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Review

Responses of Eukaryotic Cells to Oxidative Stress

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Oxidative stress is implicated in a number of diseases, in ageing of organisms, and in damage to plants that have been exposed to freezing and thawing or water stress. From the perspective of yeast as a model eukaryotic system, this article reviews the systems that are involved in the cellular responses to exposure to reactive oxygen species (ROS) generated during aerobic growth of the organism. The discussion includes the defense systems involved, the ability of cells to adapt to ROS treatment, cell-division cycle delay and the systems regulating gene expression that are activated by oxidative stress.

Key words: *reactive oxygen,* Saccharomyces cerevisiae, Schizosaccharomyces pombe.

The appearance of oxygen in the atmosphere some 3 billion years ago made possible evolution of the efficient energy generation process of respiration. However, attendant with this came the generation of ROS. These reactive oxygen species can damage most cellular constituents and ROS damage has been implicated in many diseases as well as ageing. As a consequence organisms have evolved a broad range of responses to detoxify ROS, to reduce the rate of their production, or to repair the damage caused by them.[1,2] Oxidative stress is a result of an imbalance that occurs when the cellular antioxidant defenses are unable to cope adequately with the ROS present. It arises endogenously from side reactions of normal aerobic metabolism; one of the main primary species being the generation of the superoxide free-radical from electron leakage from the respiratory chain.[3] Other sources of ROS include environmental insults such as exposure to ionizing radiation, or freezing and thawing of tissues[4] or from the re-exposure to oxygen that follows recovery from waterlogging.

Figure 1 illustrates the main reactive oxygen species that are generated in the cell, together with the detoxification mechanisms, and the genes in the yeast *Saccharomyces cerevisiae* that encode the enzymes involved. Transition metal ions also play a major role in the generation of ROS, mainly through the Fenton reaction leading to the generation of the very reactive hydroxyl radical, and hence metal ion homeostasis (for Cu and Fe ions in particular) also plays an important part in the cellular defenses against oxidative stress.[5] There have been many reviews of the effects of ROS on cellular constitutents, and on the cellular defense systems that operate to protect cells against ROS damage.[6-9]

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; ROS, reactive oxygen species.

Less is known in eukaryotic systems of the mechanisms whereby cells respond to oxidative stress, although there has been some progress made with the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*.

These are very useful model eukaryotes due to their ease of genetic manipulation, compact genomes, and the current rate of progress in gene function research. For *S. cerevisae* this also includes the availability of the entire genome sequence, the availability of deletion mutants for most genes in the genome, and the remarkable insight in to transcriptional regulation emerging from genome-wide DNA microarray technology. This article reviews recent developments in the understanding of the cellular responses to ROS, and it identifies genetic mechanisms that can be

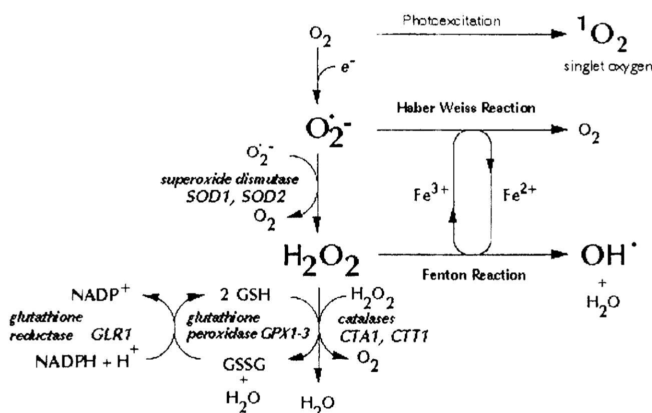

Fig. 1. Reactive oxygen species and defence systems. Major ROS are printed in large bold type. These include the superoxide anion (O_2.-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH.). Enzymic defences are indicated in italics, together with the Saccharomyces cerevisae genes that encode each enzyme. The involvement of metal ions in the Fenton and Haber-Weiss reactions is illustrated use Fe ions as examples.

exploited to boost cellular defenses.

Antioxidant Defences.

S. cerevisiae, contains many effective defence mechanisms which detoxify ROS as they are formed and these maintain the intracellular redox environment in a reduced state. In general these systems are often inducible by ROS treatment, but it is clear that the cell seems to depend on many protective systems each of which is only induced to a relatively small extent, in the range two- to ten-fold. Protective enzymes include the cytosolic (Ctt1p) and peroxisomal (Cta1p) catalases,[10] the cytoplasmic Cu/Zn (Sod1p) and mitochondrial manganese (Sod2p) superoxide dismutases,[11] three glutathione peroxidases (Gpx1p, px2p, Gpx3p),[12] and the thioredoxins (Trx1p, Trx2p)[13] and glutaredoxins (Grx1p, Grx2p)[14] or glutaredoxin-related genes (Grx3p - 5p)[15] some of which play other roles in ribonucleotide reduction and sulphur metabolism. Metal ion homeostasis systems including the metallothionein metal ion-scavenging proteins have been reviewed.[5]

Non-enzymic defences typically consist of small molecules which can act as thiol-reducing agents or free radical scavengers. Across a range of organisms these include glutathione (GSH), ubiquinol, uric acid and vitamins C and E. *S. cerevisae* and some other fungi synthesise D-erythroascorbic acid instead of L-ascorbate (vitamin C). GSH is the most abundant low-molecular-weight thiol in most organisms and also provides the reducing power for detoxification of hydroperoxides by glutathione peroxidases. The biological importance of GSH depends on the redox-active sulphydryl of its cysteine moiety and it is maintained in the reduced state via the action of glutathione reductase (Glr1p) using NADPH generated from the pentose phosphate pathway. Since the oxidation of sulphydryl groups is one of the earliest observable events during ROS-mediated damage, GSH has provided a major area of antioxidant research,[8] although there is increasing interest in ubiquinol and D-erythroascorbate.[16,17]

Yeast has provided a simple system in which to evaluate the role of each of the enzymes and compounds in protection against ROS, since it is feasible to delete genes that encode the enzymes or that are essential for the synthesis of low molecular mass antioxidants and determine the outcome in terms of the effect on resistance of cells to various oxidants. In most cases deletion of a gene encoding an antioxidant enzyme is not lethal, but often renders the cell more sensitive to oxygen or specific oxidants such as hydrogen peroxide, superoxide generating agents such as menadione or paraquat, or organic hydroperoxides. Strains lacking GSH (*gsh1*), or glutathione reductase (*glr1*) are sensitive to peroxides, the superoxide anion and to the toxic products of lipid hydroperoxides.[18-21] The *glr1* mutants are viable and have no requirement for glutathione.[22,23] Strains lacking GSH also have a very low absolute requirement for GSH for growth (about 200-fold lower than the cellular levels normally present; Lee et al. manuscript submitted) and generate petites at relatively high frequency when grown on non-fermentable substrates. Hence GSH is probably serving non-essential but important roles in the cell as an antioxidant, and probably also as a store for nitrogen and sulphur.[24,25] Depletion of GSH from mammalian cells results in cell damage due to oxidative stress in various tissues including the liver and brain.[26] Thus, the findings made in yeast provide a framework for understanding the molecular basis of the observations made in other organisms. Disruption of the *gsh* 2 gene leads to the accumulation of the dipeptide g-glutamyl cysteine in cells. This compound is an antioxidant in its own right and these mutants show little sensitivity to oxidants.[27] GSH forms mixed disulphides with proteins during oxidative stress and this may represent a post-translational modification that can regulate protein activity in response to growth or other cellular signals.

In order to serve an antioxidant function, protein S-thiolation must be reversible and studies on mammalian systems have shown that protein dethiolation can be catalyzed in vitro by the dithiol proteins glutaredoxin and thioredoxin. One of the three isozymes (Tdh3p) of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has recently been identified as the major target of protein S-thiolation in yeast.[28] GAPDH activity of both the Tdh2p and Tdh3p isoenzymes was reduced following exposure to hydrogen peroxide, but only Tdh3p activity was restored within an hour after exposure, indicating that S-thiolation of Tdh3p polypeptide was readily reversible. This protein modification was physiologically important since mutants lacking TDH3 were more sensitive to a lethal dose of the oxidant. In contrast, the non-thiolated Tdh2p was needed during exposure to continuous low levels of oxidants, conditions in which the Tdh3 polypeptide would be S-thiolated and hence inactivated. A model has been proposed in which both enzymes are required during conditions of oxidative stress, but play complementary roles depending on their ability to undergo S-thiolation. Mutants lacking the lipid-soluble coenzyme Q (ubiquinol) have been generated;[29] these have a respiratory-deficient (petite) phenotype due to disruption of the respiratory chain. Of these, the coq3 mutant is reported to be hypersensitive to unsaturated fatty acids, and since this can be rescued with synthetic antioxidants or a-tocopherol, this may be due to the loss of protection afforded by ubiquinol against the products of lipid oxidation.[16]

The potential role of D-erythroascorbate as an antioxidant in yeast (presumably in a similar way to L-ascorbate in higher eukaryotes) has been the subject of somewhat conflicting reports possibly arising due to the use of different strains. Huh *et al.*[17] identified the ALO1 gene encoding the final step in the pathway catalysed by D-arabinono-1,4-lactone oxidase. Mutants lacking this enzyme lacked D-erythroascorbate and showed an increased sensitivity to

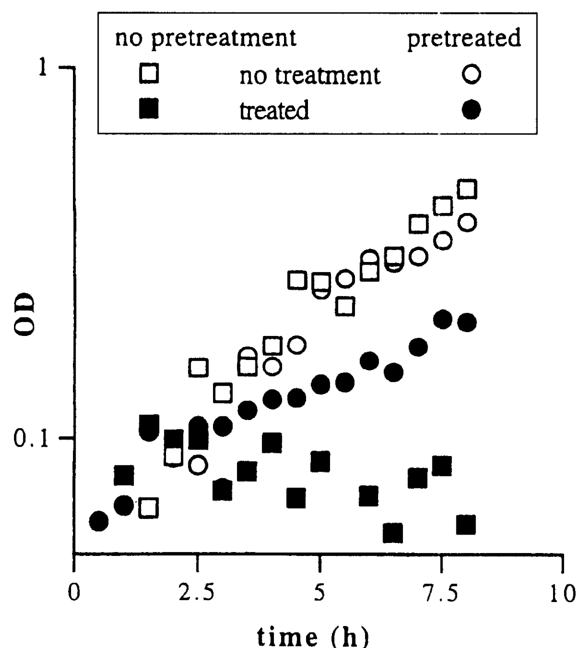

Fig. 2. Adaptive response of cells to pretreatment with a low dose of linoleic acid hydroperoxide. Cells from mid-exponential phase were either pretreated for one hour with 6 mM linoleic acid hydroperoxide (circles) or untreated (squares). The cultures were then challenged with a higher concentration (40 mM linoleic acid hydroperoxide, filled symbols) or with water (open symbols) for one hour, then inoculated in to fresh growth medium and the OD measured at 600 nm to follow growth.

oxidative stress. Spickett *et al.*, however, found that cells unsupplemented with precursors to ascorbate-like compounds did not produce detectable levels of either ascorbate or D-erythroascorbate, and that preloading with erythroascorbate did not protect glutathione levels during oxidative stress.[30]

Adaptation to Oxidative Stress

Cells of *S. cerevisiae* can adapt to oxidative stress, this was shown by their becoming more resistant to a subsequent high dose following exposure to a low, non-lethal dose of a particular ROS (including hydrogen peroxide or superoxide anion)[31-33] or product of oxidation (linoleic acid hydroperoxide or malondialdehyde;[21,34] similar adaptive responses occur in the fission yeast *S. pombe*[35] and in cells of higher eukaryotes. Cells become more resistant to hydrogen peroxide within 45 minutes of the challenge, and gradually lose the protection within four hours of its removal.[36]

Figure 2 illustrates the adaptive response of yeast cells to linoleic acid hydroperoxide. There has been considerable research on the cellular oxidative defense systems but much less is known about how this adaptation occurs. The nature of adaptation depends on the treatment: thermal shock induces cells to become resistant to most oxidants, but oxidative stress does not induce resistance to hypothermia. Cells adapted to H_2O_2 treatment become resistant to menadione (a superoxide generator), but not vice-versa.[32] This hierarchical response to stress may indicate the existence of several different adaptation systems which have overlapping components.

In most cases adaptation does depend on gene activation and de novo protein synthesis, and from two dimensional proteomic analysis a substantial number of proteins were found to be induced as a result of H_2O_2 treatment.[37] There are conflicting reports about whether or not glutathione is involved in the adaptive response.[19,38] In our hands a grande *gsh*1 mutant has been shown to undergo adaptation, despite its lower oxidant resistance; in fact most of the individual mutants in specific antioxidant functions that we have tested are still able to adapt. This may be due to the fact that there are multiple antioxidant systems activated as a result of prior exposure, and deletion of any one system leaves others intact. To some extent this is supported by the finding that mutations affecting YAP1 or YAP2 genes encoding oxidant-responsive transcription factors reduce the ability of cells to adapt to hydrogen peroxide, but not to superoxides. Mutants devoid of catalase activity (*ctt*1 & *cta*1) are also reported to lack the ability to adapt to hydrogen peroxide.[39]

The number of genes involved in the adaptive response may be quite large. Genome-wide analysis of transcription on yeast treated with hydrogen peroxide and also for a sod1 mutant lacking the cytosolic superoxide dismutase indicated that many genes were activated in response to each of these conditions.[40] There were differences as well as similarities in the sets of genes responding to hydrogen peroxide or to elevated superoxide generation in a sod1 strain. The activation or repression of a gene under these conditions is not necessarily indicative of its function in the cell's response, since changes in one regulon can have marked effects on other control systems in cells, nonetheless there are many condidate genes for future analysis.

A second approach taken in our laboratory (V. Higgins & N. Alic; personal communication) has been to analyze gene function by screening the sensitivity to a range of oxidants of deletants in the Eurofan collection of strains that have individual genes of unknown function deleted. Of over 600 strains tested for their ability to grow in the presence of three different oxidants (each at a range of concentrations) about 14% were affected in their resistance to either hydrogen peroxide, cumene hydroperoxide, or linoleic acid hydroperoxide. There were some genes that were specific to only one of the three oxidants, while others were involved in resistance to only two of the three in any combination (Fig. 3). A core set of genes were needed for resistance to all three peroxides.

A third strategy to identify genes that are involved in contributing to protection against ROS involves selecting genes that when overexpressed confer resistance to the various compounds. When using transformation of a host

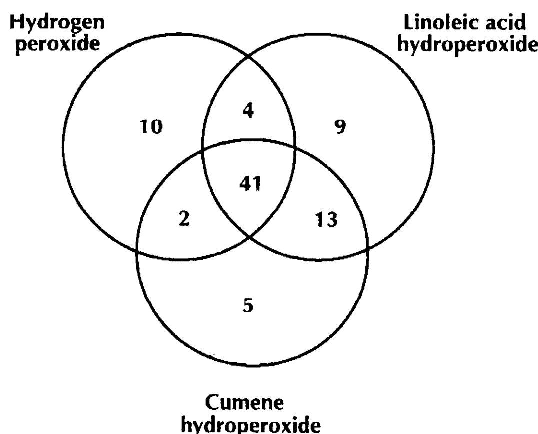

Fig. 3. Overlap of gene sets involved in the response to different hydroperoxides. Results of a preliminary survey of the resistance to hydrogen peroxide, cumene hydroperoxide and linoleic acid hydroperoxide of 600 different deletant Saccharomyces cerevisae strains from the Eurofan collection. Approximately 80 mutants were sensitive to any one of the three hydroperoxides, of these some mutants were sensitive to only one, while others were sensitive to different combinations of the oxidants as illustrated by the above diagram which gives the numbers of strains sensitive to each combination.

strain with a plasmid library of yeast sequences one usually selects only one gene, that is the one capable of conferring the highest level of resistance, but more genes can be identified if lower levels of the oxidant are used. With this approach we have identified a set of genes involved in conferring resistance to linoleic acid hydroperoxide when they are over-expressed. Interestingly, in some cases these do not include genes identified by the other techniques. Clearly each method identifies a different spectrum of genes, some are needed to maintain cells in a robust state, while others are more concerned with detoxification, or responding to ROS when a challenge occurs. These results further emphasize that cells have evolved very specific systems for dealing with each ROS and its toxic reaction products, and that parts of these individual systems overlap. A major challenge now is to determine the cellular function of these many unknown genes and how their products interact with each other in cellular maintenance or responses.

Oxidative Stress-Induced Cell Division Cycle Delay

Cells of most organisms delay progression through the cell division cycle after they have been subjected to DNA-damaging agents. It is now clear that similar delays occur in response to a range of ROS and products of oxidative stress including the superoxide anion, H_2O_2, lipid hydroperoxides and 4-hydroxynonenal.[32,41-43] The responses to superoxide anion and hydrogen peroxide again differ since superoxide-generating compounds arrest cells in the G1 phase of the cell cycle in independently of the RAD9 gene product, whereas hydrogen peroxide leads to an accumulation of G2/M phase cells.[44] G1 arrest in response to the superoxide anion is mediated through inhibition of transcription of the cyclin genes CLN1 and CLN2.[41] It is not yet clear whether the response to hydrogen peroxide requires any components unique to oxidative damage, or if it is merely a response to DNA damage initiated by the ROS. Recently we have shown that other peroxides, especially the highly toxic lipid hydroperoxide linoleic acid hydroperoxide also lead to cell cycle arrest, but in this case the arrest occurs in the G1 phase. This work has been extended to identify genes encoding signalling pathways and metabolic steps that are required for this arrest (N. Alic, personal communication).

Control of Gene Expression in Response to Oxidative Stress

Yeast cells have at least six transcriptional control systems that regulate the expression response of genes in response to oxidative stress. Some act in more general stress-response systems while others are relatively specific to oxidative stress, or to specific metal ion homeostasis. Those genes encoding antioxidant defenses that have been studied in most detail (e.g. CTT1, CTA1, GLR1, SOD2) are in most cases controlled by a combination of these systems, the promoters of these genes having several motifs for binding of the different transcription factors involved. One system that is relatively more specific to oxidative stress, or at least to glutathione and redox metabolism, depends on the yAP transcription factors belonging to the AP1 family found in eukaryotes in general. Yeast has eight members of this family, of which two at least (Yap1p and Yap2p) are involved in the response of cells to stress caused by oxidants, heavy metals or toxic compounds. Yap1p binds to the consensus TTA(G/C)TAA motif in promoters to activate genes (GSH1, GLR1, TRX2, YCF1, SSA1, PDR5, SNQ2, and ATR1) concerned with the metabolism of GSH, heat shock, metal ion resistance, or ABC transporter proteins involved in multidrug resistance.[45] In response to stress, Yap1p is activated in a similar way to its human counterpart: the protein is located in the cytoplasm of non-stressed cells, and is transported to the nucleus following the imposition of stress.[46]

Interestingly, the protein has different regions for responses to the thiol-oxidising agent diamide, and to hydrogen peroxide, and hence the transcription factor appears to be activated by separate pathways depending on the nature of the stress.[47] Pos9p/Skn7p is a transcription factor that has some functional relationship with Yap1p, since pos9 and yap1 mutants have a very similar phenotype with respect to hydrogen peroxide sensitivity, and the double mutant has the same sensitivity.[48] Yap1p and Pos9p/Skn7p cooperate to activate two genes involved in thioredoxin action (TRX2 encoding one of the thioredoxins and TRR1, the thioredoxin reductase). By proteomic analysis it has been shown that Yap1p controls a large oxidative response

regulon of at least 32 proteins, and that 15 of these proteins also require the presence of Pos9p/Skn7p for their induction. This therefore leads to the definition of two subsets of genes on the basis of whether they are coregulated by Yap1p and Pos9p/Skn7p or not. This appears to separate the antioxidant scavenging enzymes from the metabolic pathways regenerating reducing power.[49] The activation of Pos9p/Skn7p requires the protein kinase A pathway, and this may be the more direct site of action of this pathway compared with the Yap1p function. Hap2p/3p/4p/5p is a heteromeric transcription factor mainly involved in activating genes involved in respiration, although there are other genes that respond to it. It is required for the haem-dependent response of the SOD2 gene to growth under respiratory conditions, and as cells undergo nutrient shift as they enter the respiratory phase of growth.[50,51]

Since the onset of respiration leads to an increased flux of the superoxide anion it is not surprising that some genes for antioxidant defenses respond to this activator. MSN2 and MSN4 are two highly homologous genes encoding redundant transcription factors that mediate a more general stress response. They interact with the STRE element (consensus T/AAGGGA) in the promoters of many genes.[52] Msn2p and Msn4p are also translocated from the cytoplasm to the nucleus following a range of stress signals including oxidative stress, heat shock, low pH, ethanol or sorbate. The nuclear localization of these factors is also regulated by the activity of the protein kinase A signal transduction pathway.[53] Since transition metal ions play a very significant role in cellular generation of the very reactive hydroxyl radical via the Fenton reaction, homeostasis with regard to cellular uptake and metabolism of heavy metal ions is crucial to cellular integrity. This is especially the case for Cu and Fe ions. Several transcriptional control systems have been identified: Ace1p is a cuprous ion-activated transcription factor that binds to a motif (consensus: TTTTGCTG) in the promoters of copper ion-inducible genes while its homologue Mac1p also binds copper ions and regulates expression of the cytosolic catalase gene CTT1 in response to hydrogen peroxide. AFT1 encodes the Fe-responsive transcription factor controlling the Fe regulon. The signalling pathways and reactions involved in the metabolism of Cu and Fe ions has been reviewed.[5]

Future Perspectives

The cellular responses to oxidative stress are obviously complex and several important areas remain to be resolved. Functional genomic approaches to identifying genes involved in responding to oxidative stress (including DNA microarray analysis, genome-wide deletion mutant screening and overexpression analysis) has identified many more genes of poorly understood or unknown function that contribute to the cellular defense systems. Determining the role of these unknown genes in cellular physiology, and how they interact in regulons will be a major challenge. Much also remains to be learned about the redox-sensing systems in eukaryotes and how they interact with the signal transduction pathways leading to activation of antioxidant gene expression. How these signaling systems overlap and interact to fine-tune the response to different stresses remains to be determined.

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