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Candida albicans erythroascorbate peroxidase regulates intracellular methylglyoxal and reactive oxygen species independently of D-erythroascorbic acid

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ABSTRACT

Candida albicans **D-erythroascorbate peroxidase (EAPX1), which can catalyze the oxidation of D-erythroascorbic acid (EASC) to water, was observed to be inducible in *EAPX1*-deficient and *EAPX1*-overexpressing cells via activity staining. *EAPX1*-deficient cells have remarkably increased intracellular reactive oxygen species and methylglyoxal independent of the intracellular EASC content. The increased methylglyoxal caused *EAPX1*-deficient cells to activate catalase-peroxidase and cytochrome *c* peroxidase, which led to defects in cell growth, viability, mitochondrial respiration, filamentation and virulence. These findings indicate that EAPX1 mediates cell differentiation and virulence by regulating intracellular methylglyoxal along with oxidative stresses, regardless of endogenous EASC biosynthesis or alternative oxidase expression.**

1. Introduction

L-ascorbic acid (ASC), which is biosynthesized in higher plants or almost every type of animal, except for some rodents, birds or primates, plays a pivotal role in antioxidant defense [1,2]. However, certain eukaryotic cells use an isomeric D-form of erythroascorbic acid (EASC), which is a five-carbon homologue of ASC [3]. We previously reported crucial roles for *Candida albicans* or *Saccharomyces cerevisiae* EASC synthesized from D-arabinose [4–7], which is different from animal or plant ASC biosynthesis [8]. EASC-deficient *Candida* cells have filamentous growth defects, indicating that intracellular EASC has a morphological regulator function [9]. EASC can stimulate cyanide-resistant respiration by triggering two-electron transfer reactions during cellular respiration through the ubiquinone-consuming alternative oxidase (AOX) [10,11]. AOX gene expression is only induced by the intracellular EASC content, independently of cellular H_2O_2 [11]. Additionally, the reduced cellular susceptibility against the oxidative stresses, particularly including superoxide radicals and H_2O_2, induced metabolic genes, such as *AOX*, ascorbate peroxidase (*APX*), cytochrome *c* peroxidase (*CCP*) or catalase-peroxidase (*KatG*) in plants [12]. Therefore, ASC seems to regulate cell morphology and oxidoreductase gene expression levels, such as with *AOX* or heme-containing peroxidases.

ASC, which is able to achieve recalcitrant organogenesis in vitro, is currently recognized as a new type of cell growth regulator together with methylglyoxal [13]. Moreover, advanced glycation end product (AGE) formation is driven by carbonyl molecules, such as glucose, ASC and methylglyoxal [14]. These intracellular α-dicarbonyls are involved in AGE formation via various mechanisms, which can be preceded by enzyme reactions, Schiff base formation, and Amadori adducts that are particularly relevant to the interaction between ASC and methylglyoxal [15,16].

Herein, to uncover a reciprocal relationship between EASC and methylglyoxal that is based on previous investigations, *C. albicans* erythroascorbate peroxidase homologous gene (*EAPX1*) was disrupted or overexpressed. We intended to verify the physiological

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Abbreviations: EAPX1, D-erythroascorbate peroxidase; EASC, D-erythroascorbic acid; GSH, glutathione; MG, methylglyoxal; CCP1, cytochrome *c* peroxidase; KatG, catalase peroxidase; rpm, revolutions per minute; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame; ROS, reactive oxygen species

Author contributions: M.-K. K., S.-H. S., M. Ku, and S.-O. K. designed the research; M.-K. K., S.-H. S., and M. Ku performed the research and experiments; M.-K. K., S.-H. S., and S.-O. K. analyzed the data; M.-K. K., M. Ku, and S.-O. K. contributed new reagents/analytic tools; and M.-K. K., S.-H. S., and S.-O. K. wrote the paper.

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Table 1
Strains, primers, and plasmids used in this study.

Strain, primer, or plasmid	Genotype, sequence, or description	Source or reference
C. albicans		
SC5314	Wild type isolate	Fonzi and Irwin [25]
CAI4	*Δura3::imm434/Δura3::imm434*	Fonzi and Irwin [25]
MK901	*Δura3::imm434/Δura3::imm434 Δeapx1::hph-URA3-hph/MGD1*	This study
MK902	*Δura3::imm434/Δura3::imm434 Δeapx1::hisG/EAPX1*	This study
MK903	*Δura3::imm434/Δura3::imm434 Δeapx::hisG/Δeapx1::hph-URA3-hph*	This study
MK904	*Δura3::imm434/Δura3::imm434 Δeapx1::hisG/Δeapx1::hisG*	This study
MK905	*Δura3::imm434/Δura3::imm434* (YPB1-ADHPt)	This study
MK906	*Δura3::imm434/Δura3::imm434* (pMK6E-ADHPt)	This study
Primers		
MK3a-*Apa*I	5′-GAGCTCGGATAACTCTTCGTTTTATCCGTC-3′, *Sac*I site of pMK5F	This study
MK3b-*Sac*I	5′-GGTACCATATGAAGATATTTTTTAATTGAT-3′, *Kpn*I site of pMK5R	This study
MK3c-*Bgl*II	5′-AGATCTATGTCTTCATCTACTACAGTTTTC-3′, *Bgl*II site of pMK5E	This study
MK3d-*Xho*I	5′-CTCGAGTTAACCAATAATTTGAGCAACCGA-3′, *Xho*I site of pMK5E	This study
Plasmids		
P5922	4.1 kb HindIII/SacI-digested hisG-URA3-hisG of p5921 in pGEM-7Z(+)	Hwang et al. [26]
YPB-ADHPt	Expression vector containing promoter and terminator regions of ADH1 gene in YPB1	Bertram et al. [27]
pMK5F	pGEM-T Easy vector containing MK5a-MK5b fragment	This study
pMK5R	pMK5F vector containing *hisG-URA3-hisG*	This study
pMK5E	pGEM-T Easy vector containing MK3c-MK3d fragment, *EAPX1* ORF	This study
pMK6E	YPB-ADHPt vector containing MK3c-MK3d fragment, *EAPX1* ORF	This study

roles of EAPX1, including its antioxidative function, morphological changes or virulence by regulating the intracellular EASC or methylglyoxal levels, reciprocally. We elucidated that *EAPX1* gene expression might be induced concurrently with KatG and CCP only in the methylglyoxal- or reactive oxygen species (ROS)-increased conditions in budding yeast. However, EAPX1 contributes the morphological switch from budding yeast to hyphae, independently of KatG or CCP, and it has APX and class I peroxidase activity similar to the APX in plants [17]. This is the first report demonstrating that intracellular methylglyoxal, which is changed by *APX* gene disruption or overexpression, affects cell differentiation and virulence, independently of intracellular EASC content in *C. albicans*.

2. Materials and methods

2.1. Yeast strains and growth conditions

The *C. albicans* strains used in this study are listed in Table 1. To grow *Candida* cells containing disrupted or overexpressing genes, cells were cultured in YPD (1% yeast extract, 2% peptone, and 2% glucose) or minimally defined SD (2% glucose, 0.5% ammonium sulfate, and 0.17% yeast nitrogen base without amino acids and ammonium sulfate) in liquid or on 1.8% agar-containing plates [18,19]. All Ura^+- and Ura^--derivatives were inoculated (2×10^5 cells/mL) and grown at 28 °C, respectively.

To induce hyphal growth, budding *Candida* cells were mid-exponentially grown at 28 °C in SD liquid broths. Approximately fifty to one hundred cells per plate were inoculated for 6 days on different medium. Solid Spider [20] and Lee's media [21] and were used and cells were grown at 37 °C and 28 °C as proposed [9], respectively.

2.2. EAPX1 and peroxidase activity assays

The EAPX activity was spectrophotometrically measured by EASC or ASC oxidation using H_2O_2 at 290 nm (absorption coefficient $e = 2.8\ mM^{-1}\ cm^{-1}$) as proposed [19]. The 0.5 mL EAPX assay mixture for enzyme electron donors consisted of 50 mM potassium phosphate, pH 7.0, when using substrates of 0.05 mM ASC or EASC and 0.1 mM H_2O_2.

EAPX1 activity detection in native gels was performed using 25 mM TEMED and 2.5 mM NBT as described with minor modifications [22]. The unknown peroxidase activity staining with *o*-dianisidine in the presence of H_2O_2 was performed as previously described [23]. Additionally, catalase activity in native gels was performed as previously described [24].

2.3. Candida EAPX1 disruption and overexpression

EAPX1 disruption was performed with the URA blaster technique using p5922, a derivative p5921, as previously described [9,25,26]. The occurrence of the desired integration events in all of the transformants was verified with Southern blot analysis.

To overexpress *EAPX1* in *C. albicans*, the YPB1-ADHPt vector containing the *C. albicans ADH1* promoter and terminator regions was used [27]. The primers and plasmids used in this study are listed in Table 1.

2.4. CCP1 purification

Wild-type SC5314 was used for the peroxidase purification. Approximately 2×10^5 cells were used as a seed inoculation for cell growth, and the *Candida* cells were grown until the late exponential phase (24 h). Approximately 100 g (wet mass) of *Candida* cells were harvested by centrifugation at 4000×g for 15 min, washed twice with ice-cold sterilized distilled water, resuspended in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM phenylmethylsulfonyl fluoride and homogenized with four 30 s pulses at 4 °C in a Bead Beater Cell Homogenizer (Biospec Products). Unbroken cells and cell debris were removed by centrifugation at 8000×g for 30 min. The resulting supernatant was transferred and used as the crude extract during the enzyme purification procedures, which were performed with open column chromatography or a fast protein liquid chromatography system (GE-healthcare). The *Candida* CCP1 purification was performed using column chromatography [7,28] based on both the plant APX purification and activity staining, as previously described with modifications [19,22–24].

2.5. Electroblotting and staining for the N-terminal and internal sequencing analysis

For the N-terminal and internal sequencing analysis of the purified peroxidase, the protein was subjected to SDS–PAGE and the nitrocellulose membrane (Pharmacia) protein transfer was performed with a proposed method that was previously described