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Cytochrome *c* peroxidase regulates intracellular reactive oxygen species and methylglyoxal via enzyme activities of erythroascorbate peroxidase and glutathione-related enzymes in *Candida albicans*

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ABSTRACT

D-erythroascorbate peroxidase (*EAPX1*) deficiency causes glutathione deprivation, leading to the accumulation of methylglyoxal and reactive oxygen species (ROS), and especially, induction of cytochrome *c* peroxidase (Ccp1) in *Candida albicans*. Nevertheless, reciprocal effects between changes in Ccp1 activity and the anti-oxidative D-erythroascorbic acid- and glutathione-dependent redox status, which reflects methylglyoxal biosynthesis altering pathophysiology are unclear in eukaryotes. To elucidate the effect of *CCP1* expression on *EAPX1* and glutathione reductase (Glr1) activity-mediated D-erythroascorbic acid biosynthesis and redox homeostasis, the *CCP1* gene was disrupted and overexpressed. First, we demonstrated both glutathione-independent and-dependent metabolite contents and their corresponding gene transcripts and enzyme activities (i.e., Ccp1, catalase-peroxidase [KatG], superoxide dismutase [Sod], Eapx1, and Glr1) in *CCP1* mutants. Second, methylglyoxal-oxidizing alcohol dehydrogenase (Adh1) and methylglyoxal-reducing oxidoreductase activity on glycolytic methylglyoxal and pyruvate production and NAD(P)H content were determined in these mutants. Contrary to our expectation, *CCP1* disruption (42.19 ± 3.22 nmol O_2 h^{-1} mg wet $cell^{-1}$) failed to affect cell respiration compared to the wild-type strain (41.62 ± 7.11 nmol O_2 h^{-1} mg wet $cell^{-1}$) under cyanide treatment, and in contrast to hydrogen peroxide (H_2O_2) treatment (21.74 ± 1.03 nmol O_2 h^{-1} mg wet $cell^{-1}$). Additionally, Ccp1 predominantly detoxified H_2O_2 rather than negligible scavenging activities towards methylglyoxal and other oxidants. *CCP1* deficiency stimulated Sod and Adh1 activity but downregulated Glr1, Eapx1, catalase, and peroxidase activity while enhancing *KatG*, *EAPX1*, and *GLR1* transcription by decreasing glutathione and D-erythroascorbic acid and increasing pyruvate. Noticeably, the ROS-accumulating *CCP1*-deficient mutant maintained steady-state levels of methylglyoxal, which was revealed to be regulated by methylglyoxal-oxidizing and −reducing activity with drastic changes in NAD(P)H. We confirmed and clarified our results by showing that *CCP1/EAPX1* double disruptants underwent severe growth defects due to the D-erythroascorbic acid and glutathione depletion because of pyruvate overaccumulation. These observations were made in both budding and hyphal-growing *CCP1* mutants. The revealed metabolic network involving Ccp1 and other redox regulators affected ROS and methylglyoxal through D-erythroascorbic acid and glutathione-dependent metabolites, thereby influencing dimorphism. This is the first report of the Ccp1-mediated D-erythroascorbic acid and glutathione biosynthesis accompanying methylglyoxal scavengers for full fungal virulence.

Abbreviations: Adh1, alcohol dehydrogenase 1; Alo1, D-arabinono-1,4-lactone oxidase; Cat, catalase; DCFH-DA, 2′7′-dichlorofluoroscein diacetate; Eapx1, erythroascorbate peroxidase; Gcs1, γ-glutamylcysteine synthetase; Glr1, glutathione reductase; GR, glutathione reductase; HPLC, high-performance liquid chromatography; KatG, catalase-peroxidase; mBBr, monobromobimane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; $NAD(P)^+$, nicotinamide adenine dinucleotide (phosphate); NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate); NBT, nitro blue tetrazolium salt; ODS, octadecyl silica; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PMS, phenazine methosulfate; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; Sod, superoxide dismutase

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1. Introduction

Candida albicans is the most frequently isolated fungal pathogen of humans, which causes mucosal and cutaneous infections in immunocompromised individuals (Gow et al., 2002). This fungal pathogenicity that affects growth, morphogenesis, or both is controlled by intrinsic mediators including naturally occurring intracellular stresses, their detoxifying oxidoreductases, and dimorphism-regulating protein kinases (Ku et al., 2017), along with extrinsic conditions (e.g., pH, temperature, and media) (Hwang et al., 2003).

Among cellular stresses, reactive oxygen species (ROS) are formed by the one or two electron reduction of molecular oxygen resulting in the formation of the hydroxyl radical (·OH), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) (Aguirre et al., 2005). ROS easily react with nucleic acids and proteins, causing cellular damage including endogenous DNA mutation and genomic instability in yeast (Yu et al., 2009). The ROS production induced by cell metabolism is controlled by well-characterized intracellular scavengers (Herrero et al., 2008). Specifically, the detoxification of superoxide anions is catalyzed by superoxide dismutase (Sod), yielding H_2O_2 the simplest peroxide, which is consecutively metabolized by catalase (Cat) and glutathione reductase (GR) to produce water and molecular oxygen (Emri et al., 1997; Westwater et al., 2002). Both ROS and their stimulating antioxidative enzyme activities have been mainly studied on redox regulatory mechanisms of cell proliferation and differentiation in eukaryotic microorganisms (Aguirre et al., 2005) and in *C. albicans* (Dantas Ada et al., 2015; Kwak et al., 2014).

However, increasing evidence supports an alternative hypothesis that the production of cellular ROS concomitantly induces methylglyoxal-mediated physiological alterations by the accompanying cellular changes in methylglyoxal contents (Ku et al., 2017). Furthermore, methylglyoxal and ROS likely reciprocally contribute to cell metabolism-related signaling pathways in *C. albicans* (Ku et al., 2017; Kwak et al., 2014) and act as molecular signals that directly reflect cell cycle progression, development, or both in *Dictyostelium discoideum* (Lee et al., 2017; Park et al., 2017). This reactive α-ketoaldehyde, methylglyoxal, is regarded as a toxic metabolic byproduct mainly produced by the spontaneous nonenzymatic elimination of phosphate from glyceraldehyde phosphate and dihydroxyacetone phosphate during glycolysis (Cooper, 1984). Methylglyoxal has an aldehyde and a ketone moiety as an efficient crosslinking agent and causes nucleic acid and protein damage by forming *in vivo* advanced glycation end products (AGEs) during glucose and fructose catalytic metabolism in eukaryotes (Matafome et al., 2017). Interestingly, some bacteria use a methylglyoxal shunt as an alternative pathway via glyoxalase systems that produce D-lactate to recover protein glycation-induced growth defects (Nguyen et al., 2009). *In vitro* experiments have shown that methylglyoxal reacts with amine-containing biomolecules including amines, amino acids, and proteins to generate active free radicals (Lee et al., 1998; Yim et al., 1995).

In energy metabolism, methylglyoxal inactivates glyceraldehyde-3-phosphate dehydrogenase (GAPDH or Tdh3), glucose 6-phosphate dehydrogenase, or both, thereby inhibiting glycolysis specifically in human malignant cells (Ray et al., 1997). Methylglyoxal-derived embryonic malformations reduce the rate of oocyte maturation, fertilization, and *in vitro* embryonic development (Chang and Chan, 2010). Importantly, these metabolic traits commonly induce methylglyoxal-induced ROS formation, which has been attributed to cytochrome *c* release, caspase activation, and subsequent apoptotic biochemical changes (Chan et al., 2005). Furthermore, methylglyoxal-derived apoptosis of human vascular endothelial cells has been revealed to be repressed by inhibiting ROS production (Phalitakul et al., 2013). In addition, *in vitro* studies have demonstrated that superoxide anion production by glycation reaction of amino acids and methylglyoxal generates cross-linked radical cations and methylglyoxal radical anions (Yim et al., 1995). Methylglyoxal accumulation by glutathione depletion impairs glycolysis, resulting in energy deprivation (de Arriba et al., 2007) as well as induction of the methylglyoxal- or ROS-scavenging system, or both. Conversely, minimal production of methylglyoxal and ROS maintains a high steady-state glutathione levels and glutathione reductase (Glr1) activity in budding *C. albicans* (Ku et al., 2017). These reports suggest that methylglyoxal-induced glutathione deficiency possibly affects glutathione-dependent redox regulation, relevant metabolite pools, and methylglyoxal- and ROS-scavenging systems that can alter pathophysiology (Michán and Pueyo, 2009; Swoboda et al., 1994).

Similarly, we previously found that D-erythroascorbate peroxidase (Eapx1) activity correlates with the detoxification of cellular methylglyoxal and ROS, independently of the antioxidative activity of D-erythroascorbic acid (Kwak et al., 2015). The accumulation of methylglyoxal and ROS caused by the *EAPX1* deficiency, results in the remarkable consumption of glutathione, induced the production of catalase-peroxidase (KatG; CaO19.6229) (Román et al., 2016) and cytochrome c peroxidase (Ccp1; CaO19.7868) (Kwak et al., 2015). This type of Ccp1 (ferrocytochrome-c:hydrogen-peroxide oxidoreductase, EC 1.11.1.5) is known to protect cells from the effects of H_2O_2 (Spangler and Erman, 1986; Yonetani and Ohnishi, 1996). It is localized in the mitochondria of aerobically grown yeasts (Erman and Vitello, 2002; Yonetani and Ohnishi, 1996). However, there is no literature report supporting the contribution of *C. albicans* Ccp1 to pathophysiological process. Moreover, the reciprocal effect of Ccp1 activity on its relevant redox metabolites (i.e., ROS [such as superoxide], glutathione, D-erythroascorbic acid, and pyruvate) that influence methylglyoxal metabolism in the presence or absence of Ccp1 activity are also unknown.

Therefore, we sought to elucidate the effect of Ccp1 activity induced by *EAPX1* deficiency on glutathione-dependent redox regulation and methylglyoxal-detoxifying mechanism during budding growth and differentiation in *C. albicans*. To this end, *CCP1* was disrupted and overexpressed, and we discovered that the *CCP1*-deficient MK909 strain with a steady-state level of methylglyoxal was more susceptible to the increase in H_2O_2 than methylglyoxal. The *CCP1* disruptant exhibited decreased D-erythroascorbic acid and Eapx1 activity, which was mediated by decreased glutathione content and Glr1 activity. The deficiency of *EAPX1* and Glr1-mediated Ccp1 activities induced KatG and Sod activities, leading to H_2O_2 detoxification and methylglyoxal-oxidizing/reducing activity induction, which decreased the methylglyoxal content. We noted that Ccp1 activity regulated the cellular pyruvate content partially by its methylglyoxal-scavenging enzyme activity and, thereby contributed to glycolytic homeostasis. To the best of our knowledge, this is the first report of the involvement of Ccp1 in the enzyme activities of Eapx1 as well as methylglyoxal and ROS scavengers in combination with glutathione-dependent defenses.

2. Materials and methods

2.1. Yeast strains and growth conditions

The *C. albicans* strains used in this study are listed in Table 1. The *C. albicans* cells were cultured in yeast extract peptone dextrose (YPD; 1% yeast extract, 2% peptone, and 2% glucose) and minimally synthetic defined (SD; 2% glucose, 0.5% ammonium sulfate, and 0.17% yeast nitrogen base without amino acids and ammonium sulfate) media (Sherman, 2002). All Ura^+- and Ura^--strains were routinely grown in SD and YPD broth, respectively, and inoculated at an optical density of 1×10^6 cells/mL at 28 °C. Ura^- auxotrophs were selected by supplementation with 625 mg 5-fluoroorotic acid and 25 mg uridine/L, as described previously (Fonzi and Irwin, 1993).

To induce hyphal growth, budding yeast cells were grown overnight at 28 °C in YPD medium. Then, 50–100 cells/plate were cultured on a solid medium mixture containing Spider medium (1% nutrient broth, 1% mannitol, and 0.2% potassium hydrogen phosphate [K_2HPO_4]), Corn meal agar, and synthetic low ammonium dextrose (SLAD)