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Lipase of *Candida albicans* induces activation of NADPH oxidase and L-arginine pathways on resting and activated macrophages

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ABSTRACT

Candida albicans secretes various hydrolytic enzymes which are considered to be an integral part in the pathogenesis. However, the role of lipases is far from being completely understood and the direct effects of these fungal enzymes during the host–pathogen interaction remain to be established. We recently isolated and characterized an extracellular *C. albicans* lipase (CaLIP), and demonstrated the ability of this fungal enzyme to interact directly with macrophages (Mϕ) and hepatocytes and to operate as a virulence factor. Herein, we explored the effects of CaLIP on Mϕ functions such as oxidative burst and L-arginine metabolism. The study was performed in cells with different activation status: normal-resting Mϕs and Mϕs primed in vivo or in vitro with *C. albicans*. The ability of this fungal factor to modulate the above-mentioned parameters was dependent on cells status, dose, and microenvironment, where the interaction took place. These results constitute a new finding in the biology of candidiasis and could illustrate an additional evolutive advantage for the fungus in the framework of the bidirectional host–pathogen interaction.

Introduction

The pathogenicity of *Candida albicans* depends on its ability to deal effectively with the host defenses, particularly with the oxidative burst of phagocytic cells. Activated macrophages (Mϕs) and polymorphonuclear leukocytes (PMN) develop an efficient tissue reaction to control the fungus and limit its growth [1]. The reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), hypochlorous acid (HOCl), and reactive nitrogen intermediates as nitric oxide ($NO^{\cdot}$) produced at high concentrations are directly involved in the killing of this microorganism [1–3]. The powerful host-protective effects of $NO^{\cdot}$ are counterbalanced by the immunopathological role of this compound, as high levels of $NO^{\cdot}$ can affect Mϕ integrity by lytic action or apoptosis induction [4]. Consequently, a tight regulation of $NO^{\cdot}$ synthesis appears to be crucial for the host. In this respect growing interest is focused on arginase up-regulation, an enzyme that competes with NO synthase for the substrate L-arginine and is related with the alternate activation pathway in Mϕs [5].

Pathogenic fungi, including *C. albicans,* secrete various hydrolytic enzymes [6]. While secreted phospholipases are well characterized [7,8], other lipolytic enzymes such as lipases and esterases have been widely neglected [6]. The direct effects of these fungal enzymes during the host–pathogen interaction remain to be established [6,9–12]. An important contribution in this field was recently made by our group. We isolated and characterized an extracellular *C. albicans* lipase (CaLIP), and established its ability to interact with host cells actively compromised during *C. albicans* dissemination [13]. We demonstrated, for the first time, that *C. albicans*-released lipase induces cytotoxicity and promotes the deposition of lipid droplets in the cytoplasm of Mϕs and hepatocytes after 48–72 h of incubation [13]. These findings correlate with our previous reports on the induction of hepatic steatosis and the impaired Mϕ function 72 h after *C. albicans* infection [14,15].

The aim of the present study was to investigate the effects of a secreted *C. albicans* lipase on NADPH oxidase and L-arginine pathways in resting or primed Mϕs. Our in vitro results demonstrate that this fungal factor *per se* can modify the host response throughout the modulation of ROS production, $NO^{\cdot}$ release and Mϕ alternative pathway activation.

Materials and methods

Strain and lipase purification. C. albicans strain No. 387 from the collection of Mycology Division of National Clinical Hospital was used for infection and lipase purification [14–17]. Extracellular lipase was purified from cell-free supernatants obtained from 72 h-cultures in liquid nutritive broth as described [13]. The purity

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of the preparation was tested in SDS–PAGE on a 13% polyacrylamide slab gel using Miniprotean II electrophoresis apparatus (Bio-Rad, Richmon, CA), and the activity of the purified enzyme was expressed as U of lipolytic activity [18]. The secreted lipase, designed as CaLIP, exhibited its maximal activity at 37 °C and pH 7–7.5 [13].

Animals, infection and macrophage purification. Adult female Wistar rats were housed in the Animal Resource Facilities, CIBICI-CONICET. For infection, rats were inoculated i.p. (3×10^8 yeasts) and after 72 h the animals were killed and peritoneal cells were recovered [15]. The cell suspension was centrifuged and washed with RPMI and adjusted to 2×10^6 cells/ml in RPMI–10% FCS–0.1% gentamicin. Mϕs were purified by adherence in six-well flat-bottom plates (Corning). After 2 h of incubation (37 °C, 5% CO_2), plates were washed with cold RPMI to remove non-adherent cells. Mϕ monolayer was >90% pure according to morphologic analysis or non-specific esterase staining (Sigma) [16]. Viability was assessed by the Trypan blue.

Experimental design. Three different Mϕ populations were used: (1) normal-resting Mϕs obtained from untreated rats (NR-Mϕs); (2) activated Mϕs obtained from infected rats 3 days after fungus inoculation (Ca-Mϕs); and (3) Mϕs obtained from normal rats and activated in vitro for 2 h with heat-killed *C. albicans* (100 °C/15 min) at a 1:10,000 ratio (NR-Mϕ + Ca). Cells were cultured with medium alone, or with 25 U or 100 U CaLIP, two doses tested previously [17]. As positive control, 1 mg/ml phorbol myristate acetate (PMA, Sigma) or 1 μg/ml LPS from *Escherichia coli* (055:B5, Sigma) was used.

Cell damage. Cell damage was measured by the release of lactate dehydrogenase (LDH) with UV-optimized method (Wiener Lab, Argentina) [13]. The release of LDH was expressed as LDH index calculated as the ratio between the LDH released by CaLIP-treated cells versus LDH released in basal conditions for each experimental protocol.

Oxidative metabolism of Mϕs. The intracellular and extracellular production of ROS were tested in 18 h cultures by the reduction of nitro blue tetrazolium (NBT) [19]. Briefly, 10^5 Mϕs in 0.1 ml HBSS were incubated with 0.1 ml of CaLIP and 0.5 ml of NBT (1 mg/ml) at 37 °C for 30 min. The reaction was stopped with 0.1 ml of 0.1 M HCl; the samples were centrifuged, and the pellet was treated with 0.4 ml dimethyl sulfoxide and 0.8 ml HBSS. Optical density was measured at 540 nm.

The extracellular ROS production was also detected by chemiluminescence (CL) produced after luminol oxidation by $O_2^{\cdot-}$ [19]. Briefly, the CL assay was assessed at 6, 18, and 24 h in polypropylene tubes containing 10^5 Mϕs, 0.1 ml of 3.36 μM luminol (Sigma), and 0.1 ml of purified CaLIP in a final volume of 0.5 ml of HBSS. CL was measured at room temperature in a BioOrbit model 1253 luminometer. The spontaneous CL was determined by incubating Mϕs without CaLIP, and the CL background of each vial was checked before use. The light emission was expressed as relative units of light (RUL) per 10^6 cells. PMA (0.1 mg/ml) and heat-inactivated (100 °C) CaLIP were used as controls [19].

Assessment of L-arginine metabolism. Mϕs were cultured with medium or exposed to different treatments. After 24 or 48 h, the supernatants and the cells were sampled for NO· measurement and arginase activity. The NO· was evaluated as nitrite by a microplate assay method using the Griess reagent and $NaNO_2$ as standard [15]. Each sample was tested by triplicate, and the results were expressed in micromolar (μM).

To assess arginase activity cell monolayers were washed with PBS and treated with 0.15 ml of 0.1% Triton X-100 containing protease inhibitors. After 30 min, cell lysates were mixed with 10 mM $MnCl_2$ 1:1, and the enzyme was activated by heating for 10 min at 56 °C. Arginine hydrolysis was conducted by adding 0.5 M L-arginine, pH 9.7, to the activated lysates [20]. The urea formed was quantified at 540 nm after the addition of 25 μl of 9% α-isonitrosopropiophenone. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per minute. Results are expressed as microgram (μg) urea/min.

Immunoblot analysis. iNOS expression was assessed in Mϕs stimulated for 48 h. Whole cell extracts were prepared as described previously [17]. Equal amounts of protein (30 μg/lane) were fractionated in a 10% SDS–polyacrylamide gel electrophoresis, electrotransferred onto nitrocellulose membranes, and incubated with 2 μg/ml anti-iNOS polyclonal antibody (Santa Cruz Biotechnol). Immunodetection was performed with the enhanced chemiluminescence kit followed by exposure to Amersham Hyperfilm (Uppsala, Sweden).

Statistical analysis. Differences between means were assessed using ANOVA followed by Student–Newman–Keuls test. A p value <0.05 was considered significant.

Results

Effect of CaLIP on ROS production in normal-resting Mϕs

To determine the ability of CaLIP to induce oxygen-free radical formation in NR-Mϕs, we evaluated the production of intracellular and extracellular ROS by NBT reduction after 18 h of incubation with 25 or 100 U of CaLIP (Fig. 1A). In agreement with the resting status, NR-Mϕs exhibited a low spontaneous release of oxidant species; after the stimulation with PMA, the cells produced and released significant amounts of ROS (100% of stimulation). Interestingly, CaLIP was able to induce ROS increase at both concentrations tested, although a stronger effect was observed at 25 U. Comparatively, intracellular and extracellular host stimulated with CaLIP represent 58% and 52% for 25 U, and 40.5% and 36.2% for 100 U, of the values obtained with PMA.

To assess ROS production with a more sensitive technique, NR-Mϕs were cultured with both doses of CaLIP and evaluated by CL after 6, 18, and 24 h of culture. PMA was used as positive control. While ROS were absent at 6 h, NR-Mϕs produced significant levels of the oxygen radicals after 18 and 24 h of contact with CaLIP; the highest production was detected for 25 U at 18 h of culture (Fig. 1B). Considering the lower levels of ROS detected with 100 U of CaLIP or after prolonged treatment, we evaluated the toxic activity of the fungal factor by LDH release. Fig. 1C shows that at both doses and at different times the integrity of Mϕs was preserved.

Effect of CaLIP on NO· production, iNOS expression, and arginase activity in normal-resting Mϕs

In response to infection with several pathogens and inflammatory cytokines, noticeable changes occur in Mϕ arginine metabolism [5,20]. These include, for instance, increment in NO· synthesis via iNOS and catabolism of arginine to ornithine and urea via arginase. To evaluate the effect of CaLIP on L-arginine pathways, we treated NR-Mϕs (25 and 100 U) during 24 or 48 h. PMA and LPS triggered a significant NO· production (Fig. 2A). We found a dose-dependent stimulation of NO· production with higher levels after 48 h of culture. Accordingly, the iNOS protein (~130 kDa) evaluated by Western blot (inset) exhibited a stronger expression after the incubation with the highest dose tested.

The L-arginine metabolism regulation in cells that possess both iNOS and arginase activities is poorly understood. Then, we evaluated the effect of 25 and 100 U of CaLIP on Mϕ arginine metabolism after 48 h of culture. PMA and LPS were used as activation controls [21]. Interestingly, a significant increment in urea levels was detected after CaLIP contact and the major effect was observed with