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REVIEW ARTICLE

Molecular targets of oxidative stress

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Aerobic life requires organisms to resist the damaging effects of ROS (reactive oxygen species), particularly during stress. Extensive research has established a detailed picture of how cells respond to oxidative stress. Attention is now focusing on identifying the key molecular targets of ROS, which cause killing when resistance is overwhelmed. Experimental criteria used to establish such targets have differing merits. Depending on the nature of the stress, ROS cause loss of essential cellular functions or gain of toxic functions. Essential targets on which life pivots during ROS stress include membrane lipid integrity and activity of ROS-susceptible proteins, including proteins required for faithful translation of mRNA. Protein oxidation also triggers accumulation of toxic protein aggregates or induction of apoptotic cell death. This burgeoning understanding of the principal ROS targets will offer new possibilities for therapy of ROS related diseases.

Key words: actin, iron–sulfur cluster, lipid peroxidation, mistranslation, protein aggregation, protein oxidation.

INTRODUCTION

The environments of most organisms are rarely constant, so some resilience to environmental stress is essential in order for organisms to persist. Chemical stressors such as organic and inorganic pollutants can have different modes of action, but one effect common to many of these, as well as certain natural stressors such as radiation, is an association with oxidative damage in cells [1,2]. ROS (reactive oxygen species) are a necessary evil of aerobic life, being generated continuously during the process of respiration, but with the potential to cause oxidative deterioration of protein, lipid and DNA. ROS generation is elevated by a range of different stress conditions. ROS damage is linked to serious degenerative conditions in humans, including ALS (amyotrophic lateral sclerosis), Alzheimer's disease, Friedreich's ataxia and cancer [3].

Organisms have evolved a number of strategies to counter oxidative stress, and these responses have received considerable research attention in recent years. Typical responses involve the up-regulation of antioxidant proteins, such as the ROS-scavenging peroxidases and superoxide dismutases, or enzymes that reverse oxidative damage, such as methionine sulfoxide dismutases. The oxidative stress responses of a diverse range of organisms are now well characterized [4], efforts which have been facilitated by the advent of microarrays and related technologies.

Despite the above progress, a key question remains: what is the principal cellular target(s) of ROS that accounts for their toxicity? It is well known that ROS cause oxidative modification of each of the major cellular macromolecules – lipid, DNA and protein – and that damage to each can be detected during oxidative stress. What is less well known is which putative target first accumulates damage of a severity that precludes cell recovery, i.e. what target accounts for loss of viability? Obviously there can be more than one such target, the identity of which may depend on the nature of the oxidative stress [4,5], the organism, its physiological status and possibly the viability end-point in question, e.g. loss of cell integrity or reproductive capacity. Nevertheless, identification of such target(s) is now a priority for advancing understanding of the critical events during oxidative stress and so, potentially, for devising ways to combat these events in ROS-related disease.

Whereas ROS-mediated damage to cellular constituents is very widely described in the literature, the present review focuses on the specific minority of these studies where oxidative damage to particular types of target has been linked causally to loss of cell integrity and/or viability. One emphasis is on protein targets, as this area has received the most attention recently. Because of the nature of the question, the evidence discussed is drawn mostly (but not exclusively) from laboratory studies with highly tractable organisms. These models lend themselves to the types of manipulation necessary to establish causal associations. The nature of oxidative stress shares many common key features across prokaryotes, lower and higher eukaryotes [4,6].

ROS-MEDIATED LOSS OF ESSENTIAL FUNCTION

Lipid peroxidation and membrane function

The essential function of biological membranes as selectively impermeable barriers and in cellular transport processes means that severe membrane dysfunction is usually associated with loss of viability. Among the ROS, the protonated form of the superoxide anion and the hydroxy radical commonly initiate the process of autocatalytic lipid peroxidation [7]. Transition metals also catalyse lipid peroxidation. The net result of lipid peroxidation is conversion of unsaturated lipids into polar lipid hydroperoxides, which can cause increased membrane fluidity, efflux of cytosolic solutes and loss of membrane–protein activities. Extensive lipid peroxidation has been correlated with the ultimate disintegration of membrane integrity and cell death, but it has rarely been resolved whether it is a cause or effect of death.

Abbreviations used: ALS, amyotrophic lateral sclerosis; BER, base excision repair; eIF, eukaryotic initiation factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; UFA, unsaturated fatty acid.

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The use of lipid peroxidation inhibitors such as α-tocopherol (vitamin E) has provided evidence for a role of lipid peroxidation in ROS-mediated killing [8,9], although the specificity of such inhibitors remains open to question. Nonetheless, work with α-tocopherol that implicated a specific role for lipid peroxidation in killing by cadmium- (but not copper-) generated intracellular ROS [9] has been borne out by other incisive approaches. Among lipid molecules, PUFAs (polyunsaturated fatty acids) are particularly ROS-sensitive, and these can be readily enriched in membranes of yeast and certain other organisms by culturing in PUFA-supplemented medium. This approach was exploited to show that lipid peroxidation-susceptible (PUFA-rich) cells are sensitized to the toxic effects of cadmium, measured as lipid peroxidation, loss of membrane integrity and loss of viability [10,11].

Damage mediated by ROS to membrane lipids of bacteria is comparatively unlikely, as most bacteria lack PUFAs [12]. The pathogenic bacterium *Borrelia burgdorferi* is unusual in that it incorporates exogenous PUFAs. Consequently, lipid peroxidation (but not DNA oxidation) is readily detected in this organism, although a causal association with lethality was not tested [13]. *Helicobacter pylori* can also incorporate PUFAs and expresses a thiol peroxidase [BCP (bacterioferritin comigratory protein)] which preferentially reduces lipid hydroperoxides. A *bcp* mutant was sensitive to pro-oxidants and exhibited decreased host colonization, implicating membrane lipids as a major ROS target in this pathogen [14].

A genetic tool was developed to probe lipid peroxidation as a toxicity mechanism [15]. This exploits different constructs of PHGPx (phospholipid hydroperoxide glutathione peroxidase) enzymes, the principal enzymatic repair mechanisms available to cells for countering lipid peroxidation. The main yeast PHGPx protein, Gpx3, is a member of the peroxiredoxin family and has diverse antioxidant activities. However, genetic dissection of these activities revealed that it was the lipid peroxidation repair activity, specifically, which determined cadmium resistance (Figure 1). This involved exclusion of the enzyme's non-phospholipid peroxidase and signalling activities (see the legend to Figure 1). A similar conclusion was reached for the toxicity of an exogenous PUFA (linolenic acid). In contrast, membrane lipids were not a primary target of H_2O_2 or Cr(VI) action [15–17].

Mitochondria as the sites of respiration in eukaryotes are expected to be particularly prone to attack from ROS. Cadmium-induced dissipation of the mitochondrial membrane potential in a mammalian cell line was correlated with ROS production. However, the hydroxy-radical scavenger mannitol suppressed detectable ROS but not membrane disruption, indicating that the latter did not result from oxidative damage [18]. Instead, ROS formation and associated lipid peroxidation in such cases could reflect the proposed role for lipid oxidation, especially in mitochondria, as a trigger for signalling pathways responsive to oxidative damage; including those leading to programmed cell death (see the section entitled 'ROS-dependent apoptosis').

Although lipid peroxidation evidently may not contribute directly to killing in all instances of oxidative stress, products of oxidized lipids may themselves initiate further oxidative damage which could prove fatal. Thus reactive products such as malondialdehyde and 4-hydroxynonenal may attack amino acid side chains in proteins [19] and cause fragmentation of DNA [20].

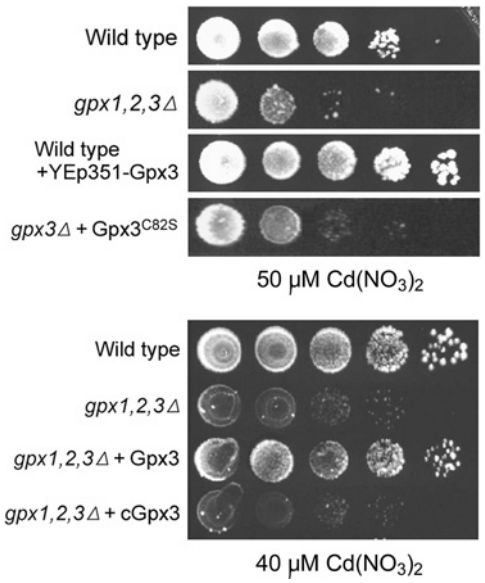

Figure 1 Genetic dissection of lipid peroxidation as a toxicity mechanism

The indicated combination of *Saccharomyces cerevisiae* strains was used to establish that formation of phospholipid hydroperoxides causes cadmium toxicity. The observed cadmium-sensitivity and -resistance respectively, of *gpx3Δ* and *GPX3*-overexpressing (+YEp351-Gpx3) strains established that a Gpx3p-dependent function confers cadmium resistance. Failure to complement the mutant with the $Gpx3^{C82S}$ construct pointed to a peroxidase activity of Gpx3p, whereas complementation with Gpx3p but not cGPx3p pinpointed the protein's phospholipid hydroperoxidase activity. Control growth in the absence of $Cd(NO_3)_2$ was similar for all strains (not shown). Figure taken from Avery, A.M., Willetts, S.A. and Avery, S.M. Genetic dissection of the phospholipid hydroperoxidase activity of yeast Gpx3 reveals its functional importance. J. Biol. Chem. 2004; 279: 46652–46658. © the American Society for Biochemistry and Molecular Biology.

DNA oxidation

It is thought that the hydroxy radical ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) are the principal ROS directly affecting DNA, so different pro-oxidants may damage DNA via generation of these species [21]. DNA may be particularly prone to iron-catalysed oxidation, as Fe binds directly to the phosphodiester backbone where $^{\bullet}OH$ radicals are subsequently generated. Most oxidative DNA damage in *Escherichia coli* appears to be Fe-catalysed [22].

Although DNA damage is commonly detectable during oxidative stress, it is not clear that such damage is a major contributor to ROS-induced killing, especially in eukaryotic cells. (Note that this section concentrates primarily on the situation of toxicity in single cells, but it must not be overlooked that mutation in a single cell within a higher animal can give rise to a dominant cell population which ultimately causes whole-organism toxicity via cancer.) Genome-wide screens for functions important in yeast resistance to five different pro-oxidants did not yield a large number of DNA repair functions, suggesting that DNA is not the primary target [5]. Furthermore, analysis of the mutant phenotypes of yeast and other organisms defective for the major N-glycosylases involved in BER (base excision repair) of oxidized DNA, has yielded mixed effects on resistance to ROS stress [23–25]. For example, yeast *ogg1*Δ mutants defective for repair of a major oxidatively modified base, 7,8-dihydro-8-oxoguanine, have a mutator phenotype but do not exhibit increased lethality when exposed to DNA-damaging agents, including H_2O_2 and Cr(VI) [17,25,26]. Even mutator phenotypes associated with deletion of antioxidant genes may not be ROS driven; the mutability and genetic instability resulting from the absence of Tsa1p, a major yeast peroxiredoxin, has recently been assigned primarily to elevated dNTP levels rather than elevated ROS in this mutant [27]. Elsewhere, simultaneous inactivation of functions involved in BER and in NER (nucleotide

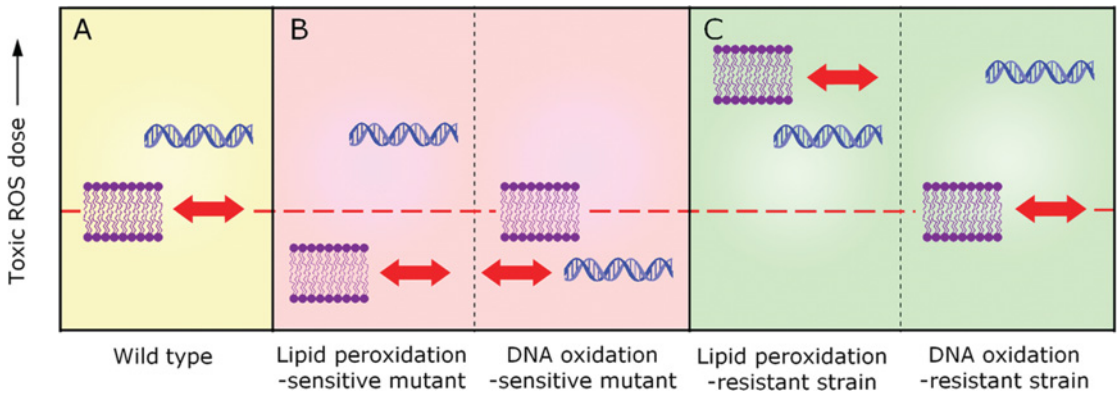

Figure 2 Scheme depicting how different manipulations may yield different indications of the principal ROS targets

The vertical positions of the lipid bilayer and DNA helix in the scheme indicate these macromolecules' relative ROS sensitivities in the different scenarios. In this example, lipid bilayer function is a more ROS-sensitive target than DNA in wild-type cells (**A**). Consequently the ROS dose causing lipid dysfunction (the principal target) is the ROS dose that determines whole cell toxicity (↔) in the wild-type. The broken line depicts the resistance level of wild-type cells. (**B**) Manipulation of wild-type cells from (**A**) to sensitize these to lipid peroxidation will lower the ROS dose that causes toxicity to cells (↔). Such a lowered toxic dose could also be achieved by sensitizing cells to DNA oxidation, despite the fact that DNA is not the principal ROS target of wild-type cells in this example. (**C**) Only manipulations that preserve function of the principal ROS target of wild-type cells will raise cellular ROS resistance above the wild-type level.

excision repair) did yield strains that were sensitive to lethal mutagens, presumably via oxidative DNA lesions [28]. Many other studies have demonstrated that DNA repair-related mutants are ROS sensitive, linking this lethality to oxidative DNA damage including gross chromosomal rearrangements and instability [23,29–37]. Many of these results were obtained with bacteria, especially *E. coli*, suggesting that DNA may be a more important ROS target in organisms where membrane lipid oxidation is less likely (see the preceding section). A diverse range of co-operative functions help to prevent oxidative DNA damage in yeast [38].

The latter examples invoke a more general issue concerning the extrapolation of resistance/sensitivity phenotypes from deletion strains to infer primary targets of stressors that are real to wild-type cells. The concern is that gene deletion can lower the threshold of resistance to an agent by sensitizing a new principal cellular target to that agent; the products of these same genes in wild-type cells may be entirely effective in protecting that purported 'target' (Figure 2). In contrast, only genes that help to preserve function of the normal toxicity target(s) can raise the lower resistance threshold, e.g. when overexpressed [15,17]. Therefore deletion strain phenotypes should be treated with caution in this regard, unless accompanied by evidence at least of the inverse phenotype in an overexpressing strain [39,40]. It may be telling that there are few reports of increased resistance to pro-oxidant lethality resulting from elevated DNA repair activity, including among the bacteria. Indeed, even strains defective for the repair of oxidative DNA damage and which are hypermutable can persist in the wild [41].

In cases where lethal DNA damage is linked to pro-oxidant toxicity, the primary target can in fact be protein(s) required for preserving DNA integrity. Here, elevated DNA damage is a secondary outcome of direct protein inactivation [42–44]. From the genome-wide study of pro-oxidant-sensitive yeast deletion strains, it was concluded that damage to proteins was probably the more important factor in the ROS-induced lethality than DNA damage [5]. A similar conclusion was reached in explaining the different radiation resistances of bacteria [45,46]. Finally, DNA damage itself can result in elevated ROS generation [47], with the potential to attack other targets which may be more pivotal for cell viability.

PROTEIN OXIDATION

Major metabolic pathways, chaperones and actin

The mechanisms of protein oxidation and of its impact on protein structure and function have been reviewed elsewhere [48]. The potential role of oxidative protein damage in ROS-mediated cell killing has been less well covered. The available data establish that certain proteins are more susceptible to oxidative targeting than others. The factors determining such selectivity include the relative content of oxidation-sensitive amino acid residues, the presence of metal-binding sites, protein localization in the cell, molecular conformation and rate of degradation. Mounting evidence also shows that newly synthesized proteins are the most prone to oxidative damage, indicating that complete folding and incorporation into protein complexes confers protection from oxidation-driven degradation [49,50].

Oxidation-sensitive proteins tend to be associated with particular metabolic pathways or functions. These include energy metabolism, mitochondrial proteins, chaperones and members of the ubiquitin–proteasome system (Table 1). Vital pathways of energy metabolism are perturbed by protein oxidation at very early stages of several human degenerative diseases [51]. Oxidized proteins accumulate in patients with supranuclear palsy [52] and age-related disorders [53], among other conditions.

In some cases, the specific amino acid residues whose modification affects the function of oxidatively modified proteins have been identified. For example, defects in protein secretion have been traced to the oxidation of critical methionine residues in component proteins of the SRP (signal recognition particle) complex [54]. Methionine is one of the most oxidation-prone amino acid residues, and nearly all organisms express methionine sulfoxide reductase enzymes to reverse this modification. Oxidized cysteine and tryptophan residues are other useful markers of oxidatively modified proteins.

A number of additional proteins have been shown to be inactivated during oxidative stress and/or by oxidative damage, including Crm1p which is required for nuclear export in HeLa cells [55], alcohol dehydrogenase [56] and a number of Fe-binding proteins [57]. Another abundant protein, the Cu,Zn superoxide dismutase (Sod1p) is commonly reported to be vulnerable to

Table 1 Protein targets of ROS

General functions	Specific proteins*	Consequences of oxidation/other comments	Reference(s)
Energy metabolism, including mitochondrial and FeS proteins	GAPDH	Potential inhibition of glycolysis and growth arrest	[58,64,136]
	Alcohol dehydrogenase (AdhE)	Chaperone defects accentuate ROS-dependent AdhE inactivation	[56,67]
	FeS proteins of amino acid biosynthesis: dihydroxyacid dehydratase, homoaconitase, isopropylmalate isomerase	Causes requirements for exogenous amino acids	[62,63,66]
	Citric acid cycle enzymes: α-oxoglutarate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase (FeS), aconitase (FeS)	Growth defects with particular C sources	[6,48,66,67,109]
Translation	Threonyl-tRNA synthetase	Errors in translation and growth impairment	[84]
	Translation initiation factor eIF4E	Essential target, potentially ROS-mediated	[83]
Protein degradation	Proteasome function	Defects in protein degradation	[137]
Chaperones	Hsp104 (heat-shock protein of 104 kDa)	Sir2 defects accentuate Hsp104 damage and loss of function	[78]
Stress resistance	Sod1	Exacerbation of oxidative stress	[58,59]
Cytoskeleton	Actin	Modification of actin structure and function	[130]
	Cofilin (actin-binding protein)	Triggers apoptosis	[128]
Other	Ffh	Defects in SRP function due to methionine residue oxidation	[54]
	Crm1	Nuclear export defect	[55]
	Serine/threonine protein phosphatases	Linked to apoptosis	[65]

*This list is not intended to be comprehensive, but highlights some of the key proteins known to have ROS-sensitive function.

oxidative damage [58,59]. This is unfortunate for organisms, considering the antioxidant properties of Sod1 and, therefore, the potential for a downward spiral of ROS sensitivity arising from Sod1 inactivation. The function of certain peroxiredoxins is also susceptible to hyperoxidation [60].

Despite the insights described above, few studies have linked protein targeting to oxidative cell killing. Furthermore, few, if any, of the above proteins have been shown to be essential, so it is likely that their oxidative inactivation modifies particular metabolic pathways without necessarily resulting in cell death. Of course, the importance of a metabolic pathway for cell vitality can depend strongly on the environmental situation, so some oxidation-sensitive proteins may be dispensible under some conditions, while required under others. This is illustrated by certain examples of metabolic enzymes, such as dehydratases that require FeS (iron–sulfur) clusters for activity. The extreme oxygen lability of FeS clusters predisposes these enzymes to oxidative inactivation, as well as the metabolic pathways that they occupy [61]. Certain amino acid biosynthetic pathways in bacteria and yeasts rely on FeS enzymes, and their influence on cell vitality during oxidative stress becomes discernible in media that do not provide an alternative exogenous supply of the affected amino acids [62,63].

GAPDH (glyceraldehyde-3-phosphate dehydrogenase) has received particular attention because it is inactivated by mild H_2O_2 stress in eukaryotic cells [58,64]. Interestingly, pro- as well as anti-apoptotic roles have been ascribed to GAPDH, so oxidative modification of GAPDH could help to regulate entry to apoptosis [48]. Similarly, cadmium-induced apoptosis in neuronal cells has been assigned to the inhibition of serine/threonine protein phosphatases 2A and 5 by cadmium-induced ROS, although it was not distinguished whether the mechanism involved direct oxidative damage or ROS-mediated down-regulation of these proteins [65]. Regarding the targeting of metabolic pathways, GAPDH and several enzymes of the citric acid cycle have been identified as the major oxidized protein species during oxidative stress in lower (yeast, bacteria) as well as higher organisms. Oxidatively targeted proteins of the citric acid cycle include α-oxoglutarate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase and aconitase, the latter two being FeS enzymes [6,48,66,67]. Potential oxidative inactivation of respiratory function will of course be most detrimental to those organisms that rely exclusively on respiration for energy generation, whereas organisms such as yeast and certain bacteria can fall back on fermentative pathways.

Several amino acid residues (e.g. arginine, proline, histidine and lysine) form carbonyl products during oxidation. Convenient assays have been developed for identifying carbonylated proteins, and these approaches are used widely for characterizing oxidized protein species. Carbonyl formation is irreversible, unlike thiol oxidation which affects protein function more transiently during signalling and similar processes. The pattern of proteins that become carbonylated during oxidative stress appears to be quite conserved across distantly related organisms. Protein carbonylation has a biochemical consequence, as the modification provides an irreversible marker for damaged proteins to be inactivated by proteasomal degradation. However, there is a limit to the cells' capacities to process carbonylated proteins, particularly as the proteasome itself may be a target for oxidative inactivation [68]. Carbonylated proteins that are not degraded may form potentially toxic aggregated species (see the section entitled 'Formation of toxic protein aggregates'). Thus elevated levels of carbonylated proteins can be linked to loss of cell viability [69].

The identification of a number of citric acid cycle enzymes as oxidation targets (above) was largely achieved with the carbonyl assay. In addition, several carbonylated chaperones have been identified in *E. coli* [67,70] and yeast [71]. This vulnerability to oxidation could be part and parcel of these proteins' protective functions against ROS. Actin is also a commonly reported target of oxidative carbonyl damage [72–74]. It has been suggested that this could merely reflect the high abundance of actin in cells, making it a more easily detectable substrate. Nevertheless, the actin cytoskeleton is required for normal retention of carbonylated and aggregated proteins in mother cells [75,76] and segregation

of catalase to daughter cells [77] during replicative aging of yeast. Therefore oxidative actin damage could perturb the imbalance in oxidative burden between mother and daughter cells. Protein chaperones are also required for this retention of oxidatively damaged proteins by yeast mother cells, an activity that may become undermined by the carbonylation of chaperones themselves during aging [78]. Preservation of chaperone function for this asymmetric inheritance of damaged proteins requires the histone deacetylase, Sir2p [78]. Interestingly, copper stress inhibits histone acetylation by a mechanism that at least partially involves oxidative stress [79]. Specifically, copper inhibits HAT (histone acyltransferase) activity and this action appears to contribute to copper toxicity, as rescue of histone acetylation rescues copper toxicity.

Protein synthesis as a ROS target

In addition to directly damaging proteins post-synthesis, certain pro-oxidants cause defects in protein function by targeting the process of mRNA translation. Decreased translation initiation and protein synthesis occur anyway as part of the response to mild oxidative stress. In yeast, this is achieved partly through phosphorylation of the translation initiation factor eIF2 (eukaryotic initiation factor 2) by the Gcn2 kinase [80,81]. This response is thought to help preclude the potentially deleterious effects of continued mRNA translation under the error-prone conditions of oxidative stress, while allowing time for a reprogramming of the proteins being expressed by the cell after stress is sensed [81]. The strategy appears to work in the case of mild H_2O_2 stress, which was not associated with mistranslation [49]. In contrast, the redox-active metal chromate caused mRNA mistranslation in an oxygen-dependent manner, and this was a primary mechanism of Cr(VI) toxicity. This action was correlated with chromium-induced protein carbonylation and the formation of toxic protein aggregates [17,49]. Chromate is known to compete with sulfate for uptake into cells, and a resultant depletion of the sulfur-containing amino acids cysteine and methionine is a cause of chromium-induced mistranslation [82]. There is currently no evidence that chromium directly targets a component of the translational machinery. However, the essential translation initiation factor eIF4E in human cell lines is a key target for another toxic metal, cadmium, via a mechanism suggested to be ROS-mediated [83]. A recent breakthrough has identified a specific protein target responsible for H_2O_2-induced protein mistranslation in *E. coli* [84]. H_2O_2 was found to oxidize Cys^{182} of threonyl-tRNA synthetase, which is critical for the translation editing function of this protein. Resultant mistranslation was associated with protein misfolding and caused impaired growth of *E. coli*. It will be of great interest to see whether the same mechanism underpins other examples of ROS-mediated mRNA translation.

Current and future approaches to identify protein targets

Some of the above studies have provided excellent, albeit rare, examples of the type of experimental strategy needed to ascertain an essential protein target of a stressor. Under oxidative stress, such a protein should show elevated oxidative damage and decreased function (which cannot be accounted for by decreased expression). Furthermore, knockdown of the gene should produce a sensitive phenotype and, moreover (see the section entitled 'DNA oxidation'), overexpression should confer resistance. Very few studies have sought to establish all of these criteria for putative protein targets of ROS, so many such targets remain to be validated.

Satisfying the above criteria experimentally requires prior knowledge of candidate target proteins of ROS. Where that knowledge is lacking, recent technologies are capitalizing on the abundance of genome sequence data now available to help mine for targets. These techniques have been developed primarily to identify essential protein targets of drugs, but they are equally applicable to identifying ROS targets. The heterozygous yeast deletion strain collection is one such powerful resource as it encompasses essential protein functions. According to the principle of haplo-insufficiency, heterozygosity in a gene that encodes an essential target of a stressor should produce a growth defect in the presence of that stressor. Molecular tagging of the entire yeast mutant collection has allowed such phenotypes to be determined from genome-wide microarray-based screens. Lum et al. [85] identified essential genes (*GIM1* and *RPN10*, with actin- and proteasome-related functions) which produced haplo-insufficiency phenotypes during growth in the presence of menadione. Menadione has a superoxide-based mode of action, like chromate [17]. Accordingly, a subsequent comparable screen of the yeast heterozygotes against chromate yielded an over-representation of actin and proteasomal functions among the annotations of haplo-insufficient genes [49]. In this case, the involvement of the actin cytoskeleton and proteasome in chromium resistance was for reasons other than being essential targets of chromium action [49,86]. Nevertheless, these studies illustrate the power of haplo-insufficiency screens for identifying essential determinants of ROS resistance.

Such strategies for target identification continue to be refined, through the deployment of newer technologies such as deep sequencing [87] and improved approaches for systematic alteration of gene expression level and gene dosage [88–90]. These types of techniques are also being applied to organisms other than yeast [40]. It seems inevitable that these chemical-genetic approaches will markedly accelerate the identification of protein targets of ROS during the coming years.

ROS-MEDIATED GAIN-OF-FUNCTION

An alternative cause of ROS toxicity is where oxidative modification of a cellular target triggers a toxic reaction in the cell. While underpinned by similar redox reactions, this mode of ROS-mediated killing is clearly distinct from inactivation of the essential functions addressed above. Several potential targets may acquire a toxic action during oxidative stress, as discussed below.

Formation of toxic protein aggregates

Carbonylated and other oxidatively damaged proteins are marked for proteasomal degradation. Accordingly, ubiquitin-dependent protein catabolism via the multivesicular body pathway was identified as a key function from screens for genes conferring ROS resistance [91]. However, degradation of oxidized proteins is not completely efficient and damaged proteins which escape degradation can form high molecular mass aggregates which accumulate with age [91–94]. Oxidative stress itself may impair the proteolytic systems responsible for removal of oxidized macromolecules, thus accelerating the accumulation of damaged and aggregating proteins [48]. Autophagic destruction of protein aggregates provides a last line of defence against these toxic species, but evidently is not wholly efficient [95,96].

Protein aggregates can be highly cytotoxic [97]. Furthermore, increased levels of carbonylated aggregates are observed in

patients with age-related disorders such as Parkinson's disease, Alzheimer's disease and cancer. Oxidative aggregation of mutant versions of the Sod1 superoxide dismutase protein can occur in ALS [98–100]. Even wild-type Sod1p is prone to oxidative destabilization and aggregation *in vitro* [59,100], consistent with this protein's susceptibility to carbonylation (see the section entitled 'Protein oxidation'). Specific oxidative modifications that cause protein aggregation have also been identified. Methionine oxidation was reported to contribute to neuronal cell death and protein aggregation induced by a mutant α-synuclein protein associated with Parkinson's disease [101], and to the formation of amyloid fibrils by apolipoprotein A-1 [102].

Oxidative chromate toxicity has also been attributed to protein aggregates formed during chromium-induced mistranslation under aerobic conditions [49]. In that study, aggregated proteins isolated from chromium-treated cells had growth-inhibitory effects. The formation of abnormal proteins is also involved in the toxicity of cadmium, another metal that provokes oxidative stress [103]. Copper provokes aggregation into toxic species of the amyloid-β peptide associated with Alzheimer's disease [104]. In this case, however, ROS generation appeared to be a consequence rather than cause of aggregation. Similarly, antibiotic-induced mistranslation and protein misfolding lie upstream of ROS formation and ROS-dependent killing during antibiotic stress [105]. Evidently, the ROS dependency of aggregation-mediated toxicity is condition specific.

Finally, it should be noted that some forms of protein aggregation may be beneficial. H_2O_2 and certain other stressors provoke the aggregation of the Sup35 translation termination factor in yeast, forming the [*PSI*$^+$] prion [106]. Recent work indicates that certain peroxiredoxins determine the level of ROS stress required to induce this transition [107]. [*PSI*$^+$] formation causes translational read-through of stop codons. This loss of fidelity uncovers genetic variation which promotes phenotypic diversity. Under conditions of stress, such diversity can confer an adaptive advantage [107,108].

Targeting of FeS clusters

As discussed above (see the section entitled 'Protein oxidation'), the extreme ROS lability of FeS clusters makes several of the proteins whose function depends on these co-factors highly ROS-sensitive. Besides loss of FeS protein function, oxidative denaturation of FeS clusters is thought to elicit a gain of toxic function, as labile iron is released from the clusters into the cellular environment. Such a ROS-induced increase in cellular iron availability can accelerate catalysis of the Fenton reaction, provoking additional oxidative damage and killing [66,109]. Antibiotics also stimulate iron release from FeS clusters [34], with Fenton catalysis exacerbating antibiotic action [34,110]. The relative contribution to cell killing made by iron from denatured FeS clusters during oxidative stress is difficult to establish. This is particularly so as simultaneous loss of FeS protein function can itself have phenotypic consequences. Increasing the gene dosage of target FeS protein(s) should provide one way to distinguish these effects: elevated FeS protein expression would counteract loss of function; conversely, the same manipulation would exacerbate the potential for gain of toxic function during ROS stress, by elevating the relative pool of cellular iron that is oxygen-labile.

ROS-dependent apoptosis

Apoptosis (a form of programmed cell death) is inducible by pro-oxidants such as H_2O_2, among other stressors, and is usually activated at stressor doses lower than those leading to necrotic cell killing. Pro-oxidant stress induces the intrinsic (also termed 'mitochondrial') apoptosis pathway, involving the release of pro-apoptotic factors from damaged mitochondria [111,112]. Moreover, a role for ROS in apoptosis is not limited to the ROS derived from environmental pro-oxidants, as increased ROS production resulting from respiratory dysfunction is a signature common to many types of apoptosis. It has not been resolved in every case whether such ROS accumulation is a cause or effect of the apoptotic cell death, but there is good evidence for the former in many apoptotic scenarios [111,113]. Thus antioxidant molecules and enzymes modulate progression of diverse apoptotic pathways, and specific ROS such as H_2O_2 or superoxide have been implicated as crucial mediators of apoptotic cell death [111,114,115]. Several apoptotic signalling pathways are modulated by cellular redox status, primarily via ROS-responsive protein kinases [116,117]. Intracellular glutathione (GSH) is a major buffer of cellular redox status and elevated ROS during apoptosis can deplete mitochondrial GSH, leading to mitochondrial membrane permeabilization and release of cytochrome *c* during the prelude to cell death [118].

ROS-dependent gain of apoptotic function does not appear to rest on a single ROS target. Cells do sustain progressive lipid peroxidation during apoptosis, which could aggravate mitochondrial membrane permeabilization. Lipid peroxidation products such as 4-oxo-2-nonenal have also been shown to trigger apoptosis in a variety of systems [27,119]. Lipids may be the primary target of some forms of ROS-mediated apoptosis. By increasing the proportion of oxidation-sensitive UFAs (unsaturated fatty acids) in mitochondrial lipids, cells were sensitized to Bax-induced death, while lipid peroxidation inhibitors blocked the effect [120]. However, interpretation of such data in the context of lipid peroxidation is complicated by the phenomenon of lipotoxicity, cell death due to lipid imbalance. Both saturated fatty acids and UFAs may provoke apoptotic lipotoxicity, in the latter case via activation of serine/threonine protein phosphatases such as PP2Cα/β, for example [121]. These same proteins in neuronal cells have been identified as mediators of ROS-dependent apoptosis induced by cadmium [65], a metal whose necrotic toxicity is tied closely to the process of lipid peroxidation (see the section entitled 'Lipid peroxidation').

Oxidation of protein targets can be important for propagation of the apoptotic signal. As mentioned above, GAPDH oxidation has been implicated in the regulation of apoptosis in lower and higher eukaryotes. GAPDH expression and aggregation have been reported to increase during apoptosis, whereas treatment of cells with antisense GAPDH blocked apoptosis [122,123]. GAPDH is also a target of nitric oxide, another molecule linked to apoptosis [124,125]. Alteration of GSH redox status via oxidation of glutamine, a precursor for GSH biosynthesis, is also reported to activate apoptosis [126]. In addition, the caspase cysteine proteases that are central to execution of the apoptotic response can be modified by ROS, modifications which have been suggested to regulate apoptosis [127].

A recent study of oxidant-induced apoptosis revealed that the actin-binding protein cofilin is a key oxidation target required for this process [128]. Oxidation of cysteine residues in the protein cause cofilin to lose its affinity for actin and translocate to mitochondria, where the oxidized protein exerts damage. Besides cofilin, specific cysteine residues in the actin polypeptide itself are highly prone to oxidation, acting as potential sensors of oxidative stress [129]. Actin oxidation certainly effects actin structure and function [130]. Studies with yeast have revealed further links between ROS, the actin cytoskeleton and apoptosis. Stabilization or aggregation of F-actin (filamentous actin) through the use of

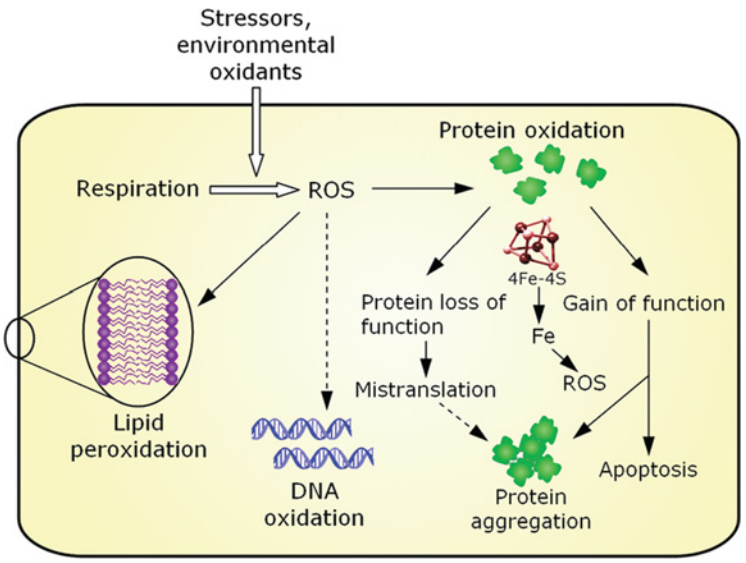

Figure 3 Scheme depicting major routes of ROS action in cells

The most important routes are indicated by solid lines. See the main text for a full description.

drugs or specific mutants, is accompanied by the accumulation of apoptotic markers in yeast and higher cells [131,132]. Such evidence has led to a model of actin-mediated apoptosis, in which actin stabilization triggers an apoptotic signal involving the Ras-cAMP-PKA (protein kinase A) pathway. cAMP signalling is thought to be linked to actin organization by the cyclase-associated protein Svr2/CAP, and this leads to mitochondrial dysfunction, ROS accumulation and apoptosis [131,133,134]. The involvement of ROS at this apparently late stage of the actin-mediated apoptosis pathway does not, in itself, suggest an action of ROS any different to that described above in other apoptotic pathways. However, an additional potential consequence of ROS production is further actin stabilization, caused by the formation of disulfide linkages between the cysteine residues in actin [135]. Such targeting of actin structure by ROS would accelerate apoptotic cell death.

CONCLUDING REMARKS

As consensus is approached on the most appropriate criteria for establishing the identity of a stressor target, and the experimental tools available to do this become increasingly powerful, progress in characterizing these targets is catching up with our understanding of the attendant cellular responses. The studies discussed above show that the molecular targets of pro-oxidant mediated killing are variable (Figure 3), depending on the nature of the stressor and on physiological parameters such as cellular PUFA content, growth conditions (e.g. iron availability, oxygen concentration) and cellular antioxidant status. Lipid peroxidation tends to be more important than DNA oxidation for oxidative cell death in eukaryotes, whereas the reverse appears to be true for many prokaryotes. Protein oxidation is a growing theme in the most recent studies [46,49,84] and identification of ROS-labile essential proteins that determine loss of cell viability remains a crucial goal. Oxidized proteins may also gain a toxic function through the formation of cytotoxic aggregates. Protein oxidation additionally modulates induction of apoptotic pathways, alongside other oxidative mechanisms. Progress in the field of the present review should continue to accelerate, as increasing numbers of studies are embracing the need to establish causality between specific oxidative events and loss of cell function. This typically requires appropriate genetic manipulations and/or genome-wide screens in conjunction with biochemical and toxicological assays in any particular study. Reassuringly, some of the most recent genomic technologies developed to identify drug targets [40,87–90] are eminently transferable to screening for pro-oxidant targets. These advances are critical considering the wide range of degenerative conditions in humans that are linked to oxidative stress. Identification of the principal ROS targets in cells will open important new possibilities for therapy of ROS-related diseases in the future.

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