Oxidative Stress and Cell Function

4

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Abstract

In order to cope with an aerobic lifestyle, organisms must resist the damaging effects of reactive oxygen species (ROS). This problem is exacerbated during environmental stress, which promotes ROS formation in cells. Oxidative damage exerted at the cellular level may ultimately be manifested at the macroscopic or whole-organism level, emphasizing the importance of cellular events during ROS stress. Extensive research has established a detailed picture of how cells respond to oxidative stress. It is only relatively recently that attention has been turning to identifying the key molecular targets of ROS. That is, what is the actual cause of killing when ROS resistance mechanisms are overwhelmed? The relative merits of experimental criteria used to establish such targets are discussed. Essential targets on which life may pivot during ROS stress include

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the activities of ROS-labile proteins, such as proteins required for faithful mRNA translation. Toxic protein aggregates may also arise from protein oxidation events, as can initiation of apoptotic cell death pathways. Nonprotein targets include membrane lipids, as lipid peroxidation is a prelude to loss of membrane integrity and possible cell death. Thus, depending on the nature of the stress, ROS cause loss of essential cellular functions or gain of toxic functions. This evolving appreciation of the principal ROS targets will offer new possibilities for therapy of ROS-related diseases.

Keywords
Actin • Iron–sulfur clusters • Lipid peroxidation • Mistranslation • Oxidative damage • Oxidative stress • Protein aggregation • Protein oxidation • Yeast

Background

Reactive oxygen species (ROS) 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. 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, like radiation, is an association with oxidative damage in cells (Avery 2001; Limon-Pacheco and Gonsebatt 2009). ROS damage is linked to serious degenerative conditions in humans, including amyotrophic lateral sclerosis, Alzheimer's disease, Friedreich's ataxia, and cancer (Roberts et al. 2009; Jomova et al. 2010).

Typical responses by organisms to ROS involve the upregulation of antioxidant proteins, such as the ROS-scavenging peroxidases and superoxide dismutases, or enzymes that reverse oxidative damage, such as methionine sulfoxide reductases. The oxidative stress responses of a diverse range of organisms are now well characterized (Imlay 2008). Despite this progress, one key unanswered question relates to 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 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 cell viability? There can be more than one such target, the identity of which may depend on the nature of the oxidative stress (Imlay 2008; Thorpe et al. 2004), the organism, its physiological status, and possibly the viability end point in question, e.g., loss of cell integrity or capacity to grow. 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, this chapter focuses on the particular studies where oxidative

damage to specific 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 and tend to be single-celled organisms such as bacteria or yeast. The nature of oxidative stress shares many common key features across prokaryotes and lower and higher eukaryotes (Cabiscol et al. 2000; Imlay 2008). Moreover, events at the single cell level ultimately may give rise to tissue and organ damage and clinical consequences of oxidative damage in higher organisms. Therefore, insights to ROS targets obtained at the cellular level feed our understanding of the nature of ROS impact on animal health.

Identification of ROS Targets

A limited number of studies have generated the range of evidence necessary to ascertain the pro-oxidant target(s) accounting for loss of viability. Under oxidative stress, such a target should (1) show elevated oxidative damage and (2) decreased function (which cannot be accounted for by decreased expression). Furthermore, (3) knockdown of the relevant protein or a protein conferring a protective function should produce a sensitive phenotype and, moreover, (4) overexpression should confer resistance.

The latter point invokes 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" (Fig. 4.1). 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 (Avery et al. 2004; Sumner et al. 2005). 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 (Chan et al. 2010b; Ericson et al. 2010). As mentioned earlier, few studies have sought to establish all of the above criteria for putative 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. Tools such as the heterozygous yeast deletion strain collection provide powerful resources, in this case exploiting the principle of haploinsufficiency to elucidate essential targets of stressors (Holland et al. 2007; Lum et al. 2004). Strategies for target identification continue to be refined, through the deployment of newer technologies like deep sequencing (Smith et al. 2009) and improved approaches for systematic alteration of gene expression level and gene

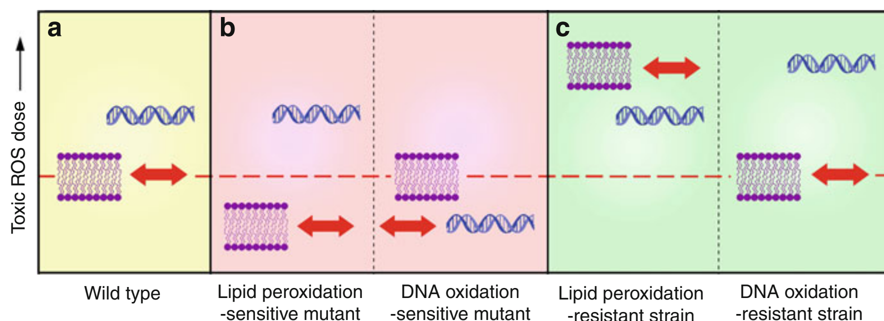

Fig. 4.1 Different types of genetic manipulation may yield different indications of the principal ROS targets. The vertical positions of the lipid bilayer and DNA helix in the scheme indicate these macromolecules' relative ROS sensitivities in the different scenarios. In this example, lipid bilayer function 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 dashed 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 (Reproduced from Avery, 2011 with permission)

dosage (Ericson et al. 2010; Hoon et al. 2008; Jones et al. 2008; Yan et al. 2009). It seems inevitable that these chemical–genetic approaches will accelerate markedly the identification of ROS targets during the coming years.

Protein Oxidation

Oxidation-Sensitive Proteins

The potential role of oxidative protein damage in ROS-mediated cell killing has been less well covered than the mechanisms of protein oxidation and of its impact on protein structure and function (Cecarini et al. 2007). Certain proteins are more susceptible to oxidative targeting than others, according to their relative content of oxidation-sensitive amino acid residues and metal-binding sites, molecular conformation and rate of degradation, and protein localization in the cell. In some cases, the specific amino acid residues whose modification affects 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 signal recognition particle (SRP) complex (Ezraty et al. 2004). Methionine is one of the most oxidation-prone amino acid residues, and nearly all organisms express methionine sulfoxide reductase enzymes to reverse that modification. Oxidized Cys and Trp residues are other useful markers of oxidatively modified proteins.

A number of amino acid residues (e.g., Arg, Pro, His, Lys) form carbonyl products during oxidation. Convenient assays have been developed for identifying carbonylated proteins. Such techniques are used widely for characterizing oxidized protein species. Carbonyl formation is irreversible, unlike thiol oxidation which impacts protein function more transiently during signaling 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 (Wu et al. 2009). Carbonylated proteins that are not degraded may form potentially toxic aggregated species (see the section "Toxic Protein Aggregates Formed During Oxidative Stress"). Thus, elevated levels of carbonylated proteins can be linked to loss of cell viability (Desnues et al. 2003).

Several further proteins are known to be inactivated during oxidative stress and/or by oxidative damage, including Crm1p which is required for nuclear export in HeLa cells (Crampton et al. 2009), alcohol dehydrogenase (Matuszewska et al. 2008), and a number of Fe-binding proteins (Drake et al. 2002). Another abundant protein, the Cu,Zn superoxide dismutase (Sod1p) is commonly reported to be vulnerable to oxidative damage (Costa et al. 2002; Yin et al. 2010). 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 hyper-oxidation (Woo et al. 2010).

Essential Protein Functions Targeted by ROS

Metabolic Enzymes

Proteins involved in metabolism are commonly reported to be oxidation sensitive. This includes proteins involved in energy metabolism, mitochondrial proteins, chaperones, and members of the ubiquitin–proteasome system (Table 4.1). Vital pathways of energy metabolism are perturbed by protein oxidation at very early stages of several human degenerative diseases (Martinez et al. 2010). Oxidized proteins accumulate in patients with supranuclear palsy (Martinez et al. 2008) and age-related disorders (Levine 2002), among other conditions. Despite such insights, few studies have linked protein targeting to oxidative cell killing. Furthermore, few if any of the proteins discussed above have been shown to be essential, so it is likely that their oxidative inactivation modifies particular metabolic pathways without necessarily resulting in cell death. Nonetheless, the importance of a metabolic pathway for cell vitality can of course depend strongly on environmental situation. Some oxidation-sensitive proteins may be dispensible under some conditions while necessary under others. This is illustrated by examples of metabolic enzymes such as dehydratases, which require iron–sulfur clusters for activity. The extreme

Table 4.1 Protein targets of ROS

General functions	Specific proteins[a]	Consequences of oxidation/other comments	References
Energy metabolism, including mitochondrial and FeS proteins	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Potential inhibition of glycolysis and growth arrest	(Costa et al. 2002; Grant et al. 1999; Weber et al. 2004)
	Alcohol dehydrogenase (AdhE)	Chaperone defects accentuate ROS-dependent AdhE inactivation	(Matuszewska et al. 2008; Tamarit et al. 1998)
	Ribulose-5-phosphate 3-epimerase (Rpe)	Impacts the pentose phosphate pathway, due to oxidation of Fe in Rpe	(Sobota and Imlay 2011)
	FeS proteins of amino acid biosynthesis: dihydroxyacid dehydratase, homoaconitase, isopropylmalate isomerase	Causes requirements for exogenous amino acids	(Carlioz and Touati 1986; Jang and Imlay 2007; Wallace et al. 2004)
	Citric acid cycle enzymes: α-ketoglutarate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase (FeS), aconitase (FeS)	Growth defects with particular C sources	(Cabiscol et al. 2000; Cecarini et al. 2007; Gardner and Fridovich 1991; Jang and Imlay 2007; Tamarit et al. 1998)
Translation	Threonyl-tRNA synthetase	Errors in translation and growth impairment	(Ling and Soll 2010)
	Translation initiation factor eIF4E	Essential target, potentially ROS mediated	(Othumpangat et al. 2005)
	Rli1/ABCE1 (FeS)	Essential, conserved target of ROS action	(Alhebshi et al. 2012)
Protein degradation	Proteasome function	Defects in protein degradation	(Zhang et al. 2008)
Chaperones	Hsp104	Sir2 defects accentuate Hsp104 damage and loss of function	(Erjavec et al. 2007)
Stress resistance	Sod1	Exacerbation of oxidative stress	(Costa et al. 2002; Yin et al. 2010)
Cytoskeleton	Actin	Modification of actin structure and function	(Lassing et al. 2007)
	Cofilin (actin-binding protein)	Triggers apoptosis	(Klamt et al. 2009)

(*continued*)

Table 4.1 (continued)

General functions	Specific proteins[a]	Consequences of oxidation/other comments	References
Other	Ffh	Defects in SRP function due to Met-residue oxidation	(Ezraty et al. 2004)
	Crm1	Nuclear export defect	(Crampton et al. 2009)
	Serine/threonine protein phosphatases	Linked to apoptosis	(Chen et al. 2008)

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

oxygen lability of FeS clusters predisposes these enzymes to oxidative inactivation as well as the metabolic pathways that they occupy (Imlay 2006). Certain amino acid biosynthetic pathways in bacteria and yeasts require FeS enzymes, and their influence on cell vitality during oxidative stress becomes detectable in media that do not provide an alternative, exogenous supply of the amino acids that are susceptible (Carlioz and Touati 1986; Wallace et al. 2004).

A metabolic enzyme that has received particular attention because it is inactivated by mild H_2O_2 stress in eukaryotic cells is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Costa et al. 2002; Grant et al. 1999). Interestingly, oxidative modification of GAPDH could help to regulate entry to apoptosis; pro- as well as anti-apoptotic roles have been ascribed to the enzyme (Cecarini et al. 2007). Similarly, cadmium-induced apoptosis in neuronal cells has been assigned to inhibition of serine/threonine protein phosphatases 2A and 5 by Cd-induced ROS, although it was not distinguished whether the mechanism involved direct oxidative damage or ROS-mediated downregulation of these proteins (Chen et al. 2008). 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-ketoglutarate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase, and aconitase, the latter two being FeS enzymes (Cabiscol et al. 2000; Cecarini et al. 2007; Jang and Imlay 2007; Tamarit et al. 1998). If respiration is defective, organisms such as yeast and certain bacteria can fall back on fermentative pathways. Potential oxidative inactivation of respiratory function will of course be most detrimental to those organisms that rely exclusively on respiration for energy generation.

Chaperone Function and the Actin Cytoskeleton

It is the assay of protein carbonyl groups that has led to the identification of several of the citric acid cycle enzymes discussed above as oxidation targets. In addition, several carbonylated chaperones have been identified in *E. coli* (Dukan and Nystrom 1999; Tamarit et al. 1998) and yeast (Irazusta et al. 2008).

This vulnerability to oxidation could be part and parcel of these proteins' protective functions against ROS. Protein chaperones are also required for the retention of oxidatively damaged proteins by yeast mother cells, an activity that may become undermined by the carbonylation of chaperones themselves during aging (Erjavec et al. 2007). Preservation of chaperone function for this asymmetric inheritance of damaged proteins requires the histone deacetylase, Sir2p (Erjavec et al. 2007). Interestingly, copper stress inhibits histone acetylation by a mechanism that at least partially involves oxidative stress (Lin et al. 2005). Specifically, copper inhibits histone acyltransferase (HAT) activity and this action appears to contribute to Cu toxicity, as rescue of histone acetylation rescues Cu toxicity.

Actin is also a commonly reported target of oxidative carbonyl damage (Butterfield et al. 2006; Dalle-Donne et al. 2001; Shanmuganathan et al. 2004). It has been suggested that this could merely reflect the high abundance of actin in cells, making it a more easily detectable substrate. Nevertheless, like chaperone function, the actin cytoskeleton is also required for normal retention of carbonylated and aggregated proteins in mother cells (Aguilaniu et al. 2003; Liu et al. 2010) and segregation of catalase to daughter cells (Erjavec and Nystrom 2007) during replicative aging of yeast. Therefore, oxidative actin damage could perturb the imbalance in oxidative burden between mother and daughter cells.

Protein Synthesis

Mounting evidence 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 (Holland et al. 2007; Medicherla and Goldberg 2008). 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 by the Gcn2 kinase (Mascarenhas et al. 2008; Shenton et al. 2006). 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 (Shenton et al. 2006). The strategy appears to work in the case of mild H_2O_2 stress, which was not associated with mistranslation (Holland et al. 2007). 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 (Fig. 4.2). This action was correlated with Cr-induced protein carbonylation and the formation of toxic protein aggregates (Holland et al. 2007; Sumner et al. 2005). Chromate is known to compete with sulfate for uptake into cells, and a resultant depletion of the S-containing amino acids cysteine and methionine is a cause of Cr-induced mistranslation (Holland et al. 2010). There is currently no evidence that Cr 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 (Othumpangat et al. 2005).

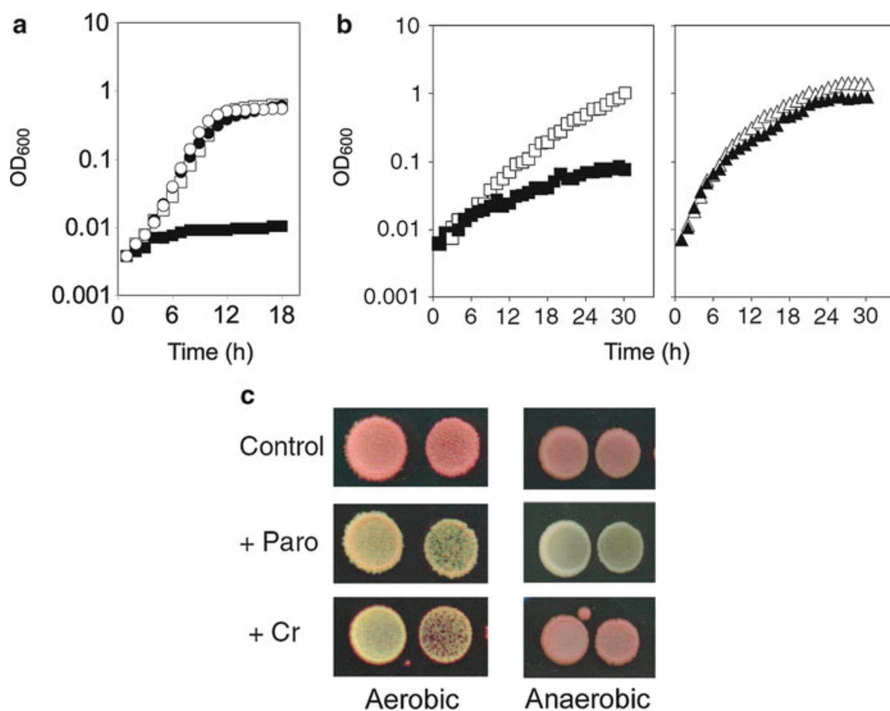

Fig. 4.2 Oxygen-dependent mRNA mistranslation causes Cr toxicity. (**a**, **b**) mRNA mistranslation causes Cr toxicity. (**a**) Synergistic action of Cr with the ribosome-targeting drug paromomycin: growth of *S. cerevisiae* in standard medium (○), or with 0.1 mM Cr (●), or 100 μg ml^{-1} paromomycin (□), or 0.1 mM Cr + 100 μg ml^{-1} paromomycin (■). (**b**) Dependence of Cr resistance on translational fidelity: growth of *S. cerevisiae* L1583 (error-prone translation) (□, ■) or L1597 (high translational fidelity) (Δ, ▲) in the absence (open symbols) or presence (closed symbols) of 0.1 mM CrO_3. (**c**) Oxygen is required for Cr-induced mistranslation. *S. cerevisiae* L1494 (*ade1-14*) cells were spotted (two dilutions) on to agar supplemented or not with 150 μg ml^{-1} paromomycin ("Paro") or 0.15 mM CrO_3. Read through of the *ade1-14* UGA codon suppresses the red pigmentation associated with this allele, yielding pale colonies to indicate mistranslation. Plates were incubated as indicated (Adapted from Holland et al. 2007)

A specific protein target responsible for H_2O_2-induced protein mistranslation has been identified in *E. coli* (Ling and Soll 2010). The Cys182 residue of threonyl-tRNA synthetase was oxidized by H_2O_2, this residue being essential for the protein's translation editing function. Resultant mistranslation was associated with protein misfolding and caused impaired growth. Further research should reveal whether other examples of ROS-mediated mRNA translation are caused by the same mechanism. Recently, the essential FeS protein Rli1 was found to be a key target accounting for ROS inhibition of yeast growth (Alhebshi et al. 2012). Rli1p has roles in translation initiation and ribosome recycling, as well as maintaining translation fidelity. It is one of the most conserved proteins known in biology.

Toxic Protein Aggregates Formed During Oxidative Stress

Besides inactivation of the essential functions addressed above, an alternative cause of ROS toxicity is where oxidative modification of a cellular target triggers a toxic reaction in the cell. These two general modes of ROS action are clearly distinct, albeit underpinned by similar redox reactions. Affected targets that may acquire a toxic action during oxidative stress include carbonylated and other oxidatively damaged proteins. These are usually 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 (Doostzadeh et al. 2007). However, degradation of oxidized proteins is not completely efficient, and damaged proteins which escape degradation can form high molecular weight aggregates which accumulate with age (Doostzadeh et al. 2007; Dunlop et al. 2009; Grune et al. 1997; Seifert et al. 2010). Autophagic destruction of protein aggregates provides a last line of defense against these toxic species, but also evidently is not wholly efficient (Madeo et al. 2009; Pan et al. 2008). Oxidative stress itself may impair the proteolytic systems responsible for removal of oxidized macromolecules, thus accelerating the accumulation of damaged and aggregating proteins (Cecarini et al. 2007).

Increased levels of carbonylated aggregates are observed in patients with age-related disorders such as Parkinson’s and Alzheimer’s diseases and cancer. Oxidative aggregation of mutant versions of the Sod1 superoxide dismutase protein can occur in amyotrophic lateral sclerosis (ALS) (Banci et al. 2008; Furukawa and O’Halloran 2005; Rakhit et al. 2004). Even wild-type Sod1p is prone to oxidative destabilization and aggregation in vitro (Rakhit et al. 2004; Yin et al. 2010), consistent with this protein’s susceptibility to carbonylation (see section “Oxidation-Sensitive Proteins”). The clinical evidence ties in with the fact that protein aggregates can be highly cytotoxic (Campioni et al. 2010). 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 (Liu et al. 2008) and to the formation of amyloid fibrils by apolipoprotein A-1 (Wong et al. 2010).

Oxidative chromate toxicity has also been attributed to protein aggregates formed during Cr-induced mistranslation under aerobic conditions, as mentioned above. Aggregated proteins isolated from Cr-treated cells had growth-inhibitory effects (Holland et al. 2007). The formation of abnormal proteins is also involved in the toxicity of cadmium, another metal that provokes oxidative stress (Jungmann et al. 1993). Copper provokes aggregation into toxic species of the amyloid-β peptide associated with Alzheimer’s disease (Smith et al. 2007). 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 (Kohanski et al. 2008). It is apparent that there is a condition specificity to the ROS dependency of aggregation-mediated toxicity.

Some forms of protein aggregation may in fact be beneficial. Hydrogen peroxide and certain other stressors provoke the aggregation of the Sup35 translation termination factor in yeast, forming the [PSI^+] prion (Tyedmers et al. 2008). Certain peroxiredoxins determine the level of ROS stress required to induce this transition (Sideri et al. 2010). [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 (Sideri et al. 2010; True et al. 2004).

Apoptosis During Oxidative Stress

Hydrogen peroxide, among other pro-oxidant stressors, can induce apoptosis, a form of programmed cell death. Apoptotic cell death 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 release of pro-apoptotic factors from damaged mitochondria (Circu and Aw 2010; Mates et al. 2008). 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 hallmark 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 (Circu and Aw 2010; Perrone et al. 2008). 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 (Carmona-Gutierrez et al. 2010; Circu and Aw 2010; Madeo et al. 1999). Several apoptotic signaling pathways are modulated by cellular redox status, primarily via ROS-responsive protein kinases (Chan et al. 2010a; Noguchi et al. 2005). 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 (Franco and Cidlowski 2009).

Induction of apoptosis during oxidative stress does not appear to rest on a single ROS target. Protein targets can be important for propagation of the apoptotic signal. As mentioned earlier, 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, while treatment of cells with antisense GAPDH blocked apoptosis (Nakajima et al. 2009; Sirover 1997). GAPDH is also a target of nitric oxide, another molecule linked to apoptosis (Almeida et al. 2007; Ortiz-Ortiz et al. 2010). Alteration of GSH redox status via oxidation of glutamine, a precursor for GSH biosynthesis, is also reported to activate apoptosis (Obrador et al. 2001). Additional modifications that have been suggested to regulate apoptosis include those of the caspase cysteine proteases that are central to execution of the apoptotic response and which can be modified by ROS (Marnett et al. 2003).

The actin-binding protein cofilin has also been revealed to be a key oxidation target required for oxidant-induced apoptosis (Klamt et al. 2009). Oxidation of Cys residues in the protein causes 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 prone to oxidation, acting as potential sensors of oxidative stress (Leadsham et al. 2010). Actin oxidation certainly impacts actin structure and function (Lassing et al. 2007). Studies with yeast have revealed further links between ROS, the actin cytoskeleton, and apoptosis. Stabilization or aggregation of F-actin through the use of drugs or specific mutants is accompanied by the accumulation of apoptotic markers in yeast and higher cells (Gourlay et al. 2004; Posey and Bierer 1999). Such evidence has led to a model of actin-mediated apoptosis, in which actin stabilization triggers an apoptotic signal involving the Ras–cAMP–PKA pathway. cAMP signaling 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 (Gourlay and Ayscough 2005, 2006; Gourlay et al. 2004; Leadsham and Gourlay 2010). The involvement of ROS at this apparently late stage of 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 Cys residues in actin (Franklin-Tong and Gourlay 2008). Such targeting of actin structure by ROS would accelerate apoptotic cell death.

Cells 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 (Cerbone et al. 2007; Tang et al. 2009). Lipids may be the primary target of some forms of ROS-mediated apoptosis. By increasing the proportion of oxidation-sensitive unsaturated fatty acids (UFAs) in mitochondrial lipids, cells were sensitized to Bax-induced death, while lipid peroxidation inhibitors blocked the effect (Priault et al. 2002). However, interpretation of such data in the context of lipid peroxidation is complicated by lipotoxicity: cell death due to lipid imbalance. Both saturated and unsaturated fatty acids may provoke apoptotic lipotoxicity, in the latter case via activation of serine/threonine protein phosphatases such as PP2Cα/β (Schwarz et al. 2006). These same proteins in neuronal cells have been identified as mediators of ROS-dependent apoptosis induced by cadmium (Chen et al. 2008), a metal whose necrotic toxicity is tied closely to the process of lipid peroxidation (see section "Lipid Peroxidation").

ROS Stress and Iron Release from FeS Clusters

Proteins whose function depends on iron–sulfur (FeS) clusters can be highly ROS sensitive. Loss of FeS protein function was discussed earlier. In addition, oxidative denaturation of FeS clusters can elicit a gain of toxic function, as labile Fe is

released from the clusters into the cellular environment. Such an ROS-induced increase in cellular Fe availability can accelerate catalysis of the Fenton reaction, provoking additional oxidative damage and killing (Gardner and Fridovich 1991; Jang and Imlay 2007; Kell 2010; Tavares et al. 2011). Antibiotics also stimulate Fe release from FeS clusters (Kohanski et al. 2007), with Fenton catalysis exacerbating antibiotic action (Kohanski et al. 2007; Yeom et al. 2010). The relative contribution to cell killing made by Fe 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.

Lipid Peroxidation

The protonated form of the superoxide anion and the hydroxyl radical commonly initiate the process of autocatalytic lipid peroxidation (Halliwell and Gutteridge 1999). Transition metals also catalyze lipid peroxidation. The net result of lipid peroxidation is conversion of unsaturated lipids to 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. However, it has rarely been resolved whether lipid peroxidation is a cause or effect of death. 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 (Bansal and Bilaspuri 2009; Mattie and Freedman 2001), although the specificity of such inhibitors can be questioned. Nonetheless, work with α-tocopherol that implicated a specific role for lipid peroxidation in killing by cadmium- (but not copper-) generated intracellular ROS (Mattie and Freedman 2001) has been borne out by other incisive approaches.

Among the species of lipid molecules, polyunsaturated fatty acids (PUFAs) are particularly ROS sensitive. PUFAs 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 (Howlett and Avery 1997a, b). Damage mediated by ROS to membrane lipids of bacteria is comparatively unlikely, as most bacteria lack PUFAs (Imlay 2003). 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 (Boylan et al. 2008). *Helicobacter pylori* can also incorporate PUFAs and expresses a thiol peroxidase (BCP) which preferentially reduces lipid hydroperoxides. A *bcp* mutant was sensitive to pro-oxidants and exhibited decreased host colonization, implicating membrane lipids as major ROS targets in thiol peroxidase-defective cells of this pathogen (Wang et al. 2005).

The subcellular sites of respiratory activity in eukaryotes, the mitochondria, 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 hydroxyl-radical scavenger mannitol suppressed detectable ROS but not membrane disruption, indicating that the latter did not result from oxidative damage (Bolduc et al. 2004). 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 signaling pathways responsive to oxidative damage, including those leading to programmed cell death (see section "Apoptosis During Oxidative Stress").

While 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 (Requena et al. 2003) and cause fragmentation of DNA (Wang et al. 2006).

To address the problem of probing lipid peroxidation as a toxicity mechanism, a genetic tool was developed (Avery et al. 2004). This exploits different constructs of phospholipid hydroperoxide glutathione peroxidase (PHGPx) 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 Cd resistance. This involved exclusion of the enzyme's non-phospholipid peroxidase and signaling activities. 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 (Avery et al. 2004; Delaunay et al. 2002; Sumner et al. 2005).

DNA Oxidation

While DNA damage is commonly detectable during oxidative stress, it is not necessarily 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 single cells within a higher animal can give rise to reduced fertility (Aitken and Curry 2011) or a dominant cell population which ultimately causes whole-organism toxicity via cancer.) The hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($^{1}O_2$) are considered to be among the principal ROS effecting DNA damage directly, so different pro-oxidants may damage DNA via generation of these species (Dawes 1999). DNA may be particularly prone to iron-catalyzed 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 catalyzed (Macomber et al. 2007).

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 (Thorpe et al. 2004). Furthermore, analysis of the mutant phenotypes of yeast and other organisms defective for the major *N*-glycosylases involved in base excision repair (BER) of oxidized DNA has yielded mixed effects on resistance to ROS stress (Chan et al. 2009; Melo et al. 2004; Thomas et al. 1997). 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) (Kozmin et al. 2005; Sumner et al. 2005; Thomas et al. 1997). 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 been assigned primarily to elevated dNTP levels rather than elevated ROS in this mutant (Tang et al. 2009). Elsewhere, simultaneous inactivation of functions involved in BER and in nucleotide excision repair (NER) did yield strains that were sensitive to lethal mutagens, presumably via oxidative DNA lesions (Gellon et al. 2001). 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 (Ananthaswamy and Eisenstark 1977; Chan et al. 2009; Degtyareva et al. 2008; Demple and DeMott 2002; Imlay and Linn 1988; Jiang et al. 1997; Kohanski et al. 2007; Leroy et al. 2001; Park et al. 2005; Swartzlander et al. 2010; Liu et al. 2011). 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 preceding section). A diverse range of cooperative functions help to prevent oxidative DNA damage in yeast (O'Rourke et al. 2002).

Regarding the earlier discussion under "Identification of ROS Targets," 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 (Guelfo et al. 2010).

Where certain experiments have indicated that DNA damage is linked to pro-oxidant-mediated cell killing, the primary target can in fact be a protein(s) required for preserving DNA integrity. Here, elevated DNA damage is a secondary outcome of direct protein inactivation (Jin et al. 2003; Serero et al. 2008; Youn et al. 2005). 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 (Thorpe et al. 2004). A similar conclusion was reached in explaining the different radiation resistances of bacteria (Daly 2009; Krisko and Radman 2010). Finally, DNA damage itself can result in elevated ROS generation (Salmon et al. 2004), with the potential to attack other targets which may be more pivotal for cell viability.

Conclusions

A wide range of degenerative conditions in humans 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. The studies discussed above highlight that the molecular targets of pro-oxidant-mediated killing are varied (Fig. 4.3) and condition dependent, reflecting the nature of the stressor and physiological parameters such as cellular PUFA content, cellular antioxidant status, and growth conditions (e.g., Fe availability, oxygen concentration). Lipid peroxidation tends to be more important than DNA oxidation for oxidative cell death in eukaryotes, whereas the reverse appears to be true for most prokaryotes. Protein oxidation is a growing theme in the most recent studies (Holland et al. 2007; Krisko and Radman 2010; Ling and Soll 2010), and identification of ROS-labile essential proteins that determine loss of cell viability remains a crucial goal (Alhebshi et al. 2012). 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 this 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. As consensus

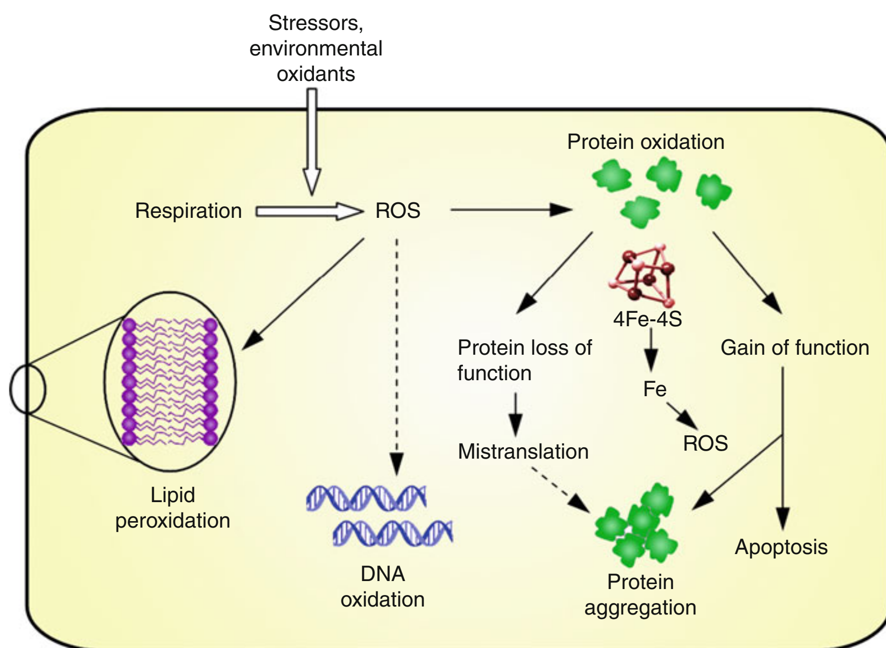

Fig. 4.3 The major routes of ROS action in cells. The most important routes are indicated by solid lines. See the main text for full accounts (Reproduced from Avery 2011 with permission)

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.

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