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Inhibitory effect of terbinafine on reactive oxygen species (ROS) generation by *Candida albicans*

Hemmender Einfluss von Terbinafin auf die Bildung reaktiver Sauerstoffspezies von *Candida albicans*

C. S. Sander[1], U. C. Hipler[1], U. Wollina[2] and P. Elsner[1]

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Schlüsselwörter. *Candida albicans*, ROS-Generation, freie Radikale, Terbinafin, Virulenz.

Summary. *Candida albicans*, the most important opportunistic fungal pathogen, is able to generate remarkable amounts of reactive oxygen species (ROS). Since ROS are highly cytotoxic, this mechanism may contribute to the pathogenicity of this yeast, including its invasiveness and the inflammatory response of the host. Terbinafine, a synthetic antifungal agent of the allylamine class, inhibits ergosterol biosynthesis at the level of squalene epoxidase. Furthermore, there is evidence that terbinafine at therapeutic concentrations can be considered a free radical scavenger *in vitro* and could exert an anti-inflammatory activity *in vivo*. In this study we investigated whether terbinafine affects the generation of ROS by *C. albicans*. Blastoconidia of the *C. albicans* strain 3153A were cultured in YEPG-medium and, subsequently, incubated with different doses of terbinafine (1, 10 and 100 μg ml^{-1}) for 10 and 60 min, respectively. ROS generation was measured by lucigenin-enhanced chemiluminescence. Formation of ROS was considerably dependent on cell number. Chemiluminescence signals were measured at a concentration $\geq 1 \times 10^6$ cells ml^{-1}, with a maximum of 1×10^8 cells ml^{-1}. Already after 10 min of incubation with terbinafine, a dose-dependent significant inhibition of ROS generation was found ($P < 0.05$), whereas after 60 min this effect was amplified. In conclusion, terbinafine reduced the ability of *C. albicans* to generate ROS. Besides the known effect on ergosterol biosynthesis, this mechanism may contribute to its antifungal action.

Zusammenfassung. *Candida albicans*, der wichtigste opportunistische Krankheitserreger unter den Pilzen, bildet große Mengen von reaktiven Sauerstoffspezies (ROS). Da ROS sehr zytotoxisch sind, kann dieser Mechanismus zur Pathogenität dieser Hefe beitragen. Terbinafin, ein synthetisches Antimykotikum der Allylamine-Klasse, inhibiert die Ergosterolbiosynthese auf der Stufe der Squalenepoxidase. Außerdem gibt es Hinweise, daß Terbinafin in therapeutischen Konzentrationen *in vitro* als Radikalfänger fungieren kann und *in vivo* eine anti-inflammatorische Aktivität ausübt. In dieser Arbeit haben wir untersucht, ob Terbinafin die ROS-Bildung von *C. albicans* beeinflußt. Blastoconidien des *C. albicans* Stammes 3153A wurden in YEPG-Medium kultiviert und anschließend mit verschiedenen Terbinafinkonzentrationen (1, 10 und 100 μg ml^{-1}) für 10 bzw. 60 min inkubiert. Die ROS-Bildung wurde mit Hilfe der Lucigenin-verstärkten Chemilumineszenz gemessen. Die Bildung von ROS war stark von der Zellzahl abhängig, Chemilumineszenzsignale wurden ab einer Konzentration von $\geq 1 \times 10^6$ Zellen ml^{-1} mit einem Maximum bei 1×10^8 Zellen ml^{-1} nachgewiesen. Schon nach einer 10-minütigen Inkubation mit Terbinafin konnte eine dosisabhängige signifikante Hemmung der ROS-Bildung gemessen werden ($P < 0.05$), während dieser Effekt nach 60 min noch verstärkt war. In dieser Arbeit konnte gezeigt werden, daß

[1]Department of Dermatology and Allergology, Friedrich Schiller University, Jena, Germany, and [2]Department of Dermatology, Friedrichstadt, Dresden, Germany.

Correspondence: Dr Christina S. Sander, Department of Dermatology, Friedrich Schiller University Jena, Erfurter Strasse 35, D-07740 Jena, Germany.
Tel.: +49–3641–937381 Fax: +49 3641937423
E-mail: christina.sander@derma.uni-jena.de

Terbinafin die Fähigkeit von *C. albicans*, ROS zu bilden, hemmt. Neben dem bekannten Effekt auf die Ergosterolbiosynthese könnte dieser Mechanismus zur antimykotischen Wirkungsweise beitragen.

Introduction

Candida albicans is the most important opportunistic fungal pathogen. The yeast is able to cause candidosis, a disease that can vary from superficial mucosal complaints to life-threatening systemic disorders. Several virulence factors of *C. albicans* are known, including dimorphism, which is the ability to grow in either a yeast-like (blastoconidial) or a mycelial (hyphal) form in response to different environmental factors [1]. The argument that hyphae represent an advantage in tissue invasion seems obvious and widely accepted, but definitive experimental evidence is lacking [2].

Reactive oxygen species (ROS) have received increasing attention as short-acting compounds involved in inflammation and tissue damage. ROS may react with polyunsaturated fatty acids in cellular membranes, nucleotides, and sulfhydryl bonds in proteins [3], and they have been related to the tissue injury in yeast infections [4].

The mitochondria in *C. albicans* are capable of generating extracellularly released ROS [5]. In our previous work we demonstrated that ROS generation by *C. albicans* is dependent on morphogenesis and that their highest levels are found in cells with hyphal form [6]. Thus, ROS generation may play a major role in tissue invasion and infection.

Terbinafine, a synthetic allylamine antifungal agent, inhibits ergosterol biosynthesis at the level of squalene epoxidase. When exposed to terbinafine, fungi accumulate squalene while becoming deficient in ergosterol, an essential component of fungal cell membranes. The inhibition of squalene epoxidase leads to fungistatic and fungicidal actions. Although earlier studies suggested that terbinafine had less activity against *Candida* species, the clinical efficacy of terbinafine in cutaneous *Candida* infections has been proven [7]. Terbinafine has demonstrated good activity against some azole-resistant *C. albicans* strains, and could therefore be a useful therapeutic option in cases of severe superficial *Candida* infections.

Furthermore, there is evidence that, at therapeutic concentrations, terbinafine can be considered a free radical interceptor *in vitro* and could exert an anti-inflammatory activity *in vivo* [8]. The substance is capable of interacting with cell membranes and inhibiting the generation of ROS [9, 10].

In this study, the effect of terbinafine on ROS generation by *C. albicans* was analysed. We investigated whether terbinafine influences the oxidant properties of this pathogen.

Materials and methods

Candida albicans strain 3153A was cultured on Sabouraud glucose (4%) agar (BAG, Lich, Germany). Cultures were prepared in yeast extract peptone glucose (YEPG) medium to obtain yeast cells (blastoconidia) in the stationary phase by incubating for 21 h at 25 °C. Samples at a concentration of 1×10^6 cells ml^{-1} to 1×10^9 cells ml^{-1} were prepared in saline and YEPG medium. *Candida* cells at a concentration of 1×10^8 cells ml^{-1} were incubated in saline with terbinafine (Novartis, Nürnberg, Germany) at concentrations of 1 μg ml^{-1}, 10 μg ml^{-1} and 100 μg ml^{-1} dissolved in dimethylsulphoxide (DMSO; Merck, Darmstadt, Germany). Incubation was performed for 10 min and 60 min at 25 °C in a shaking incubator. The duration was restricted to exclude effects of terbinafine on cell number and growth, which had been studied prior to the experiment.

ROS measurements were carried out using lucigenin-amplified chemiluminescence as described before [11]. Lucigenin solution (*N*,*N*′-dimethyl-9,9′-biacridinium dinitrate; Fluka, Buchs, Switzerland) was prepared in phosphate-buffered saline at a concentration of 0.1 mmol l^{-1} and 200 μl fungal cell suspension was measured in the tube luminometer LB 953 (Berthold, Wildbad, Germany). Chemiluminescence signals were recorded for yeast cells at different cell numbers and for 1×10^8 cells ml^{-1} incubated with terbinafine using the slow kinetic method. The calculation was performed using the average counts per minute (c.p.m.) over measurement periods of 20 min.

All data were expressed as means ± standard deviation. Data were analysed using analysis of variance (ANOVA) for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Generation of ROS was detectable in *C. albicans* blastoconidia. The results were dependent on yeast cell number and on media used for measurement. Chemiluminescence signals could be found at concentrations $\geq 1 \times 10^6$ cells ml^{-1} of *C. albicans* blastoconidia. The maximum was measured at concentrations $> 1 \times 10^8$ cells ml^{-1} (Fig. 1). At higher concentrations, ROS generation was inhibited in both media, which is likely to be a protective mechanism. The media used for measurements strongly affected the total amounts of ROS.

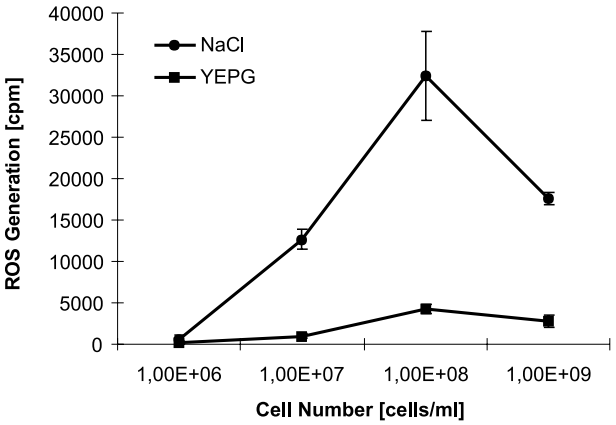

Figure 1. Effect of increasing numbers of *Candida albicans* blastoconidia on generation of ROS, measured by lucigenin-amplified chemiluminescence in NaCl solution and YEPG medium.

Measurements in saline revealed the highest values, which could be explained by the lack of nutrients in this solution and the resulting stress within the cells. Furthermore, YEPG medium presumably possesses light-quenching effects in chemiluminescence measurements [6, 12]. Due to these results, further experiments were carried out with blastoconidia at a cell number of 1×10^8 cells ml^{-1} in saline.

After 10 min of incubation with terbinafine a dose-dependent inhibition of ROS generation was already measurable. DMSO itself as the soluble reagent revealed antioxidant properties. However, the inhibition of ROS generation was considerably dependent on the terbinafine concentration used. At a terbinafine concentration of 1 μg ml^{-1} (T 1) the inhibition was 9.4% and at 100 μg ml^{-1} (T 100) it was 24% (Fig. 2a).

The inhibition was significant when the control incubated with DMSO and the highest chosen terbinafine concentration of 100 μg ml^{-1} (T 100) were compared ($P < 0.05$).

After 60 min of incubation with terbinafine the inhibition was amplified. The final inhibitory effect was 18% for 1 μg ml^{-1} terbinafine (T 1) and 41.1% for 100 μg ml^{-1} terbinafine (T 100) (Fig. 2b). The effect was significant for terbinafine concentrations of 10 μg ml^{-1} (T 10) and 100 μg ml^{-1} (T 100) ($P < 0.05$).

Discussion

Terbinafine, a synthetic allylamine antifungal agent, possesses antioxidant properties. Camera *et al.* demonstrated that at therapeutic concentrations terbinafine can be considered a free radical interceptor *in vitro* and could exert an anti-inflammatory activity *in vivo* [8]. The *in vitro* properties of the drug could be related to its chemical structure. Terbinafine possesses one double and one triple bond, conjugated where an adduct can be formed following exposure to ROS. Furthermore, this agent is capable of interacting with cell membranes and, thus, inhibiting the generation of ROS [9, 10]. Naftifine, another

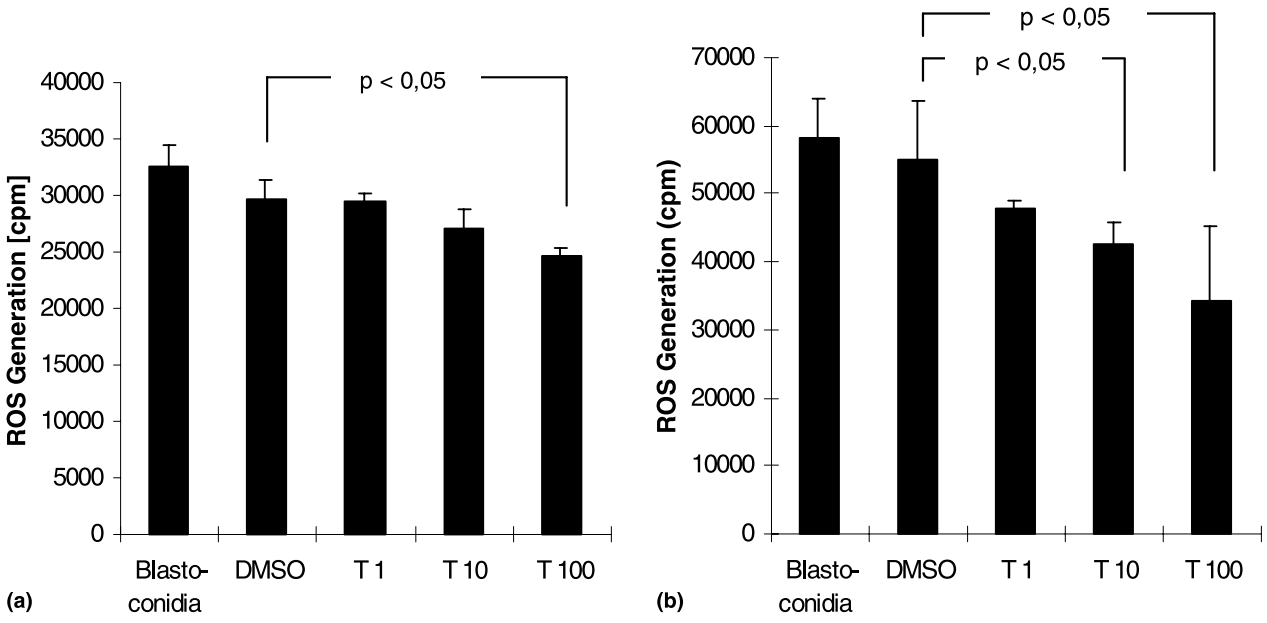

Figure 2. Dose-dependent inhibition of ROS generation by *Candida albicans* (a) after 10 min incubation with different concentrations of terbinafine, (b) after 60 min incubation with different concentrations of terbinafine. Blastoconidia: cell number 1×10^8 cells ml^{-1} in saline. All other samples contain yeast cells and additionally DMSO or terbinafine. DMSO, + 1% DMSO; T 1, + 1 μg ml^{-1} terbinafine; T 10, + 10 μg ml^{-1} terbinafine; and T 100, + 100 μg ml^{-1} terbinafine.

antifungal drug of the allylamine class, has been reported to inhibit chemiluminescence and superoxide anion production of polymorphonuclear leukocytes [13, 14].

As we demonstrated in our previous work, *C. albicans* is able to generate significant amounts of ROS [6] but this ROS generation is dependent on the morphological form of the yeast. The formation of hyphae, usually acknowledged as a major virulence factor in *Candida* pathogenicity, was shown to be associated with markedly increased ROS formation. ROS generation was not closely linked to the ability to form hyphae, but was highest in germinative cells.

In this study, the effects of terbinafine on the generation of ROS by *C. albicans* were investigated in the yeast phase. The results reveal a time- and dose-dependent inhibition of ROS generation by *C. albicans*. After 10 min incubation with 100 $\mu g\ ml^{-1}$ terbinafine a significant reduction of ROS was measured ($P < 0.05$). After 60 min a significant reduction of ROS formation was demonstrated with a terbinafine concentration of 10 $\mu g\ ml^{-1}$ (1%) which corresponds to the dose applied for topical preparations [7].

Due to this time-dependency it is likely that not only radical scavenging properties but also an interaction with the generation of ROS is responsible for the terbinafine-induced inhibition. The remarkable increase of the inhibitory effect after 60 min suggests an inhibition of the ROS generation within the *Candida* cells.

In summary, terbinafine reduces the generation of ROS by *C. albicans*. Besides the known influence on ergosterol biosynthesis, this mechanism may contribute to an antifungal action and could exert an anti-inflammatory effect within the host.

Further investigations are needed to prove whether terbinafine inhibits the generation of ROS as well in the hyphal phase, which is associated with markedly increased ROS generation.

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