PHYSIOLOGICAL AND MORPHOLOGICAL CHARACTERIZATION OF *TERT*-BUTYLHYDROPEROXIDE TOLERANT *CANDIDA ALBICANS* MUTANTS

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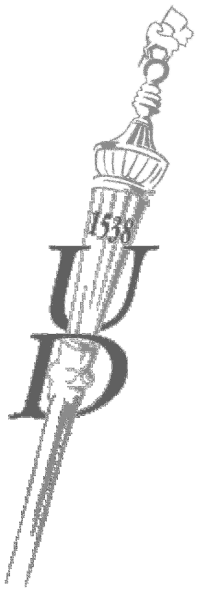

UNIVERSITY OF DEBRECEN
Doctoral school of Pharmacological Sciences

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Theses of doctoral (Ph.D.) dissertation

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Introduction

Commensally growing *C. albicans* is more resistant to oxidative stress than the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and is able to adapt to oxidative stress caused by different oxidants *in vitro*. *C. albicans* produces powerful antioxidants to cope with reactive oxygen substances (ROS; *e.g.* superoxide, peroxide, hypochlorite) and reactive nitrogen intermediates {RNIs, *e.g.* nitric oxide (NO), peroxynitrite} produced by polymorphonuclear leukocytes (PMNLs) and macrophages when fungal cells enter the bloodstream and infect deep organs. The elements of antioxidative defence include small molecular mass metabolites with high ROS quenching potential, *e.g.* D-erythroascorbic acid and 2,4-(hydroxy)phenyl-ethanol, as well as powerful enzymes neutralising both ROS (*e.g.* catalase, glutathione peroxidases, superoxide dismutases, thioredoxin, thioredoxin reductase, methionine sulfoxide reductase) and RNIs (*e.g.* NO-responsive flavohemoglobin). Importantly, the up-regulation of this enzyme system has been demonstrated in *C. albicans* cells exposed to whole blood and separated PMNLs as well as to macrophages.

Although antioxidative enzymes of fungal pathogens are regarded as "persistence factors" promoting the survival of these micro-organisms under colonization and invasion rather than virulence attributes *sensu stricto* the significance of their activities and regulation cannot be underestimated especially during penetration into deeper tissue and bloodstream - a radically changing and hostile environment. It is therefore understandable that the disruption of genes coding for key enzymes in the antioxidative defence may slow down, moderate virulence and may reduce viability in the presence of whole blood and PMNLs.

Previously, it was reported that the induction of oxidative stress responses by exposure of *C. albicans* to immune system cells inhibits the development of hyphae, an important virulence attribute, which facilitates escape of *C. albicans* from the blood stream and subsequent invasion of tissues.

We tested the hypothesis that adaptation to chronic oxidative stress would inhibit formation of hyphae and reduce pathogenicity. To reach this goal, *C. albicans* was chronically exposed to *tert*-butylhydroperoxide (*t*BOOH), an oxidative-stress generating agent with long-lasting physiological effects and accelerating lipid peroxidation chain reactions in biological membrane. Importantly, the physiological and transcriptional effects of *t*BOOH and H_2O_2, a toxic decomposition product of superoxide produced by phagocyte NADPH oxidases, seemed to be equivalent in *C. albicans* in previous oxidative stress response and sensitivity studies. This hydroperoxide was selected because oxidative injuries of biological membranes caused

by *t*BOOH and the phagocytes' NADPH oxidase-myeloperoxidase (MPO) system may be quite similar. The NADPH oxidase-MPO system generates versatile reactive oxygen species (ROS), OCl^-, tyrosyl radical and nitrating intermediates, which reactants modify and oxidize lipids effectively through lipid peroxidation pathways similar to *t*BOOH.

Specific aims

The hypothesis that the continuous induction of the antioxidative defence system in *C. albicans* and the concomitant reduction in the hypha-forming capability of the fungus may affect adversely its virulence was tested by comparing the virulence attributes, antigenicity and the pathogenicity of the *C. albicans* AF06 mutant and its parental strain (*C. albicans* ATCC 14053).

(1) Are there change in development of *t*BOOH-tolerance on the time of storage?

(2) Does the development of *t*BOOH-tolerance cause a change in a hypha-forming capability in *C. albicans*?

(3) Does the increased *t*BOOH-tolerance altere the production of virulence factors (extracellular aspartic protease production, extracellular phospholipase production, germ tube formation, pseudohypha and hypha-formation) and affect antigenicity?

(4) How does pathogenicity change in mouse?

(5) Does the increased *t*BOOH-tolerance affect cell size and morphology?

(6) How does tolerance against a series of oxidative stress generating agents and antimycotics change?

(7) Which change are detectable in GSH metabolism?

(8) Does the increased *t*BOOH-tolerance cause change lipid combination and lipid peroxidation?

(9) Does the increased *t*BOOH-tolerance affect respiration?

(10) How does characterise the reproducibility of the development of *t*BOOH-tolerant mutants?

(11) With a what kind of probability may *t*BOOH-tolerant mutants appear in the nature?

Materials and methods

Organisms

*t*BOOH-tolerant *C. albicans* strains were developed from *Candida albicans* ATCC 14053 and clinical strains. *t*BOOH-tolerant mutatnts: AF01-10, 4774T, 8387T, 10934T, 19890T, 20072T *t*BOOH tolerant mutants were developed by continuous cultivation of *C. albicans* ATCC 14053 and clinical strains and in the presence of stepwise increasing concentrations of *t*BOOH under similar conditions.

Comparative morphological and physiological characterization of the *t*BOOH-tolerant mutants and parental strains

The *t*-BOOH, H_2O_2, sodium hypoclorite and menadione minimal inhibitory concentrations (MIC) were determined by microdilution methods.

The colony formation were compared on Sabouraud dextrose agar (SDA) and the colony size were compared in Sabouraud dextrose broth (SDB).

The pseudohypha and hypha-forming capability of the *C. albicans* strains was tested on corn-meal agar and Spider agar medium. The formation of pseudohypha and chlamydospore by *C. albicans* parental and mutant strains was observed microscopically.

Determination of extracellular phspholipase activity were measured by the egg yolk agar medium.

The antigenicity of *C. albicans* AF06 and ATCC 14053 cells was characterized by the amount of superoxide produced by PMNLs in the presence of opsonized *C. albicans* cells.

LD_{50} of the strains were determined cell injected into the tail vein of mouse.

MIC valued were also determined for fluconazole, voriconazole, 5-fluorocytosine and amphotericin B in RPMI-1640 and SDB media according to the national Committee for Clinical Laboratory Standards (NCCLS, 2002) guidelines for antifungal susceptibility testing.

The specific activities of a series of antioxidant enzymes (G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase; γGT, γ-glutamyltranspeptidase; catalase; SOD, superoxide dismutase) as well as

the intracellular glutathione (GSH) and glutathione disulphide (GSSG) concentrations were determined in *C. albicans* strains.

Accumulation of intracellular ROS (peroxide and superoxide) was always detected by the formation of 2',7'-dichlorofluorescein (DCF) from 2',7'-dichlorofluorescein diacetate and ethidium (Et) from dihydroethidium.

Results

A series of *C. albicans* mutants with increased *t*BOOH tolerance was developed by continuous cultivation of *C. albicans* ATCC 14053 in the presence of increasing concentrations of *t*BOOH (2→16 mmol L^{-1}). The AF06 strain also had an increased tolerance against the major oxidants produced by phagocytes including H_2O_2, superoxide and OCl^- as demonstrated by the two-fold increases in the MIC_{H2O2}, MIC_{MSB} and MIC_{NaOCl} values, respectively

Morphological transitions are inevitably needed for successful tissue and organ invasion and dissemination within the host and are, therefore, important virulence factors. Considering other virulence attributes, the AF06 mutant produced less extracellular phospholipase, which is highly expressed in yeast cells and pseudohyphae and degrades cell membrane components effectively

PMNLs recognised both the mutant and the parental strain equally indicating that the mutation leading to the oxidative stress tolerant phenotype of *C. albicans* AF06 did not affect antigenicity. The reduced capability of the fungus to undergo dimorfic morphological transitions together with a decreased phospholipase production resulted in a less pathogenic strain with a 5-fold higher LD_{50} value in mice when compared to that of the ATCC 14053 strain ($40x10^4$ *vs.* $8x10^4$ cells). Therefore, the development of oxidative stress tolerance, which is thought to be advantageous for *C. albicans* when it interacts with immune system cells, seems to be disadvantageous when the fungus escapes from blood vessels and invades deep organs.

In terms of cell physiology, *C. albicans* AF06 possessed high specific GR, G6PD, GPx, catalase and SOD activities, which, unlike in the parental strain. The persistently high antioxidant enzyme activities coincided with high endogenous oxidant (peroxide, GSSG, lipid hydroperoxide) levels and a GSH/GSSG redox imbalance. Progressing lipid peroxidation was

also demonstrated in the mutant by increased conjugated diene and TBARS productions and by the increased SFA and decreased MUFA and PUFA contents. The long-lasting physiological changes typical of the *C. albicans* AF06 mutant (redox imbalance, lipid peroxidation) are therefore highly suitable to promote a continuous induction of the antioxidative defence system.

The origin of peroxide and lipid peroxidation products observable in high concentrations in unstressed *C. albicans* AF06 cultures has remained yet to be studied, but the unbalance between the CN^--sensitive and SHAM-sensitive respirations may be indicative of a heritable mitochondrial DNA damage resulting in an increased electron leak of the electron transport chain.

All *t*BOOH-tolerant mutants from clinical strain (4774T, 8387T, 10934T, 19890T, 20072T) possessed increased *t*BOOH and H_2O_2 tolerances, continuously elevated antioxidative enzyme (GR, G6PD, GPx) activities, intensified KCN-resistant alternative oxidase-dependent respiration, increased intracellular GSSG concentration, GSH overproduction (except the 19890T strain) and GSH/GSSG redox imbalance (except the 4774T strain). Considering virulence attributes, all mutants secreted phospholipase at lower activities than the parental strains, and had reduced capabilities to develop pseudohyphae and hyphae . Growths of the mutants were considerably delayed both on SDA surface and in SDA submerged cultures.

On the other hand, changes in the antibiotic (fluconazole, voriconazole, amphotericin B, 5-fluorocytosine) tolerances followed versatile patterns, and intracellular peroxide concentrations, total respiration and KCN-sensitive cytochrome c-dependent respiration were affected only in a few mutants

The moderated virulence of *t*BOOH-tolerant mutants can be explained with their reduced and delayed hypha formation, decreased phospholipase secretion and slower growth. Importantly, the mutants tested did not lose completely their hypha-forming capability, still secreted phospholipase and their biomass production reached the same level in the stationary phase of growth in SDB as recorded for the parental strains.

Our data with *t*BOOH-tolerant mutants indicate that some *C. albicans* cells may adapt to chronic oxidative stress and may switch to hyphal growth after a temporary cease in proliferation without the protective effect of any blood cells.

Although the *in vivo* selection of *C. albicans* mutants with an increased antioxidative potential but with a concomitantly decreased virulence seems to be unlikely some more recent findings by other research groups may challenge this view. This means that the *in vivo*

selection of oxidative stress tolerant respiratory *C. albicans* mutants even with a decreased pathogenicity cannot be excluded when *C. albicans* have to adapt to environmental stress caused by concurrent attacks of immune system cells and antimycotics.

Publications

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