hard' nucleophilic centres in purine and pyrimidine bases. On the other hand, 'soft' electrophiles include many RLS [119] and react readily with 'soft' nucleophiles such as GSH and protein cysteinyl thiols [119,121]. In comparison with the cyclopentenone lipid electrophiles, $\alpha,\beta$-unsaturated aldehydes including acrolein and isoketals are relatively harder [122], allowing them to adduct to harder nucleophiles including DNA and the amino groups on lysine and lipids. An example of a relatively soft electrophile is 15-$PGJ_2$, which reacts with thiol groups on cysteine, but does not modify other nucleophilic amino acids [123–125]. Reactivity with soft lipid electrophiles occurs mostly through the modification of cysteine residues in the more reactive thiolate anion form [23,126,127]. These modifications are biologically significant

**Table 1 Selected protein targets of RLS**

ANT, adenine nucleotide translocator; NF-$\kappa$B, nuclear factor $\kappa$B.

| Protein | Functional change | Reference |
|---|---|---|
| p50 subunit of NF-$\kappa$B | Inhibition of NF-$\kappa$B DNA binding | [183] |
| H-, N-, K-Ras | Activation of H-, N-, K-Ras | [124,184] |
| Keap1 | Release of Nrf2 | [136] |
| Thioredoxin | Serves as sensor for oxidative stress | [185] |
| Thioredoxin reductase | Disruption of the conformation of the tumour-suppressor protein p53 | [186] |
| c-Jun | Inhibition of AP1 DNA binding | [187] |
| $\beta$-Actin | Filament disruption | [188,189] |
| GSTP1-1 | Inactivation of GSTP1-1 | [30] |
| 26S proteasome | Inhibition of proteasome and impairment of proteasomal assembly | [190] |
| Hsp70 | Release of HSF1 to up-regulate the heat-shock response | [42,43] |
| Hsp90 | Release of HSF1 to up-regulate the heat-shock response | [42,43] |
| $PPAR\gamma$ | Activation of receptor; anti-inflammatory | [123,191] |
| ANT | Mitochondrial membrane permeabilization | [140,192] |
| ATP synthase | Inhibits ATP synthesis | [140,193] |
| Cytochrome *c* oxidase | Inhibited oxidase activity | [194–196] |

since thiol residues are 'redox sensors', which are important in cell signalling. In addition to modification by RLS, signalling can also be initiated by several other thiol-dependent post-translational modifications including S-nitrosylation, S-glutathionylation, formation of sulfenic and sulfinic acids, and disulfide formation [128–133]. Importantly, the local protein environment can influence the $pK_a$ value of candidate thiols influencing their reactivity with electrophilic lipids as well as with other oxidants and will be discussed in more detail below [126–128].

The differences in reactivity of softer and harder electrophiles may explain, in part, the different cellular effects of RLS. For example, cyclopentenones react specifically with thiol groups on cysteine residues, whereas acrolein can react with other amino acids. This is important when considering the ability of RLS to modify proteins thus changing their function and the cellular response. It is now appreciated that site-specific modification of cysteine residues contributes to cell signalling through cysteine rich proteins, such as Keap1, whereas modification of lysine residues is associated with toxicity [21,122,134–137].

The development of lipid- and electrophile-tagging techniques has been crucial in the identification of several key metabolic and signalling proteins which are covalently modified by RLS at reactive cysteine residues [138–140]. Some selected proteins known to be modified by RLS and their cellular effects are given in Table 1. As can be seen, the signalling pathways regulated by electrophilic adduct formation are diverse in both their cellular location and their role in pathophysiology. These data imply that there is specificity in terms of RLS signalling with respect to both the electrophilic lipid and the protein targets. This concept is shown schematically in Figure 3. The amino acid residues on proteins can confer specificity in reaction with lipid electrophiles which, in turn, are important for determining whether the lipid peroxidation products formed elicit a cell signalling response or contribute to damage [37,124,136,141].

## PROTEIN THIOL REACTIVITY AS A REGULATOR OF SIGNALLING BY RLS

The primary mechanism by which redox signalling occurs is through the post-translational modification of critical cysteine

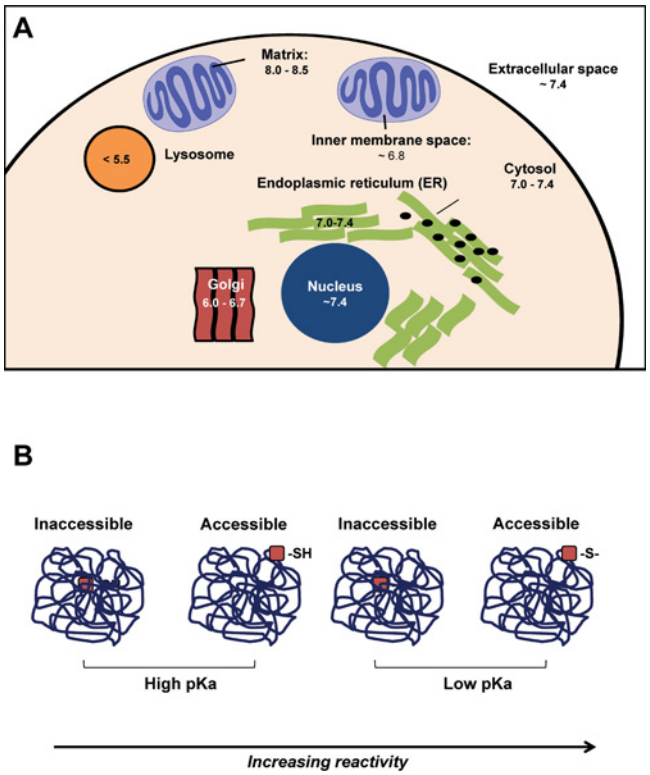


**Figure 4 Factors which determine susceptibility to thiol modification and cellular thiol targets**

Thiol residues have different susceptibilities to being modified by thiol-reactive agents. One important factor is accessibility (within the cell as within a protein) and the p$K_a$ value of the thiol target. (**A**) The pH of the environment is different, depending on the subcellular location. As shown, the average pH of the mitochondrial matrix is 8–8.5, whereas lysosomes are much more acidic, averaging a pH less than 5.5. The pH, along with the thiol p$K_a$ value determines whether a thiol is deprotonated to form thiolate anion. (**B**) The local protein environment is a very important determinant of thiol reactivity. For example, an inaccessible, high p$K_a$ protein thiol would be considered the least prone to modification. However, a low p$K_a$ accessible thiol would be a highly sensitive target.

residues (thiols) in redox-sensitive proteins. This modification can then change the structure and/or function of the modified protein and alter downstream signalling [16]. There are multiple lines of evidence which demonstrate that redox signalling occurs in a regulated and specific manner and does not simply represent non-specific oxidative damage. Conserved cysteine residues occur in almost all classes of proteins and, in many cases, are important for protein function [142–144]. For example, there are 25 cysteine residues in the redox sensor Keap1 which are selectively modified by reactive species [145]. It is for this reason that thiols are poised to mediate diverse redox signalling responses to multiple stimuli.

In Figure 4, we have integrated the factors which regulate susceptibility to thiol modification by RLS. The susceptibility of cysteine residues to modification by a defined RLS is dictated by a combination of factors including the p$K_a$ of the thiol and the local pH of the intracellular compartment (Figure 4A). Interestingly, the range of pH within the cell is likely to have a major impact on thiol reactivity. For example, the high intramitochondrial pH may be one reason mitochondrial protein thiols are particularly susceptible to modification and play a key role in cell signalling [146,147]. Other factors are the accessibility of the thiol within the protein structure, subcellular localization and the reactivity of the thiol-modifying agent. The p$K_a$ of a specific thiol is defined as the pH at which 50 % of that thiol will be deprotonated. Thus a thiol having a p$K_a$ of 7.4 will be 50 % deprotonated at physiological pH. Since deprotonated thiol (thiolate) is much more nucleophilic in nature, lower p$K_a$ thiols, which are more likely to be deprotonated at physiological pH, are favoured in their reaction with lipid electrophiles [148]. However, this factor alone is not sufficient to explain why electrophiles can activate cell signalling pathways independent of GSH.

Localization of thiol residues, either within a protein or within the cell, also seems to be important in dictating their relative susceptibilities to modification; however, these factors are less well characterized. This is shown in Figure 4(B), where the most accessible thiol residue is more likely to be modified than those less accessible. There is evidence demonstrating site-selective modification of cysteine residues within a single protein by two different RLS [124], although characterization of this type of regulation for a larger subset of proteins has not been examined to date. As shown in Figure 4(B), it is the combined properties of a low p$K_a$ thiol and accessibility which are the most consistent feature of proteins activated by electrophilic signalling.

The combination of steric and biochemical factors result in a functional hierarchy for the activation of cellular signalling pathways on exposure of cells to an electrophile. The first pathways to respond are those which are closest to the site of formation or exposure to the electrophile and with the most reactive protein thiol to the specific electrophile in question. The functional consequence of these factors is that the 'first responders to electrophile exposure' are not necessarily the most abundant thiol-containing proteins. For example, actin possesses several reactive thiol groups, but is modified by 15d-PGJ$_2$ at concentrations higher than those required for modification of Keap1, which is a lower-abundance protein than actin [149]. The consequence for cell signalling is that the RLS-dependent signalling pathways are activated sequentially according to the site of formation, specific chemistry, available signalling proteins and characteristics of the specific electrophile.

## GSH AND RELATED ENZYMES AS INSULATORS OF ELECTROPHILE SIGNALLING DOMAINS

In addition to protein thiols, GSH is an abundant low-molecular-mass thiol, with a p$K_a$ value of 8.3, which is present at micromolar levels within the cell. Interestingly, electrophiles such as 15d-PGJ$_2$ activate the Keap1 pathway at concentrations in which reaction with GSH is not detectable [150]. This lack of involvement of the GSH pathway in electrophile signalling probably occurs for two reasons. The first relates to the fact that the direct reaction of GSH with electrophiles, due to its high p$K_a$ value, is slow even though it is present in the cell in the micromolar range. It is often not appreciated that, although this may seem a high concentration, it may, in many cases, be lower than the protein thiol levels. For example, intramitochondrial protein thiol levels have been demonstrated to be 26-fold higher than GSH [151]. A further important factor is that the pH of intracellular compartments, which will modulate the amount of reactive thiols, shows great variability between cellular compartments as shown in Figure 4(A). In a compartment with elevated pH, such as the mitochondrion, electrophilic adduct formation with a reactive protein thiol on a signalling protein will be favoured over reactions with free GSH.

The major route of GSH reaction with electrophiles is catalysed by GSTs. The $K_m$ value for GSTs for electrophiles is typically in the micromolar range which suggests that if the RLS concentration is the nanomolar range, the metabolism of RLS through the GSH–GSTs pathway will be minimal [152]. In addition, the access to GSTs will be restricted by steric factors.

This is important because the generation of a lipid electrophile is likely to occur in a hydrophobic environment inaccessible to water-soluble GSH or GSTs. Evidence is now emerging in which the potential site for an electrophile formation is in close proximity to the nucleophilic redox sensor. For example, a sub-population of Keap1 has been shown to be associated with the mitochondrion and may be a mechanism through which mitochondria regulate the Keap1/Nrf2 (nuclear factor-erythroid 2-related factor) system [153]. If this is the case, what is the role of the GSH–GSTs system in cells? We suggest that the conjugation of reactive electrophiles which are formed in an uncontrolled manner are deleterious to endogenous low-level signalling, and the GSH systems 'insulate' against high levels of electrophile production which may cause cellular damage.

The reactivity of the thiol-modifying agent itself imparts an important selective pressure for the subset of proteins (or subproteome) which will be modified. Similarly, the source of an electrophile often dictates its potential protein targets, as the site of generation may promote modification within a subcellular microdomain. Indeed, it is now becoming clear that the redox characteristics of intracellular compartments are not equivalent and differ widely in their key biochemical characteristics. As alluded to above, mitochondria have emerged as both a key site of RLS-mediated signalling and a target of RLS-dependent damage. Owing to the basic microenvironment in the mitochondrial matrix, mitochondrial protein thiols are more often in the thiolate form and are particularly sensitive to modification [151]. At low levels of electrophile, the interaction with mitochondria may result in adaptive cell signalling. For example, blocking mitochondrial thiols has been shown to attenuate HO-1 (haem oxygenase-1) induction by lipid electrophiles, suggesting that the mitochondrion plays a critical permissive role in this signalling pathway [125]. This raises the possibility that localized ROS production in mitochondria can induce the formation of reactive electrophiles that participate in cell signalling. Exogenous electrophilic lipids have also been shown to localize with the mitochondrion and modify proteins in this subcellular compartment [154]. At high concentrations this can result in the promotion of apoptosis, probably through inducing the permeability transition [140,155]. Some of the strongest evidence for a localized effect of electrophilic signalling in specific domains of the cell comes from a series of experiments with 15d-$PGJ_2$. This electrophile activates the Keap1/Nrf2 system in the cytosol, as discussed below, but also targets mitochondria and induces mitochondrial ROS [154]. If a mitochondrial derivative of 15d-$PGJ_2$ is used, the cytosolic signalling is essentially repressed and the dominant effect becomes mitochondrial dysfunction followed by apoptotic cell death [156]. Other studies have shown that cardiolipin (diphosphatidylglycerol) oxidation in the mitochondria can contribute to the release of cytochrome *c* from the organelle and the initiation of apoptosis [157]. This is one of the most direct examples in which mitochondrial lipid peroxidation has been linked to cell signalling.

## LIPID ELECTROPHILES AND THE ADAPTIVE RESPONSE TO OXIDATIVE STRESS

One important protein target of electrophilic lipids mediating an adaptive response is Keap1, an adaptor protein normally bound to the transcription factor Nrf2. Modification of Keap1 by electrophilic lipids, including 15d-$PGJ_2$ and HNE, results in the release of Nrf2 and translocation of the transcription factor to the nucleus (Figure 5A). In the nucleus, Nrf2 binds to the EpRE (electrophilic-response element) and genes responsible for the antioxidant proteins HO-1 and GCL (glutamate–cysteine ligase) are transcribed. The EpRE is also called the ARE (antioxidant-response element). It is thought that many of the protective effects of dietary electrophiles such as sulforaphane and resveratrol may be indirect and lie in their ability to up-regulate endogenous cellular antioxidants through the EpRE [158,159].

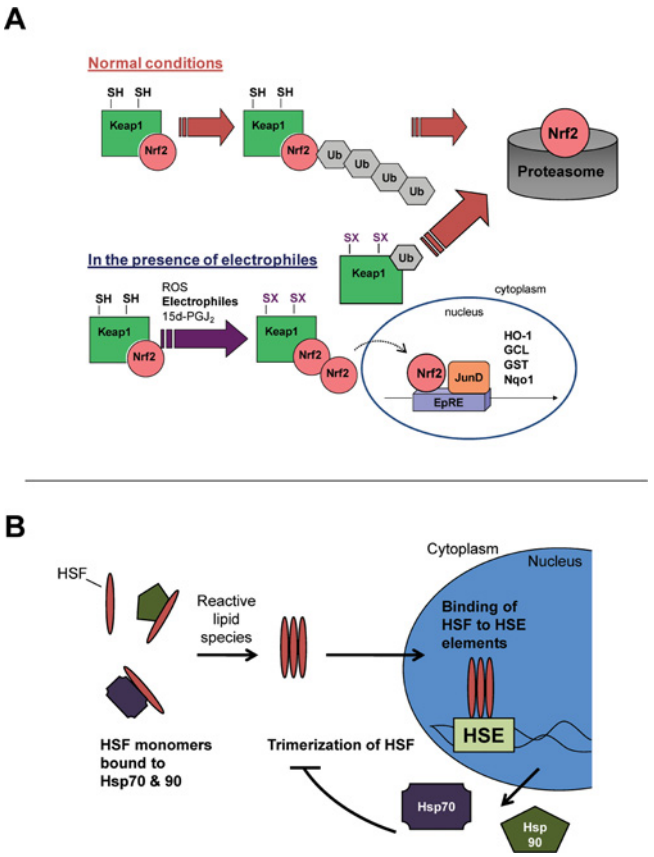


**Figure 5 Modification of protein targets involved in the adaptive response**

(**A**) Modification of Keap1 leads to the release of the transcription factor Nrf2 and its translocation to the nucleus. Upon binding to the EpRE, several genes are up-regulated including *HO-1*, *GCL* (glutamate-cysteine ligase), *GST* and *NQO1* (NADPH-quinone oxidoreductase). (**B**) The heat-shock response is also regulated by RLS. Normally present in monomeric form and bound to Hsp70 or Hsp90, HSF1 trimerizes upon exposure to several electrophilic lipids and up-regulates the expression of Hsps by binding to the heat-shock element (HSE). Ub, ubiquitin.

Electrophilic lipids have also been shown to increase protection through the up-regulation of Hsps (heat-shock proteins), particularly HSF (heat-shock factor) 1 [42,160]. As shown in Figure 5(B), the activation of HSF1 by oxidative stress is mediated by covalent modification of Hsp70 and Hsp90 [42]. These chaperone proteins normally maintain HSF1 in the cytosol [42,161–163]. Once HSF1 translocates to the nucleus, it is responsible for up-regulating Hsp110, Hsp90, Hsp70 and Hsp40, all of which are cytoprotective against toxic stressors [164]. Several oxidative stressors including hydrogen peroxide, ozone and metal toxicity have been shown to increase the heat-shock response [165–167]. It is likely that some of these pro-oxidants mediate their effects through the secondary production of RLS.

One important example of the activation of an adaptive protective response is cardiac ischaemic pre-conditioning. This describes the condition where several short periods (~5 min) of ischaemia and reperfusion protect the heart from longer ischaemic periods [168,169]. Importantly, the oxidants that have been hypothesized to play a role in the ischaemic pre-conditioning

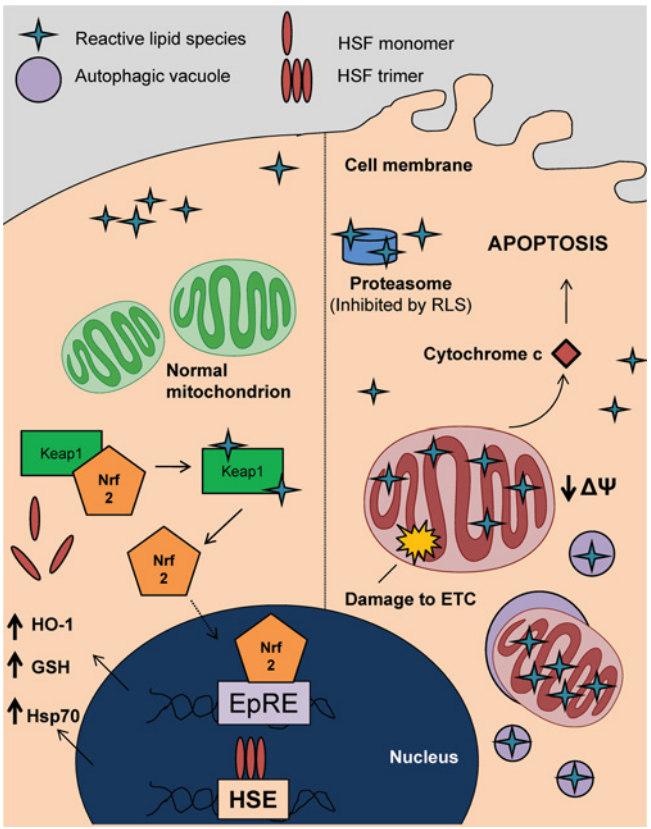


**Figure 6 Subcellular localization of protein targets governs the biological response to RLS**

The diverse biological effects of electrophilic lipids is due, in part, to their subcellular targets. On the left-hand side, cytosolic targets predominate with the modification of proteins such as Keap1 and the Hsp70/Hsp90, leading to an increase in adaptive responses. On the right-hand side, mitochondrial targets control the response, leading to changes in cellular respiration and apoptotic cell death. In addition, RLS may mediate autophagy and/or mitophagy, leading to proteasome-independent degradation of adducted proteins. ETC, electron-transport chain; HSE, heat-shock element.

process may also cause lipid peroxidation [170,171]. It is clear that some of the protection afforded by ischaemic pre-conditioning occurs though increases in EpRE- and HSF-regulated genes (e.g. HO-1 or Hsp70) [172–179], and emerging literature suggests that electrophilic nitroalkanes can induce these protective mechanisms [160]. Understanding whether the modification of protein targets by RLS contribute to pre-conditioning may help direct the development of therapeutic agents in ischaemia/reperfusion injury.

## FUTURE DIRECTIONS

A number of important questions remain to be addressed in our understanding of how RLS modulate cell function. The development of sophisticated mass spectrometry techniques for the identification of lipidomes will allow for a complete characterization of the oxylipidome, an important subset of the lipidome. The development of techniques to monitor specific lipid–protein adducts to define the electrophile-responsive proteome for specific RLS will be integral to investigate further this paradigm of covalent modification as a cell signalling mechanism. Relating the concentration-dependent biological responses to the intrinsic biochemical properties of the electrophile and the proteomes they modify is an important research problem. This has been most effectively demonstrated with the cyclopentenone 15d-$PGJ_2$ [23,149,156,180].

As markers of responses to physiology and pathophysiology, the isoprostanes have been particularly successful as indicators of oxidative stress in human subjects [7]. Perhaps surprisingly, it is now clear that the RLS react with a discrete electrophile-responsive proteome and this is domain-sensitive [23,124,156]. Although cytosolic targets, including the Hsps and Keap1, drive the adaptive response, mitochondrial targets govern apoptosis, ROS production and cellular respiration, and also participate in the adaptive response (Figure 6). For example, targeting an electrophile to the mitochondria suppresses activation of the Keap1/Nrf2 pathway and promotes mitochondria-dependent cell death [156]. This role of the mitochondrion in cell signalling is now becoming more prominent since it is a site for the controlled formation of ROS and plays an important role in the transcriptional regulation of HO-1 [125]. The reason the mitochondria plays such a central role is probably related to the extensive lipid environment within the inner mitochondrial membrane adjacent to redox active transition metals in the electron-transport chain. Is the mitochondrion the source of endogenous low levels of RLS for cell signalling? We have proposed that the mitochondrion can transduce hydrogen peroxide to form a reactive electrophilic lipid oxidation product [154,181]. Understanding the interface between the pathological effects of RLS and their cell signalling is challenging. An aspect we have not discussed in depth in the present review, but which is emerging as an important area, is the role autophagy and mitophagy play in the biological effects of RLS [182].

In summary, the current paradigm is that low levels of RLS can accumulate over time and specifically modify cysteinyl thiols to modulate protective cell signalling pathways. In contrast, high levels of RLS can modify other nucleophilic residues in a less specific manner resulting in protein damage and activation of GST-mediated GSH conjugation. Failure to decrease the RLS levels and subsequently repair or remove the damage is likely to lead to deleterious consequences for the cell and the development of pathology. It will be interesting to see how these concepts develop over the next few years as analytical techniques allow the identification of specific cellular targets for RLS in physiology and pathology.

## FUNDING

The work described in the present review was supported by the National Institutes of Health [grant numbers ES10167, AA13395, DK075867 (to V.D.U.), HL096638 (to A.L.) and HL007918 (to A.R.D.)].

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Received 30 September 2011/12 December 2011; accepted 13 December 2011
Published on the Internet 24 February 2012, doi:10.1042/BJ20111752