Effect of Nitric Oxide on the Antifungal Activity of Oxidative Stress and Azoles Against *Candida albicans*

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Abstract Nitric oxide (NO) is a small molecule with a wide range of biological activities in mammalian and bacteria. However, the role of NO in fungi, especially *Candida albicans*, is not clear. In this study, we confirmed the generation of endogenous NO in *C. albicans*, and found that the production of endogenous NO in *C. albicans* was associated with nitric oxide synthase pathway. Our results further indicated that the production of endogenous NO in *C. albicans* was reduced under oxidative stress such as menadione or H₂O₂ treatment. Meanwhile, exogenous NO donor, sodium nitroprusside (SNP), synergized with H₂O₂ against *C. albicans*. Interestingly, SNP could inhibit the antifungal effect of azoles against *C. albicans* in vitro, suggesting that NO might be involved in the resistance of *C. albicans* to antifungals. Collectively, this study demonstrated the production of endogenous NO in *C. albicans*, and indicated that NO may play an important role in the response of *C. albicans* to oxidative stress and azoles.

Keywords Nitric oxide · *Candida albicans* · Oxidative stress · Azoles

Introduction

Candida albicans is one of the most common opportunistic fungal pathogens of humans, which can cause invasive infections with high mortality rate in immunocompromised individuals [1–3]. An important fungicidal mechanism employed by innate immune cells involves the generation of reactive oxygen species (ROS), such as H₂O₂. Nevertheless, *C. albicans* employed strategies to evade the oxidative killing by innate immune cells [4]. The need for effective antifungal therapy is increasing. However, available antifungal agents are limited up to now. Azoles, fluconazole (FCZ) for example, are still widely used in clinic [5, 6]. With the increasing use of the azoles, drug resistance has become a significant challenge [7, 8]. The high mortality rate of invasive infections and increasing drug resistance of *C. albicans* make it significant to study the oxidative stress response and drug resistance mechanisms of *C. albicans* [9].

Nitric oxide (NO) is a highly diffusible free radical, which is acknowledged as an intra- and inter-cellular signaling molecule in both animals and plants [10, 11]. In mammals, NO acts as a critical regulator in nervous, immune and cardiovascular systems [12], and an important mediator in certain pathophysiologic conditions [13]. NO in animals is synthesized by a family of enzymes, known as nitric oxide synthases (NOS), which catalyze the conversion of the amino acid L-arginine to NO and L-citrulline [14]. In plants, algae, and fungi, no true NOS homologs have yet been identified. Nevertheless, there have been a number of publications reporting the existence of NOS-like effect in bacteria [15, 16], yeast [17, 18], and plants [19].

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Notably, the production and physiological activity of the bacterial endogenous NO has been deeply studied. It was reported that bacterial NOS are present in many gram-positive species, which could increase the resistance of bacteria to a broad spectrum of antibiotics, enable the bacteria to survive and share habitats with antibiotic-producing microorganisms [15, 20]. However, whether there is endogenous NO in *C. albicans* is unclear, and few study has focused on the role of NO in *C. albicans* up to now.

In this study, we focused on the production of endogenous NO in *C. albicans*, and explored the effects of NO on the antifungal activity of oxidative stress and azoles against *C. albicans*.

Materials and Methods

Strains and Media

Candida albicans strains SC5314 and Y0109 were used in this study. Strains were routinely grown in YPD (1 % yeast extract, 2 % peptone, and 2 % dextrose) liquid medium at 30 °C in a shaking incubator. DAF-FM DA (diaminofluorescein-FM diacetate) and L-NAME (N-Nitro-L-arginine Methyl Ester, Hydrochloride) were purchased from Beyotime Institute of Biotechnology. Sodium nitroprusside (SNP) FCZ and Miconazole (MCZ) were purchased from Sigma.

Measurement of NO in *C. albicans*

The intracellular levels of NO were assessed using the NO-selective probe DAF-FM DA. Exponentially growing *C. albicans* cells were harvested, washed and resuspended in PBS. After addition of a specific NOS inhibitor L-NAME (100 mM), cell suspensions were incubated at 30 °C under constant shaking (200 rpm) for 1 h [21]. Control group was incubated without L-NAME treatment. *C. albicans* cells were then harvested, washed, and resuspended in PBS containing 5 µM DAF-FM DA, pH 7.4, and incubated at 37 °C for 20 min [22]. After the incubation, cells were washed three times and resuspended in PBS. Living cells were immobilized on 0.1 % poly-L-lysine-coated slides, and observed under a Leica TCS SP2 confocal microscope equipped with argon and helium–neon lasers (Leica, Germany). The mean fluorescence intensity of 10,000 cells per sample was measured by flow cytometry.

Time-Kill Curve Assay

Exponentially growing *C. albicans* cells were harvested and resuspended in fresh YPD medium to 5×10^4 cells/ml. Various concentrations of antifungal agents were added. The cells were cultured at 30 °C under constant

shaking (200 rpm), and OD₆₀₀ was measured at the designated time points (0, 12, 16, 20, 24 h). No antifungal agent was added in the control group. Three independent experiments were performed.

Results

NO Production in *C. albicans*

To date, NO production has not been reported in *C. albicans*. Therefore, we initially investigated the production of endogenous NO in *C. albicans*. A NO-selective probe DAF-FM DA, which has been successfully used to detect NO production in yeast was used in this study [18]. After incubation with DAF-FM DA, *C. albicans* showed bright fluorescence under confocal microscope, indicating NO production (Fig. 1a). Notably, after treatment with 100 mM NOS inhibitor L-NAME, no observable fluorescence was found (Fig. 1a). The mean fluorescence intensity indicating NO was measured by flow cytometry. In accordance with the observation under confocal microscope, after treatment with L-NAME, NO production was reduced by 65 % ($P < 0.001$, Fig. 1b). Collectively, these findings suggested that NO could be produced by *C. albicans*, and the NO production was associated with a NOS-like biological effect.

NO is Involved in the Response of *C. albicans* to Oxidative Stress

We further detected the generation of endogenous NO in *C. albicans* under oxidative stress. *C. albicans* were exposed to menadione and H₂O₂ respectively. The results indicated that the generation of endogenous NO in *C. albicans* was significantly inhibited by menadione and H₂O₂ ($P < 0.001$, Fig. 2). We further investigated the effects of exogenous NO on oxidative stress response of *C. albicans*. SNP, a specific NO donor [21] was used in this work. Although 2 mM H₂O₂ could inhibit the growth of *C. albicans* to some degree, SNP ranging from 3.7 to 7.5 µg/ml didn't show significant antifungal effect (Fig. 3). Interestingly, the combination of 3.7 µg/ml SNP and 2 mM H₂O₂ killed *C. albicans* completely (Fig. 3b). Collectively, our results showed that the generation of endogenous NO in *C. albicans* could be inhibited by oxidative stress and exogenous NO could synergize with H₂O₂ against *C. albicans*.

NO Protects *C. albicans* Against Azoles

Furthermore, we investigated the effects of exogenous NO on the antifungal effect of azoles. Unexpectedly, exogenous NO could attenuate the antifungal effect of azoles. As shown

Fig. 1 Intracellular NO fluorescence of *C. albicans* SC5314 treated with 100 mM L-NAME or not. **a** Fluorescence of intracellular NO. DAF-FM DA, fluorescence field; BF, bright field. **b** The fluorescence intensity of intracellular NO was observed with excitation at 485 nm

and emission at 525 nm. Statistical significance between two groups was determined by Student *t* test. The data are shown as means ± SDs from three independent experiments. ****P* < 0.001

Fig. 2 Intracellular NO fluorescence intensity of *C. albicans* SC5314 treated with menadione (Med) or H₂O₂. The fluorescence intensity was observed with excitation at 485 nm and emission at 525 nm. Statistical significance between two groups was determined by Student *t* test. The data are shown as means ± SDs from three independent experiments. ****P* < 0.001

in Fig. 4a and c, 1 µg/ml FCZ or 0.15 µg/ml MCZ could inhibit the growth of *C. albicans* SC5314 significantly. However, the addition of 3.7 µg/ml SNP attenuated the antifungal effect of azoles. Moreover, with the concentration of SNP increasing, the effect was more obvious (Fig. 4a, c). To confirm this finding, another clinical *C. albicans* strain Y0109 was used, and the results were consistent with the finding on *C. albicans* SC5314 (Fig. 4b, d).

Discussion

To the best of our knowledge, this is the first study focusing on the generation of endogenous NO in *C. albicans*. We found that NO production in *C. albicans* could be inhibited by the specific NOS inhibitor L-NAME, indicating that *C. albicans* may produce NO by a classic NOS mechanism

Fig. 3 Time-kill curves of *C. albicans* SC5314 with the treatment of different concentrations of SNP and H₂O₂

Fig. 4 Time-kill curves of *C. albicans* SC5314 or Y0109 with the treatment of different concentrations of SNP, FCZ and MCZ

catalyzing the conversion of the amino acid L-arginine to NO and L-citrulline [14, 23]. We have carried out bioinformatic analysis on the *C. albicans* genome but failed to identify sequences that are homologous to known NOS (data not shown). It is possible that *C. albicans* may have an enzyme with NOS-like activity, but it is structurally unrelated to classical NOS, which is similar with the findings in yeast and plants [24, 25].

We demonstrate that NO is involved in the response of *C. albicans* to oxidative stress. More specifically, exogenous NO has significant synergistic effect with H₂O₂ against *C. albicans*. Some previous studies reported that NO played an important role in the process of microorganism killing through oxidative stress in macrophage, which is consistent with our findings [26, 27]. Besides, our results indicated that the production of endogenous NO was reduced in *C. albicans* upon oxidative stress, which may be a feedback protection mechanism of *C. albicans* to response to oxidative stress. Consistently, it was reported that nitric oxide dioxygenase coding gene in *C. albicans* was upregulated under oxidative stress or in macrophage, by which *C. albicans* could promote the metabolism of NO to reduce oxidative stress [28, 29].

It is interesting to find that NO could protect *C. albicans* against azoles. A previous finding indicated that NO was synergistic with azoles against *C. albicans* [30], but they did microdilution assays using DETA-NO (Diethylenetriamine NONOate) as the NO donor and 37 °C as the incubation temperature. The different results may be due to the different experimental design. Nevertheless, our findings were

consistent with recent studies in bacteria and yeast. Gusarov et al. [15] and Shatalin et al. [20] reported that in bacteria, NO generated by NOS increases the resistance of bacteria to a broad spectrum of antibiotics. Moreover, Domitrovic et al. [23] reported that low levels of NO exhibited a cytoprotective effect during stress from heat-shock or high hydrostatic pressure in *S. cerevisiae*. Generally speaking, the protection role of NO on microorganisms has been gradually discovered, while the underlying mechanism still needs further investigations [18, 31].

Collectively, we demonstrated the existence of endogenous NO in *C. albicans*, and showed that the production of NO in *C. albicans* involves a NOS-like pathway. Moreover, NO is involved in the response of *C. albicans* to oxidative stress and azoles. The regulatory role of NO in numerous physiological events of fungi is still unclear, and further studies in this area are worthwhile.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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