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Mechanisms of Resistance to Oxidative and Nitrosative Stress: Implications for Fungal Survival in Mammalian Hosts

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The ability of a fungal pathogen to cause disease requires the ability to survive in the host. Survival in the host is dependent on evasion of the host's immune system, including the microbial killing mechanisms of phagocytes. The innate immune system is comprised of macrophages and neutrophils, which phagocytize invading microorganisms and use reactive oxygen, nitrogen, and chlorine species to protect the host (127). The adaptive immune system contributes antibodies that can enhance the respiratory burst of immune effector cells. These reactive species can harm pathogens by readily altering or inactivating proteins, lipid membranes, and DNA, and they have potent immunomodulatory effects on the immune system that affect the efficacy of the host response.

Phagocytosis of microbes by neutrophils and macrophages may result in an oxidative burst, due to activation of the membrane-associated NADPH-dependent oxidase complex through an intricate biochemical signaling system from the phagosome (26). Concomitant with an increased rate of oxygen consumption by the phagocytes, the enzyme NADPH oxidase reduces oxygen to superoxide, which is secreted into the phagosome. A defect in NADPH oxidase results in chronic granulomatous disease (CGD), a severe human disorder causing recurrent bacterial and fungal infections (45). A supporting model shows that macrophages deficient in NADPH oxidase are unable to kill *Aspergillus fumigatus*, one of the most common pathogens in patients with CGD (88, 145). Similarly, in an animal model of CGD, mice are most highly susceptible to *Staphylococcus aureus* and *A. fumigatus* (78, 147). Though this crucial enzyme forms the extremely reactive superoxide anion, it is not simply this reactive oxygen intermediate that results in microbial killing (Fig. 1 shows the basic pathways in macrophages). Superoxide readily dismutates to hydrogen peroxide (H_2O_2) or combines with nitric oxide (NO) to form the strong oxidant peroxynitrite, which has been shown to be fungicidal (169) and more deleterious to microorganisms than nitric oxide alone (15). Nitric oxide, a potent antimicrobial compound, is produced in professional phagocytes after macrophage activation by appropriate cytokines through the action of inducible nitric oxide synthase on L-arginine (109). In addition, the heme enzyme myeloperoxidase can convert hydrogen peroxide and chloride to hypochlorous acid and other antimicrobial chlorinating oxidants (43). The importance of this system to host defense is portrayed in myeloperoxidase-deficient mice, which are more susceptible to *Candida albicans* or *Candida tropicalis* and show delayed clearance of *A. fumigatus* (6, 8). A myeloperoxidase deficiency is not as serious as an NADPH oxidase deficiency, as it has been shown that the hydrogen peroxide used by myeloperoxidase is mostly derived from NADPH oxidase-derived superoxide (7). Hydrogen peroxide may also break down to form the highly reactive hydroxyl radical in the presence of a transition metal catalyst, such as iron. Alternatively, the damaging hydroxyl radical may be formed from superoxide-mediated reduction of hypochlorous acid (20).

The relative roles of neutrophils or macrophages in host defenses against pathogenic fungi depend on the microbe, as reviewed elsewhere (94, 131, 170). Similarly, the relative susceptibility of the fungus to reactive oxygen or nitrogen species, and therefore to the effectiveness of a given mechanism of host defense, depends on the microbe. For example, *C. albicans* and *Cryptococcus neoformans* were shown to stimulate a macrophage oxidative burst, including superoxide release (75). Less-clear results were obtained for the pathogen *Histoplasma capsulatum*, which has been shown to stimulate the human macrophage oxidative burst but not the mouse macrophage oxidative burst in vitro, unless the yeast cells were opsonized (75, 131). *A. fumigatus* stimulates the production of nitric oxide in alveolar macrophages (57). Though it has been shown that *C. neoformans* does not induce nitric oxide synthase in a mouse macrophage cell line in vitro (126), other researchers have shown the production of nitric oxide to be stimulated by *C. neoformans* in rat alveolar macrophages in vitro (57), and recently the gene encoding the inducible form of nitric oxide synthase has be shown to be greatly expressed during murine cryptococcal meningoencephalitis (110). These results may reflect the use of different species (mouse versus rat) or different cell types (brain tissue versus primary macrophages or a macrophage-like cell line). Importantly, resistance to reactive nitrogen and oxygen species is shown to correlate with the virulence in clinical isolates of *C. neoformans* (180). Although the anticryptococcal activity of murine macrophages is more dependent on reactive nitrogen species (165), the actual killing of *A. fumigatus* by murine macrophages is mediated by reactive oxygen intermediates (145). Though there are conflicting results on whether or not the macrophage oxidative burst, when obtained, is detrimental to *H. capsulatum* (14, 131), it is well documented that nitric oxide production by macrophages causes fungistasis of *H. capsulatum* (131). Similarly, the growth of *Pneumocystis carinii* and *Paracoccidioides brasiliensis* is in-

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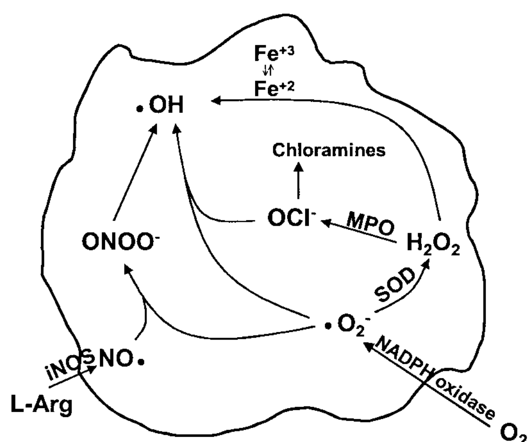

FIG. 1. Relevant pathways of phagocytes which generate reactive oxygen, nitrogen, or chlorine species. The importance of these toxic species to disease is described in the text. MPO, myeloperoxidase; iNOS, inducible nitric oxide synthase.

hibited by the induced synthesis of nitric oxide in macrophages (49, 76). In addition, it has been shown that amphotericin B enhances the synthesis of nitric oxide in macrophages, so some of its antifungal effects may be mediated through nitric oxide (121).

Both reactive nitrogen and reactive oxygen species have pleiotropic effects in the cell. Reactive nitrogen intermediates (RNI) encountered by living cells consist of the free radical nitric oxide as well as products derived from NO (nitrite, nitrogen dioxide, and nitrate) or from the reaction of NO with other molecules to generate peroxynitrite, peroxynitrous acid, or nitrosothiols. Importantly, peroxynitrite, which is generated from NO and superoxide, is more stable and is a more reactive oxidizing species than NO. It has been shown in vitro to be much more toxic to *Escherichia coli* than NO (15). RNI readily react with proteins, DNA, and metal ions and have been shown to inactivate heme-, nonheme iron-, iron-sulfur-, and Cu-containing proteins. Thiol groups, both in small molecules and in proteins, are particularly susceptible to S nitrosylation by NO. RNI can also deaminate the N-terminal and other amino groups of proteins (for a review, see reference 152). Identified targets of RNI include the nonheme, iron-dependent enzyme ribonucleotide reductase which can be rate limiting in DNA synthesis; the iron-sulfur cluster-dependent enzymes aconitase of the tricarboxylic acid cycle and complexes I and II of the mitochondrial electron transport chain; and the heme-containing enzyme cytochrome *c* oxidase, the terminal member of the mitochondrial electron transport chain (28, 128). Finally, RNI can deaminate cytosine to uracil and have been shown to be mutagenic in *Salmonella enterica* serovar Typhimurium (176).

Superoxide, produced in large amounts by the oxidative burst of phagocytes or in smaller amounts as a by-product of normal aerobic respiration of cells, is readily converted to other reactive oxygen species (ROS), such as hydrogen peroxide and the hydroxyl radical. The reaction of superoxide with lipids, which are plentiful in plasma and organelle membranes of cells, produces lipid hydroperoxide radicals, which initiate a chain reaction and hence amplification of further lipid peroxidation. NO acts as a chain-terminating molecule in this reaction. Singlet molecular oxygen can be produced as a by-product of lipid peroxidation as well as by oxidation of peroxide. ROS can oxidize all amino acid residues on proteins (reviewed in reference 11), but there is some selectivity. Thiol groups on cysteines and methionines are particularly sensitive to oxidation, which results in disulfide bridges and methionine sulfoxide, but these reactions are reversible. The oxidized thiols can be reduced to their native state by several mechanisms, including treatment with methionine sulfoxide reductase and disulfide reductases. Oxidation of other amino acid residues results in irreversible modifications that often have significant consequences for enzyme activity or regulation. Oxidation of lysine residues could be particularly harmful, since it would preclude the covalent attachment of ubiquitin to the lysine amino group, which in turn is necessary for proper targeting of damaged proteins to the proteosome, and accumulation of damaged proteins would likely have negative consequences for a cell (reviewed in reference 103). Hydroxyl radicals and singlet molecular oxygen can damage DNA by producing over 20 different base lesions (recently reviewed in reference 27), leading to high mutation rates. In a genome-wide screen of *Saccharomyces cerevisiae*, oxidative stress enzymes have been implicated in preventing high-frequency mutagenesis (69).

Since the host uses many microbicidal compounds to protect itself from invading microbes, it is incredible how many fungi survive this attack during infection. To thrive within the oxidative environment of professional phagocytes, pathogenic fungi have multiple defense systems, both enzymatic and nonenzymatic. It has been hypothesized that the ability of some fungi to survive the stress of the host defense has evolved from the environmental interactions of fungi with phagocytic unicellular organisms such as amoebae (161). Recent studies have demonstrated that pathogenic *C. neoformans* strains, but not less virulent *C. neoformans* mutants, can replicate in amoebae (161), and that *C. neoformans*, but not other closely related nonpathogenic *Cryptococcus* species, can cause disease in multicellular invertebrates like nematodes and flies (5, 123). It has been shown that nitric oxide synthase is present in invertebrates (107) and that mosquitos use NO to limit the growth of the pathogen causing malaria (108).

ENZYMATIC DEFENSES AGAINST NITROSATIVE STRESS

Several enzymes responsible for resistance to nitrosative stress have been identified through genetic studies of bacteria, including *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Mycobacterium tuberculosis* (30, 42, 127), or of the yeast *S. cerevisiae* (105). These enzymes provide an initial list of candidates for examining nitrosative stress resistance pathways of pathogenic fungi.

In bacteria, direct consumption of NO is accomplished by the flavorubredoxin nitric oxide reductase enzyme under anaerobic and microaerophilic conditions (42, 47) or the flavohemoglobin denitrosylase enzyme under all conditions (40). Direct consumption of peroxynitrite by the bacterial AhpC protein, the founding member of the peroxiredoxin family, was also demonstrated (16). Not surprisingly, these enzymes are important for bacterial resistance to nitrosative stress. Enzymes that metabolize *S*-nitroso products, resulting from the reaction of NO with thiol compounds, also protect cells from

nitrosative stress. Identified so far in this class is *S*-nitrosoglutathione (GSNO) reductase, which reduces GSNO to ammonia and glutathione (GSH; γ-glutamylcysteinylglycine) disulfide (105). Enzymes that repair or circumvent damage to DNA or proteins caused by RNI may confer resistance to nitrosative stress. The proteosome of *M. tuberculosis* was recently shown to be required for resistance to nitric oxide in vitro and for full virulence in mice (30), perhaps because cell viability depends upon the proper degradation of proteins damaged by NO.

Studies of the genetic basis of fungal resistance to nitrosative stress and the potential role of these pathways in fungal virulence are in their infancy. However, genomic analysis of *C. albicans* revealed three flavohemoglobin denitrosylase homologs, but recent studies have demonstrated that one of these, *C. albicans YHB1*, is highly induced in response to NO but not in response to superoxide or peroxide. In addition, deletion of the *C. albicans YHB1* gene resulted in increased sensitivity to NO and slightly attenuated virulence (167). Flavohemoglobin denitrosylase is also implicated in the virulence of *C. neoformans*; a recent study showed that a flavohemoglobin denitrosylase mutant (*fhb1* mutant) showed attenuated virulence in a mouse infection model (31). In the same study, it was shown that a GSNO reductase mutation (*gno1*) alone has little or no effect on virulence but that the *fhb1* mutation combined with *gno1* or *sod1* (cytoplasmic superoxide dismutase [SOD]) mutations has a greater effect on virulence than *fhb1* alone. In another study of *C. neoformans*, *TSA1*, which encodes a member of the peroxiredoxin family, was shown to confer resistance to exogenous nitrosative stress and to contribute to virulence in a mouse model (116). As noted above, other enzymes of nitrosative stress resistance in bacteria, to the extent that they are conserved in pathogenic fungi, are prime candidates for studies of fungal virulence.

ENZYMATIC DEFENSES AGAINST OXIDATIVE STRESS

All aerobic organisms are exposed to ROS and have developed enzymatic defenses against them. Fungi have enlisted several classes of antioxidant enzymatic defenses to cope with the variety of ROS that is presented by phagocytes.

SODs

SOD is an interesting oxidative defense enzyme, since it takes one harmful ROS, superoxide, and converts it to another ROS, hydrogen peroxide. Moreover, the dismutation reaction that it catalyzes occurs spontaneously in its absence. One explanation for the importance of this enzyme in oxidative defense is that it helps to shunt the superoxide in the direction of other ROS and away from the formation of lipid hydroperoxides or the RNI peroxynitrite. This shunting action seems to be beneficial to the cell, since cells have many defense systems to deal with peroxides, often overlapping or redundant in specificity, but few to deal with reactive nitrogen species.

There are four known classes of SODs: Mn, Fe, Ni, and Cu,Zn. In addition to the different cofactors, these enzymes show differential expression and/or localization in the cell. In *S. cerevisiae*, which has only two of these SODs, the Cu,Zn enzyme (Sod1) is located in the cytoplasm and the mitochondrial intermembrane space (38, 162) while the Mn enzyme (Sod2) is localized to the mitochondrial matrix (53).

A total of six SOD genes have been identified in *C. albicans. SOD1* encodes a Cu,Zn isozyme that is cytoplasmic and expressed more abundantly during exponential growth than in stationary phase (73, 99). A *sod1/sod1* mutant is sensitive to menadione (a reagent that generates superoxide) and displays reduced survival in macrophages and attenuated virulence in mice (74). *SOD2* and *SOD3* each encode Mn SODs. The Sod2 isozyme is mitochondrial, while Sod3 is an unusual cytoplasmic isozyme bearing the Mn cofactor (99, 151). A *sod2/sod2* null mutant displays heightened sensitivity to several stresses (menadione, paraquat, ethanol, or 43°C heat shock), is essential for growth under hyperoxic conditions, and shows elevated cyanide-insensitive respiration (see "Alternative oxidase" below) (72). These results are consistent with a primary role of Sod2 in scavenging superoxide generated by the mitochondrial respiratory chain. The Sod3 isozyme is expressed more abundantly during the stationary phase than exponential growth, which is the inverse of the expression pattern displayed by Sod1 (99). The recently identified *SOD4*, *SOD5*, and *SOD6* genes encode predicted proteins homologous to Cu,Zn SODs and are presumed to be cytoplasmic, since they lack mitochondrial targeting sequences (112). *SOD5*, which was studied in detail, is expressed more abundantly during the yeast-to-hypha transition, as well as after exposure of yeast cells to an increase in pH from 6.0 to 8.0 or to osmotic or oxidative stress. In addition to H_2O_2, both riboflavin and menadione induced *SOD5* transcript levels, which indicates that *SOD5* gene expression is sensitive to both external and intracellular sources of superoxide. Since the natural and principal intracellular source of superoxide is the mitochondrial electron transport chain (12), the expression levels of *SOD5* during metabolism on nonfermentable carbon sources, which requires increased respiration activity, were also tested and shown to be induced. Studies of a *sod5/sod5* null mutant indicate a role for the Sod5 isozyme in virulence in mice infected via tail vein injection but not in macrophages.

Studies of SODs in other pathogenic fungi have been initiated recently. In both *C. neoformans* var. *grubii* and *C. neoformans* var. *gattii*, the Cu,Zn Sod1 contributes to virulence in mice infected intranasally or intravenously (29, 125). The *C. neoformans* var. *grubii* mutant also shows slower growth in macrophages, relative to that of the *SOD1* parental or reconstituted control strains, and the *C. neoformans* var. *gattii* mutant shows greater susceptibility to in vitro killing by neutrophils, relative to control strains. In addition, the SOD deficiency of *C. neoformans* var. *gattii* results in defects in laccase, urease, and phospholipase expression.To date, little work has been done on the role of this enzyme in *A. fumigatus*, but the Cu,Zn SOD is known to be immunoreactive to the sera of patients with aspergillosis (66).

Catalases

Catalases are antioxidant metalloenzymes, ubiquitous among aerobic organisms, which promote the conversion of hydrogen peroxide to water and molecular oxygen. Catalase is produced by *A. fumigatus* in vivo during invasive aspergillosis (163). *A. fumigatus* encodes one conidial catalase, CatA, and two my-

celial catalases, Cat1 and Cat2 (141). Though Cat1 and Cat2 protect *A. fumigatus* from peroxide killing, they are not sufficient for protection against macrophage killing (141). A mutant deficient in any single catalase isozyme manifests virulence comparable to that of wild-type strains, but a mutant deficient in both mycelial catalases has reduced virulence (19, 141).

Four catalases in *Aspergillus nidulans* have been elucidated. Though found to be posttranscriptionally regulated, CatA is induced during conidiation and in response to multiple stresses (129, 130). CatA activity is detected in spores and protects this fungus from peroxide stress and heat shock (130, 133). Results of immunolocalization studies indicate that CatA is in the asexual spore cell wall and cytosol, whereas CatB is in the hyphal cell wall and cytosol, and that CatB is induced in growing and developing hyphae and in response to oxidative and other stresses (91, 93). CatC is a constitutively expressed small-subunit catalase with a peroxisomal targeting sequence, while CatD activity is induced during late stationary phase by glucose starvation, high temperature, and peroxide stress (91). CatD has also been described as the catalase-peroxidase product of *cpeA*, which is transcriptionally induced during sexual development as well as carbon starvation (155).

Three catalase genes in *H. capsulatum* have been found to have differential expression (86). *CATA* transcript abundance is regulated by morphology and oxidative stress, while *CATB* (M antigen) and *CATP* transcript levels are modestly regulated by the carbon source. The *CATB* catalase is extracellular (181), and catalase P (from *CATP*) is intracellular, presumably localized in the peroxisome (86). Interestingly, *C. albicans* has only one catalase gene, and studies of a deletion mutant indicate that catalase protects the organism from peroxide stress and neutrophil killing (124, 179).

Thiol peroxidases, glutaredoxins, GSH peroxidases, and GSH *S*-transferases

The thiol peroxidase and glutaredoxin proteins are broadly important for many normal cellular processes, such as metabolic reactions involving disulfide bond formation as part of the catalytic cycle and protein folding or prosthetic group insertion during protein assembly (54). In addition, some or all members are involved in responses to acute oxidative or nitrosative stress, either as direct metabolizers of organic hydroperoxides, including lipid hydroperoxides resulting from superoxide-initiated chain reactions, or as agents for protein repair and refolding. The GSH peroxidases and GSH *S*-transferases are involved in the breakdown of organic hydroperoxides with GSH as a reductant or in the conjugation of toxic lipophilic compounds to GSH, respectively (54).

Thiol peroxidases, also known as peroxiredoxins, are 20 to 30 kDa in size and are present in organisms from all kingdoms (150). As peroxidases, they act to remove peroxides and provide defense against oxidative damage. Some peroxiredoxins are known as thiol-specific antioxidants because the active site for these peroxidases is a thiol group of a conserved N-terminal cysteine. Members of the peroxiredoxin family can be divided into two subgroups: 1-Cys peroxiredoxins, which contain only the N-terminal cysteine, and 2-Cys peroxiredoxins, which contain a C-terminal cysteine in addition to the active site cysteine (90). When oxidized, the 2-Cys variety of thiol peroxidases forms an intramolecular disulfide bridge and is dependent on thioredoxin to be reduced to its active state. Thioredoxin is, in turn, dependent upon thioredoxin reductase for regeneration. Five thiol peroxidases in *S. cerevisiae* have been described. Three are of the 2-Cys variety and are located in the cytoplasm, Tsa1p and Tsa2p are highly similar to each other, and Ahp1p is an atypical 2-Cys peroxidase (142). *S. cerevisiae* also has two 1-Cys enzymes; mTPx, encoded by YBL064C, is mitochondrial and Dot5p is nuclear (142). Tsa1p is described as the principal antioxidant because it regulates intracellular oxidative stress, especially stress due to hydrogen peroxide (142). It is suggested that Tsa2p has a physiological role in cell proliferation, while Ahp1 appears to be the key antioxidant for yeast at an acidic pH.

These unique thiol peroxidases in pathogenic fungi have not been well studied. Recently, Tsa1 has been identified as being highly induced during 37°C hyphal growth in *C. albicans*, and the downstream thioredoxin system genes are induced after peroxide exposure (25, 37). In *C. neoformans, TSA1* has been shown by serial analysis of gene expression to be transcriptionally induced at 37°C (160). And further, Tsa1 has been shown to be important to the oxidative and nitrosative stress resistance of *C. neoformans*, as well as critical for virulence in a mouse model (116).

Glutaredoxins are small heat-stable proteins and are reduced in a manner similar to thiol peroxidases. Whereas thiol peroxidases are regenerated by reaction with thioredoxin, glutaredoxins are regenerated via GSH. The yeast *S. cerevisiae* encodes five glutaredoxins, Grx1 through Grx5. *S. cerevisiae* also encodes three GSH peroxidases (Gpx1 through Gpx3) and three GSH *S*-transferases (Gtt1, Gtt2, and Ure2). Single mutants with mutations in *grx1*, *grx2*, *grx5*, *gpx3*, and *ure2* are sensitive to oxidant stress (54, 149). The roles of glutaredoxins, GSH peroxidases, and GSH *S*-transferases in antioxidant defenses or the virulence of fungal pathogens have not been examined so far.

Methionine sulfoxide reductase

Methionine residues have been proposed to act as a sink for oxidative stress, since oxidation of exposed methionines does not always lead to protein inactivation (104). The enzyme methionine sulfoxide reductase was shown to reduce methionine sulfoxide residues to methionine (119). The gene encoding methionine sulfoxide reductase was deleted from *E. coli*, and the mutants were more sensitive to H_2O_2. Deletion of the homologous gene in *S. cerevisiae* results in H_2O_2 sensitivity and accumulation of methionine sulfoxide in proteins (117), while overexpression of the gene causes the yeast to be slightly more resistant to H_2O_2 (118).

NONENZYMATIC DEFENSES AGAINST OXIDATIVE AND NITROSATIVE STRESS

In addition to enzymatic defenses, there are nonenzymatic defenses against oxidative and nitrosative stress in the form of several metabolites that are important scavengers of reactive oxygen or nitrogen species. The most common of these antioxidant metabolites include melanin, mannitol, and trehalose.

Melanin

Melanins are multifunctional polymers found throughout nature and are known to reduce oxidants (63, 157). Melanins are thought to play a protective role in the virulence of human pathogenic fungi, including *C. neoformans*, *P. brasiliensis*, *Wangiella dermatitidis*, *Sporothrix schenckii*, and *A. fumigatus* (100). Two types of melanin, 1,8-dihydroxynaphthalene (DHN)–melanin and 3,4-dihydroxyphenylalanine (DOPA)–melanin, have been implicated in fungal pathogenesis (61, 79, 98, 144, 175).

The conidial pigment of *A. fumigatus* contains DHN-melanin and protects the fungus from reactive oxygen and chlorine species as well as from oxidative killing by macrophages, conferring virulence to the organism (13, 84, 85). DHN-melanin also protects *S. schenckii* from reactive oxygen and nitrogen radicals and from macrophage-mediated killing (153).

Melanin confers resistance to microbicidal oxidants in both wild-type and mutant strains of the neurotropic fungi *C. neoformans* and *W. dermatitidis* (80, 83). DHN-melanin of *W. dermatitidis*, as well as DOPA-melanin of *C. neoformans*, is important to the virulence of these pathogens (33, 98). Expressing a strong antioxidant activity, DOPA-melanin provides protection from oxidant concentrations similar to those produced by stimulated macrophages (83, 172). In addition, this melanin reduces the susceptibility of *C. neoformans* to nitrogen- and oxygen-derived oxidants, which may contribute to survival when fungal cells are attacked by macrophages (173). The importance of melanin to the oxygen sensitivity of *C. neoformans* is exemplified by the demonstration that mutants selected for albinism are sensitive to oxidants and those selected for oxygen sensitivity exhibit albinism (36, 81, 146).

In addition to phenoloxidase activity, melanization itself can be detected in both the conidia and yeast cells of the fungal pathogens *P. brasiliensis* and *H. capsulatum* in vitro and during infection (48, 132). These two dimorphic fungi are able to produce melanin without exogenous phenolic substrates, so it is hypothesized that the production of melanin could be by the DHN pathway (48, 132).

Mannitol

Polyols and polyhydroxyalcohols are present in all organisms, from bacteria to animals (154). In addition to serving as a reserve carbon source, the polyol mannitol is known to scavenge ROS in vitro (159) and to protect *C. neoformans* from oxidative killing in vivo (23). In addition, *C. neoformans* produces large amounts of mannitol during infection (23). A mutant that produces less mannitol is less virulent and more sensitive to neutrophil-mediated oxidative killing (22, 23). It has recently been shown that mannitol is required for stress tolerance in *Aspergillus niger* conidiospores (154). Likewise, a mutant deficient in a mannitol biosynthetic enzyme is hypersensitive to high temperatures and oxidative stress (154).

Trehalose

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) (41) is a nonreducing disaccharide constituting up to 15% of the dry weight of lower eukaryotic cells or spores (35). Due to its distinctive properties, such as high hydrophilicity and chemical stability, trehalose serves a unique role as a stress metabolite (9). Trehalose, which accumulates in response to heat and oxidative stress in *A. nidulans*, acts by stabilizing membranes and native proteins as well as by suppressing the aggregation of denatured proteins (39, 158). It has been thought that trehalose is most important in the adaptive acquisition of stress tolerance, but it was found that trehalose is also essential to *C. albicans* in dealing with intense stress when it is not already adapted to stress (4, 9).

In a *C. albicans* trehalose biosynthesis-deficient mutant, enzymatic antioxidant activities of catalase, GSH reductase, and Cn,Zn SOD were highly induced in response to hydrogen peroxide, suggesting synergism between antioxidant enzymes and the antioxidant metabolite trehalose (50).

MODULATION OF OXIDATIVE OR NITROSATIVE STRESS

Through compensatory reactions, functions that are inhibited by nitrosative or oxidative stress may be bypassed until reversal of inhibition or repair of damage is accomplished. As discussed below, alternative oxidase, responsible for cyanide-insensitive respiration in mitochondria, is likely to function in this manner. Similarly, homeostatic mechanisms exist to sequester free iron and thus limit production of hydroxyl radical by the Fenton reaction. Other homeostatic mechanisms maintain cellular redox balance and thus buffer oxidant stress, allowing the rapid repair of inhibited or damaged proteins. Critically important for the modulation of stress conditions is global gene regulation, allowing for the induction of stress response pathways and reprogramming of cell growth and metabolism, or even morphogenesis, during the acute phases of the stress.

Alternative oxidase

Alternative oxidase is a mitochondrial enzyme found in all plants and many fungi and protists, but it has never been found in mammalian mitochondria (89). It is a cyanide-resistant terminal oxidase of a branched electron transport chain, in which electrons from ubiquinone are transferred directly to alternative oxidase as well as to cytochrome *c* reductase (complex III) of the mitochondrial electron transport chain. Alternative oxidase, like cytochrome *c* oxidase (complex IV) of the electron transport chain, reduces oxygen to water via a two-electron transfer. Consequently, transfer of a pair of electrons from NADH along the alternative branch results in one coupling site for oxidative phosphorylation (complex I) instead of three coupling sites (complexes I, III, and IV) in the main chain, and hence production of about one-third of the ATP generated by the main chain.

Alternative oxidase is believed to function metabolically (168, 171) and as an antioxidant (140), and both functions are potentially important for fungal pathogenesis. Significantly, nitric oxide is a potent reversible inhibitor of cytochrome *c* oxidase but not of alternative oxidase (114). Upon exposure to nitric oxide, the relative insensitivity of alternative oxidase to nitric oxide allows the maintenance of electron flow through carriers of the mitochondrial electron transport chain, the generation of a mitochondrial membrane potential via proton

pumping through complex I of the electron transport chain, and ATP synthesis by oxidative phosphorylation. The maintenance of NAD/NADH redox balance, allowing high glycolytic flux and substrate level ATP production, is also potentially important. Hence, alternative oxidase maintains cellular bioenergetics under these conditions. In addition, the maintenance of mitochondrial electron flow as well as rapid consumption of oxygen by alternative oxidase at the mitochondrial inner membrane attenuates the production of ROS by a mitochondrial electron leak from ubisemiquinone directly to oxygen. The last-named reaction, resulting in the production of superoxide (12), occurs more readily when electron flux through coenzyme Q is impeded, as would be the case if neither cytochrome oxidase nor alternative oxidase were active (140). Hence, alternative oxidase performs an antioxidant function.

Though there is no alternative oxidase in *S. cerevisiae*, at least one functional enzyme has been found in many fungal pathogens, including *C. albicans*, *C. neoformans*, and *H. capsulatum* (2, 70, 71, 87). Evidence of alternative oxidase activity has also been observed in *A. fumigatus*, and an alternative oxidase RNA has been shown to be induced in the mycelial phase of *P. brasiliensis* (46, 166).

C. albicans produces one constitutive alternative oxidase, Aox1a, and another isozyme, Aox1b, inducible by cyanide, antimycin A, hydrogen peroxide, menadione, paraquat, and nonfermentable carbon sources (70). Each of the alternative oxidase genes of *C. albicans*, which are located in tandem on the same chromosome, can be functionally expressed in *S. cerevisiae* (70, 71). The alternative oxidase gene *AOX1* of *H. capsulatum* shows increased expression after peroxide stress or in the presence of respiratory inhibitors (87). In addition, exposure of live *H. capsulatum* yeast cells to physiological levels of nitric oxide (generated from 5 μM GSNO) causes rapid inactivation of cyanide-sensitive respiration but little inhibition of alternative respiration. Substantial loss of viability occurs after *H. capsulatum* yeast is cultured in the presence of both an NO donor and a chemical inhibitor of alternative oxidase, suggesting that alternative oxidase is necessary for survival of nitrosative stress by this organism (J. E. McEwen, unpublished data). A critical test of the role of alternative oxidase in fungal pathogenesis was accomplished with the recent generation and study of an *aox1* null mutant of *C. neoformans*, which displays increased sensitivity to peroxide stress and decreased virulence in mice (2).

Iron metabolism

Since hydrogen peroxide can break down to form the highly reactive hydroxyl radical in the presence of a transition metal catalyst, the regulation of transition metals, such as iron, may be of great importance to a microorganism's ability to deal with oxidative stress. Iron is essential for the viability of most organisms and is abundant, but its bioavailability is limited in an aerobic environment because it is mostly present as ferric hydroxides, which have low solubility (136). When considering pathogenic microorganisms, the acquisition of iron is recognized as a fundamental step in the infection process, since this essential nutrient is tightly sequestered by high-affinity iron-binding proteins and therefore not readily available in mammalian hosts (58, 174). Since an overabundance of iron leads to oxidative stress and allows for the deleterious oxidation of biomolecules, microbes have developed tightly regulated strategies for acquiring and storing iron (58, 60). As a lack of cellular iron storage in *A. nidulans* results in oxidative stress, including an induction of antioxidant enzymes, it is clear that stringent control of iron metabolism is crucial for resistance to oxidants (34, 134, 136).

This tight control of iron is accomplished primarily by the rate of uptake (137). Successful pathogens obtain iron by extracting it from heme or transferrin, capturing extracellular iron with secreted siderophores, or utilizing high-affinity systems for iron reduction and transport (174). Under iron starvation conditions, many fungi excrete siderophores, which are low-molecular-weight iron-scavenging ligands that chelate ferric iron with high affinity and specificity (136, 139). Numerous pathogenic fungi, including *A. fumigatus*, *H. capsulatum*, *C. albicans*, *S. schenckii*, *Microsporum* spp., *Blastomyces dermatitis*, and *Trichophyton* spp., are known to produce siderophores (59, 62, 64, 67, 68), and siderophore-mediated iron uptake is essential to the survival of *A. nidulans* as a free-living organism (34).

Iron acquisition is critical for the survival of fungi in mammalian hosts (59). *H. capsulatum* is believed to acquire iron by three mechanisms: secretion of siderophores, expression of iron-reducing activities, and acidic-pH-dependent release of iron from host transferrin (177). The conversion of the inhaled saprophytic mycelia of *P. brasiliensis* to the virulent yeast phase after phagocytosis requires iron-loaded macrophages (17, 21).

Although *C. neoformans* has not been shown to produce its own siderophores, it is able to obtain iron from deferoxamine as well as from ferric siderophore complexes utilizing its cell-associated ferric reductase (135, 174). Emphasizing the importance of iron regulation, iron-hypertransporting mutants of *C. neoformans* exhibit oxygen-sensitive phenotypes (134). In addition, the reduction of iron and melanin is linked in *C. neoformans*. Melanized cells reduce iron at a 16-fold-higher rate (135), and reduced iron may be important for maintaining melanin in a reduced state, ready to protect the cell from oxidative attack (82). Similarly, *A. nidulans* iron regulatory mutants with increased iron uptake display increased expression of antioxidant enzymes, including CatB, SodA, and SodB, and increased sensitivity to the oxidant paraquat (34, 138).

GSH, redox homeostasis, and reprogramming of metabolism to optimize NADPH generation

GSH is an abundant low-molecular-mass intracellular tripeptide thiol. Though the roles of GSH in fungal pathogens have not been thoroughly studied, much work on this molecule in *S. cerevisiae* and several other yeasts has been done (143). GSH in *S. cerevisiae* was shown to be essential as a reductant during normal growth conditions in minimal media. In rich media, strains with mutations for GSH synthesis show sensitivity to both H_2O_2 and organic hydroperoxides (56, 77). As discussed above and reviewed elsewhere (54), glutaredoxins, GSH peroxidases, and GSH *S*-transferases are important in cellular responses to oxidative stress, and all utilize GSH as a cofactor. The activities of these enzymes result in the conjugation of two molecules of GSH, to generate the GSH disulfide (GSSG). GSH is then regenerated from GSSG by the action of

GSH reductase, which has been shown to be important for oxidative stress resistance in yeast (55, 122).

GSH reductase, as well as thioredoxin reductase (see above), is in turn dependent upon NADPH as the reductant. A proteomics study of the H_2O_2 stimulon in *S. cerevisiae* shows that exposure of yeast cells to H_2O_2 results in rapid resetting of carbohydrate metabolism to redirect carbohydrates to pathways, such as the pentose phosphate shunt, that regenerate NADPH at the expense of glycolysis (44). In other studies, genes expressing glucose-6-phosphate dehydrogenase (the first enzyme of the pentose phosphate shunt) (Zwf1) and the NADP-dependent isocitrate dehydrogenase (Idp2) are shown to be important for yeast oxidative stress resistance (96, 115). In fact, the *zwf1 idp2* double mutant is impaired for growth in media containing oleate or acetate as carbon sources, presumably due to intracellular oxidants produced by metabolism of these carbon sources (115).

Although the role of GSH in oxidative stress resistance in pathogenic fungi has not been studied, there is evidence that GSH levels decline during the thermally induced transition of *C. albicans* from the yeast cell phase to the mycelium phase (164). In the transition from the mycelium phase to the yeast cell phase of *H. capsulatum*, the maintenance of a low redox potential by a sulfhydryl compound such as GSH, in addition to a specific nutritional need for cysteine, has been shown to be necessary (111). In each case, these phase transitions are associated with virulence, suggesting that perturbations in GSH (or redox) homeostasis will affect the virulence of these fungi. In addition, the data from *S. cerevisiae* pointing to the critical roles played in oxidative stress resistance by GSH and NADPH homeostasis indicate that GSH metabolism pathways may be essential for fungal virulence.

Global gene regulation

Some transcription factors are shown to be important to the specific regulation of gene expression during oxidative stress. For a complete review on the regulation of the fungal transcriptional response to oxidative stress, see reference 120. *S. cerevisiae* Yap1 of the AP-1 family is the best characterized of these transcription factors. Yap1 is able to induce the expression of many stress response genes, including a GSH biosynthetic gene, GSH reductase gene, and a thioredoxin gene (55, 97, 178). Nuclear localization of the Yap1 protein is dependent on stress-induced redox regulation of intramolecular disulfide bond formation (120). As for pathogenic fungi, the *YAP1* counterpart in *C. albicans*, *CAP1*, has been found to complement the oxidative-stress hypersensitivity of an *S. cerevisiae yap1* mutant but not to restore the wild-type pattern of gene expression (182).

One of the most important ways in which prokaryotes and lower eukaryotes adapt to stress conditions is through a two-component signal transduction system (3, 10, 18, 95, 102). Functionally different from *S. cerevisiae*, the putative response regulator of the Hog1 signal transduction system of *C. albicans*, Ssk1, was recently shown to regulate genes whose functions are associated with cell wall biosynthesis as well as the adaptation to oxidative stress (24). In addition, an Ssk1 or Hog1 deficiency results in hypersensitivity to peroxides and superoxide (3, 24). Similarly, another stress mitogen-activated protein kinase in *A. nidulans*, known as SakA, is important for the expression of catalase and resistance to peroxide stress (92).

In addition to the many pathways that result in the induced expression of specific genes under stress conditions, there also seems to be a mechanism that keeps stress-induced genes expressed at a low level during nonstress conditions. Recently, the nuclear actin-related protein of *S. cerevisiae*, Act3p/Act4p, was shown to play a role in the regulation of stress genes through repression (51). As expected, a mutation in Act3p/Act4p resulted in resistance to peroxide stress, since many stress response genes were upregulated (51).

Role of oxidant stress in stimulation of fungal cell differentiation

There is evidence from the model systems *Neurospora crassa* and *A. nidulans* that fungal morphogenesis is stimulated by a hyperoxidant state (101, 106, 113). In *A. nidulans*, an NADPH oxidase just recently discovered in fungi, NoxA, is shown to be necessary for the differentiation of sexual fruit bodies. Furthermore, the mechanism of NoxA in differentiation is directly related to ROS production by the enzyme (101). Based on the identification of oxygen-signaling mechanisms in *S. cerevisiae* and other systems, including mammalian cells, additional mechanisms for the generation of a hyperoxidant signal can be envisioned. These include changes in environmental oxygen tension (32); regulation of electron transport chain-associated ROS production by several mechanisms, including the activation of *RAS2* (65) or other signal transduction pathways that activate protein kinase A (148); and nutrient limitation (1). Given the importance of morphological-phase transitions in fungal virulence (52) and the exposure of fungi to oxidant stress even in the early stages of mammalian host infection, it is tempting to speculate that the morphogenetic mechanisms demonstrated in *N. crassa* and *A. nidulans* may extend to fungal pathogens. In fact, evidence of ROS production by *C. albicans* in relation to morphogenesis has been presented, although a causal relationship has not been demonstrated (156).

CONCLUSIONS AND FUTURE DIRECTIONS

Antioxidant mechanisms necessarily coevolved with the advent of aerobic growth of cells. Multiple mechanisms, as well as redundancy or overlap between stress resistance pathways, serve to allow maximum protection and flexibility for adaptation to changing environments. Flexibility and multiplicity of defenses seem to be particularly important in pathogenic microorganisms that exist both free in the environment and in association with a host organism that expresses innate immune responses, based on the production of reactive oxygen and nitrogen species. There is little data to suggest that fungi that frequently grow in mammalian hosts have evolved additional or specialized mechanisms to protect themselves from oxidative or nitrosative stresses. Instead, it seems likely that free-living fungi encounter many sources of stress, including nitrosative stress from denitrification, oxidative stress from free radicals formed by UV light, and various stresses from encounters with many different phagocytic microbes, such as amoebae. There is little information available regarding the oxidative and nitrosative stress environments present in the various

TABLE 1. Functions of fungal proteins or compounds important for resistance to oxidative and nitrosative stresses

Fungal product(s)	Function in ROS or RNI metabolism	Demonstration of virulence function[a]
Flavohemoglobin denitrosylases	Direct consumption of nitric oxide and O_2 to generate NO_3^-	*C. albicans* *C. neoformans*
GSNO reductases	Reduction of GSNO to ammonia and GSH disulfide	*C. neoformans*
Superoxide dismutases	Conversion of superoxide to hydrogen peroxide	*C. albicans* *C. neoformans*
Catalases	Conversion of hydrogen peroxide to water and molecular oxygen	*A. fumigatus* *C. albicans*
Thiol peroxidases	Metabolism of peroxides and/or peroxynitrite	*C. neoformans*
Glutaredoxins, GSH peroxidases, GSH *S*-transferases, GSH reductases	Metabolism of ROS, reduction of oxidized sulfhydryl groups, maintenance of cellular redox homeostasis	None studied so far
Methionine sulfoxide reductases	Reduction of methionine sulfoxide residues to methionine	None studied so far
Alternative oxidase	Bypass nitric oxide-inhibited cytochrome *c* oxidase; prevent ROS production from the mitochondrial respiratory chain	*C. neoformans*
Melanins	Scavenging ROS and RNI	*A. fumigatus* *C. neoformans* *S. schenckii* *W. dermatitidis*
Mannitol	Scavenging ROS	*C. neoformans*
Trehalose	Membrane and protein stabilization	*C. albicans*

[a] See the text for references. In most cases, fungi other than those listed have not yet been studied with respect to the role in virulence played by the relevant stress resistance protein or compound.

phagocytic microorganisms. These environments may differ among various phagocytic microbes, so fungi that are exposed to them may require a larger arsenal of defenses than fungi that specialize in a single host. Genome analyses of many fungi are under way, and the comparison of the relative numbers of enzymes functioning in the resistance to oxidative or nitrosative stress may help clarify this hypothesis.

A complete picture of the strategies and mechanisms for resistance to oxidative and nitrosative stress is not available for any of the fungal pathogens studied to date, and conversely, there is no comprehensive examination of the virulence role of a particular enzyme or compound across multiple genera of pathogenic fungi. As summarized in Table 1, many gaps remain to be filled in by future studies. Genetic studies involving screening or selection of mutants altered in stress resistance has been a powerful approach for studies of stress responses in bacterial pathogens and can be applied relatively easily to genetically tractable fungal pathogens such as *C. neoformans*. The fact that nematodes or amoeba can be used as primitive hosts to screen mutants for sensitivity or resistance to innate immunity will accelerate the process. In addition to random mutagenesis studies, candidate gene approaches will also be useful. Of the stress resistance pathways reviewed here, it is striking that GSH and redox homeostasis play central roles in the metabolisms of both reactive oxygen and nitrogen species and are known to be critical for stress resistance in *S. cerevisiae*. A goal of future studies of fungal pathogenesis is to not only identify and understand mechanisms of oxidative and nitrosative stress resistance, but also prioritize studies based on pathways likely to be essential for virulence.

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