Brazilian Journal of Medical and Biological Research (2004) 37: 1455-1461
ISSN 0100-879X

Respiration, oxidative phosphorylation, and uncoupling protein in *Candida albicans*

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Abstract

The respiration, membrane potential ($\Delta\psi$), and oxidative phosphorylation of mitochondria *in situ* were determined in spheroplasts obtained from *Candida albicans* control strain ATCC 90028 by lyticase treatment. Mitochondria *in situ* were able to phosphorylate externally added ADP (200 μM) in the presence of 0.05% BSA. Mitochondria *in situ* generated and sustained stable mitochondrial $\Delta\psi$ respiring on 5 mM NAD-linked substrates, 5 mM succinate, or 100 μM N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride plus 1 mM ascorbate. Rotenone (4 μM) inhibited respiration by 30% and 2 μM antimycin A or myxothiazole and 1 mM cyanide inhibited it by 85%. Cyanide-insensitive respiration was partially blocked by 2 mM benzohydroxamic acid, suggesting the presence of an alternative oxidase. *Candida albicans* mitochondria *in situ* presented a carboxyatractyloside-insensitive increase of $\Delta\psi$ induced by 5 mM ATP and 0.5% BSA, and $\Delta\psi$ decrease induced by 10 μM linoleic acid, both suggesting the existence of an uncoupling protein. The presence of this protein was subsequently confirmed by immunodetection and respiration experiments with isolated mitochondria. In conclusion, *Candida albicans* ATCC 90028 possesses an alternative electron transfer chain and alternative oxidase, both absent in animal cells. These pathways can be exceptional targets for the design of new chemotherapeutic agents. Blockage of these respiratory pathways together with inhibition of the uncoupling protein (another potential target for drug design) could lead to increased production of reactive oxygen species, dysfunction of *Candida* mitochondria, and possibly to oxidative cell death.

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Research supported by CNPq/PRONEX (No. 66.1192/1997-2), FAPESP (Nos. 98/13012-5 and 00/08019-2), and CAPES.
R.A. Cavalheiro and V.C. Faustinoni are supported by CAPES. F. Fortes is the recipient of a doctoral fellowship from CNPq, and J. Borecký is the recipient of Visiting Professor from CNPq (No. 305381/02-4 NV).

Received November 18, 2003
Accepted June 8, 2004

Key words
- *Candida albicans* spheroplasts
- Mitochondria
- Respiratory chain
- Mitochondrial membrane potential
- Uncoupling protein

Introduction

Candidiases are common infections of the skin, oral cavity, esophagus, gastrointestinal tract, vagina, and vascular system, and have become a major cause of mortality in immunocompromised patients, including those with AIDS (1-3) or debilitated in some other way (4). From 2001 to 2003, a total of 2803 *Candida* spp strains were isolated from various tissues of patients from the University of Campinas Hospital, UNICAMP, Brazil (5). The strains were identified and classified into the following species: *C. albicans*

(57%), *C. tropicalis* (13%), *C. glabrata* (10%), *C. parapsilosis* (6%), and *C. krusei* (3.4%), and 10.6% belonged to other yeast genera (5). The chemotherapy currently used against these fungi has a number of deficiencies such as low specificity, toxicity, and drug resistance. Most antifungal drugs react with ergosterol or inhibit its production (6); however, there is an alarming increase in resistance to antifungal agents (3,7). Therefore, it is very important to search for biological targets that could be exploited for the rational development of improved therapies.

Mitochondria are the major source of cellular aerobic energy generated by oxidative phosphorylation. *C. parapsilosis* mitochondria possess three types of respiratory pathways (8): i) the classical respiratory chain, ii) a secondary parallel respiratory chain (PAR) consisting of alternative ubiquinone, cytochromes ($cytb_{PAR}$ and $cytc_{PAR}$), and a terminal oxidase (oxc_{PAR}) insensitive to antimycin A but inhibited by amytal (9) and by high concentrations of salicylhydroxamic acid (SHAM), myxothiazol (10), and KCN (11), and finally iii) the constitutive alternative oxidase (12,13). The high resistance of *C. parapsilosis* to drugs (14) has been attributed to the presence of these two alternative electron transfer systems (8). Recently, Helmerhorst et al. (13) reported that *C. albicans* also contains a variety of respiratory systems whose expression depends on growth conditions. The authors reported that the *C. albicans* ability to regulate the expression of individual complexes and the partitioning of electrons between both respiratory chains may be the reason why the cells can survive under conditions of oxidative stress.

Another mechanism of cell protection against oxidative stress may involve the recently described isoforms of uncoupling protein (UCP, now UCP1; 15). The demonstration of various UCP homologues in plants (16-18) and animals (17-18) raised the question of their true physiological role (18-21) that should be more general than that of UCP1 - the production of heat in brown adipose tissue of hibernating mammals (15). Evidence has been presented that the new UCPs are involved in cell defense against oxidative stress (22-27). They are able to induce mild uncoupling, thus increasing the rate of respiration and decreasing the production of reactive oxygen species (ROS) (24-27).

A UCP was also detected recently in *C. parapsilosis* (28). Therefore, it is of great interest to study *C. albicans* UCP in order to understand its role in the ability of these cells to resist stress. In the present study, we demonstrated that spheroplasts obtained from cultures of *C. albicans* in the middle of their exponential phase of growth possess intact mitochondria able to phosphorylate ADP and an uncoupling protein (CaUCP) homologous to the previously described *C. parapsilosis* UCP (CpUCP) (28).

Material and Methods

Candida albicans

C. albicans ATCC 90028 was obtained from the American Type Culture Collection. It is a quality control strain for antifungal susceptibility testing and was maintained in sterile water and Sabouraud Dextrose Agar (BD, Difco, Franklin Lakes, NJ, USA). The cell cultures were grown in YEPG medium (29) at 37ºC and 200 rpm. To determine the inflexion point of exponential growth, samples were collected at 1-h intervals and culture density was determined spectrophotometrically at 530 nm. Experimental points were fitted using a logistic dose-response model and the maximum growth rate during the exponential phase was calculated as its power parameter.

Spheroplast preparation

One liter of culture was harvested by

centrifugation for the standard preparation (29) and cells were washed once with cold water and once with buffer A (1 M sorbitol, 10 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.8). Cells were resuspended in buffer A (3 ml/g of cells) supplemented with 30 mM dithiothreitol. After 15 min at room temperature with shaking, cells were harvested, resuspended in buffer A containing lyticase (1 mg/g of cells) and 1 mM DTT, and incubated at 30ºC until about 90% of cells were converted to spheroplasts (~60 min). The reaction was stopped with an equal volume of ice-cold buffer A and spheroplasts were washed twice with buffer A. The protein concentration of the final suspension was determined by the biuret method (30) in the presence of 0.2% deoxycholate.

Isolation of mitochondria

The spheroplasts were resuspended in buffer B_1 (0.6 M mannitol, 1 mM EDTA, 0.5% BSA, 1 mM PMSF, and 10 mM Tris-HCl, pH 7.4). Spheroplasts were broken mechanically using a Dounce homogenizer with a maximum of 10 strokes and cell debris were removed by centrifugation at 1,000 *g* for 10 min. Mitochondria were pelleted by 10-min centrifugation at 10,500 *g* and washed with buffer B_2 (0.6 M mannitol, 1 mM EDTA, 1% BSA, 10 mM Tris-HCl, pH 7.0). The last wash was made in buffer B_2 medium without BSA and EDTA (28) and mitochondrial protein concentration was determined by the biuret method (30).

Mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\psi$) of permeabilized cells was monitored by measuring the fluorescence spectrum of safranine O with a Hitachi F4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at excitation-emission wavelength of 495-586 nm (31). All experiments were performed at 28ºC in 2 ml of standard incubation medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH 7.2, 2.5 mM KH_2PO_4, and 1 mM $MgCl_2$, plus 1 mg mitochondrial protein.

Oxygen uptake

Oxygen uptake was measured polarographically at 28ºC using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) in 1.3 ml of standard incubation medium containing 1 mg mitochondrial protein (28).

Immunodetection of *Candida albicans* UCP

Fifty micrograms of total proteins extracted from spheroplasts prepared from cultures of *C. albicans* and *C. parapsilosis* (positive control) was separated on 10% polyacrylamide gel by standard SDS-PAGE. Protein bands were transferred to a nylon membrane (Hybond N) with a semi-dry blotting apparatus (Amersham Biosciences AB (Pharmacia) Uppsala, Sweden). The membrane was blocked overnight in 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween 20, and 10% (w/v) non-fat dry milk and incubated with the anti-AtPUMP1 polyclonal antibody (1:1000 dilution). After incubation with an anti-rabbit IgG alkaline phosphatase conjugate (1:5000 dilution), the membrane was developed in the dark in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 12.5 µM CSPD for 5 min. The bands were visualized by autoradiography and scanned with the Eagle-Eye photo documentation system (Eagle Eye Photo, Homer, AK, USA) (32).

Chemicals

ATP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone, succinate, ethylene ethylenediaminetetraacetic acid, antimycin A, myxothiazol, carboxyatractyloside (CAT), BSA, linoleic acid, KCN, N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride

(TMPD), and lyticase were purchased from Sigma (St. Louis, MO, USA). Disodium 3-(4-methoxyspiro{1,2-dioethane-3,2'-(5'-chloro) tricyclo [3.3.1.1^{3,7}) decane}-4-yl] phenylphosphate (CSPD) was from Tropix (Applied Biosystems (Tropix), Foster City, CA, USA). All other reagents were of analytical grade.

Results and Discussion

In the present study we characterized mitochondrial bioenergetic pathways in *C. albicans* ATCC 90028 using a method previously applied to *C. parapsilosis* (29).

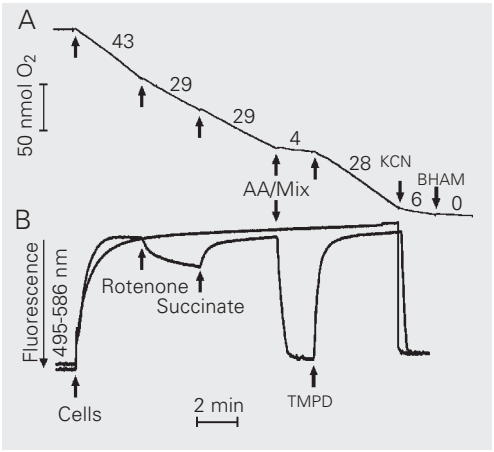

Figure 1. Effect of substrates and inhibitors on *Candida albicans* spheroplast oxygen consumption (A) and mitochondrial membrane potential (B). *C. albicans* spheroplasts (1 mg/ml) were incubated in reaction medium containing 125 mM sucrose, 10 mM HEPES, pH 7.2, 2 mM P_i, 1 mM $MgCl_2$, 65 mM KCl, and 5 mM cocktail (malate, glutamate, pyruvate, and α-ketoglutarate). The additions of the substrates 5 mM succinate and 100 µM TMPD/ascorbate (TMPD), and the inhibitors 4 µM rotenone, 2 µM antimycin A (AA) and/or 2 µM myxothiazol (Mix), 1 mM KCN, and 2 mM BHAM, are indicated by the arrows. Numbers on the trace indicate O_2 consumption rates in nmol O_2 min^{-1} mg protein^{-1}. TMPD = tetramethyl-p-phenylenediamine dihydrochloride; BHAM = benzohydroxamic acid.

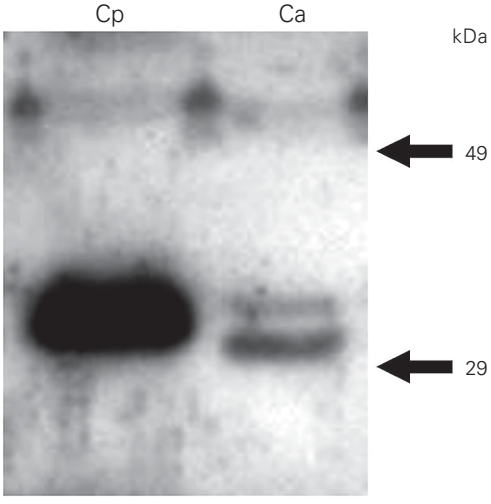

Figure 2. Immunodetection of uncoupling proteins in a total protein extract of *Candida albicans* (2 bands in lane Ca) with polyclonal antibody raised against *Arabidopsis* PUMP1. A *C. parapsilosis* cell protein extract containing CpUCP (single band in lane Cp) was used as a positive control. The positions of molecular mass standards are indicated by the arrows.

In order to search for the presence of UCP and to examine its activity in these mitochondria we characterized the classical respiratory chain and its ability to sustain $\Delta\psi$ by oxidizing different respiratory substrates. Figure 1 shows that spheroplasts prepared from cell cultures grown to the middle exponential phase were permeable to mitochondrial substrates and inhibitors without affecting the functional integrity of the spheroplast mitochondria. These spheroplast mitochondria suspended *in situ* in reaction medium containing a cocktail of NAD-linked substrates generated and sustained a stable membrane potential and respiration rate of 43 nmol O_2 min^{-1} mg protein^{-1} (Figure 1). The subsequent inhibition of complex I by 4 µM rotenone resulted in a decrease of about 30% in the respiration rate (29 nmol O_2 min^{-1} mg protein^{-1}) and $\Delta\psi$. This suggests a significant contribution of this complex to $\Delta\psi$ generation, in agreement with the results obtained by Helmerhorst et al. (13). They found the presence of a rotenone-sensitive and a proton pumping NADH-Q oxidoreductase similar to complex I in *C. albicans* ATCC 10231. The addition of 5 mM succinate restored $\Delta\psi$ but total oxygen consumption level was unchanged (29 nmol O_2 min^{-1} mg protein^{-1}). The increase in $\Delta\psi$ by succinate addition was certainly the consequence of an increased electron flux through the classic respiratory pathway (coupled respiration - the only one that increases $\Delta\mu_H+$) associated with some alteration in the partitioning of electrons among the three respiratory pathways (classic, parallel, and via alternative oxidase) present in *Candida* yeast (13), resulting in a decrease in electron flux through the uncoupled pathways. Therefore, the total respiration rate was not modified by the addition of succinate. Blockage of complex III by 2 µM antimycin A or 2 µM myxothiazol promoted collapse of $\Delta\psi$ and a reduction of more than 90% in respiration (4 nmol O_2 min^{-1} mg protein^{-1}). Addition of 100 µM

TMPD/1 mM ascorbate restored the respiration rate (28 nmol O_2 min^{-1} mg $protein^{-1}$). Finally, inhibition of complex IV by 1 mM KCN was accompanied by a complete loss of $\Delta\psi$ and by a decrease of about 85% in respiration rate (6 nmol O_2 min^{-1} mg $protein^{-1}$). Drastic changes in $\Delta\psi$ after inhibition of complexes III and IV suggest the essential role of these complexes in energy conservation by *C. albicans* mitochondria. The CN^--insensitive respiration was inhibited by 2 mM benzohydroxamic acid, suggesting the presence of alternative oxidases, as previously described (8,13). In contrast, Helmerhorst et al. (13) reported that respiration by *C. albicans* ATCC 10231 cells was almost insensitive to 5 mM SHAM when grown in the absence of antimycin A. On the other hand, respiration by cells grown in the presence of 10 µM antimycin A was completely inhibited by 5 mM SHAM, indicating that the alternative pathway was the only pathway utilized by *C. albicans* mitochondria when the classical cytochrome pathway was inhibited at the cytochrome *c* oxidase level.

An uncoupling protein, another energy-dissipating system that can also modulate $\Delta\psi$ and the respiration rate, was recently identified in the fungus kingdom as CpUCP from *C. parapsilosis* (28). Interestingly, UCP is absent in *Saccharomyces cerevisiae* (33). In the present study, the existence of a CpUCP homologue (CaUCP) was also detected in *C. albicans* mitochondria by immunoblot analysis using anti-AtPUMP1 antibody (32) and CpUCP (line Cp) as a positive control. The immunoblot revealed 2 protein bands of about 32 kDa (Figure 2, lane Ca) in a sample of total protein extract from *C. albicans* cells. The existence of two bands in *C. albicans* suggests the presence of more than one UCP, as observed in mammals and plants (17,18,34,35).

Uncoupling proteins are known to be activated by free fatty acids (FFAs) and inhibited by ATP (36-39). Accordingly, *in situ* $\Delta\psi$ measurements in *C. albicans* mitochondria demonstrated an increase of $\Delta\psi$ after consecutive additions of 5 mM ATP and 0.5% BSA, which binds non-covalently FFAs (Figure 3A, trace b) as compared to control experiments without additions (trace a). The dotted line (c) shows the $\Delta\psi$ generated by *C. albicans* mitochondria in the presence of ATP and BSA from the beginning of the experiment. In contrast, the addition of linoleic acid caused a decrease in $\Delta\psi$ that was reversed by BSA (Figure 3B, trace e), reaching levels higher than control at the end (trace d), probably because BSA also binds the endogenous FFAs. These experiments were performed in the presence of 1 µM CAT to prevent the contribution of the ADP/ATP carrier to the FFA-induced H^+ re-uptake through FFA anion translocation (23). Indeed, it is known that the ADP/ATP carrier in the absence of its substrates ADP and ATP can translocate FFA (23). In addition, the presence of glutamate, pyruvate, and malate/α-ketoglutarate prevented fatty acid anion transport through the corresponding carriers (23).

Accordingly, in experiments with isolated *C. albicans* mitochondria, the ADP/O

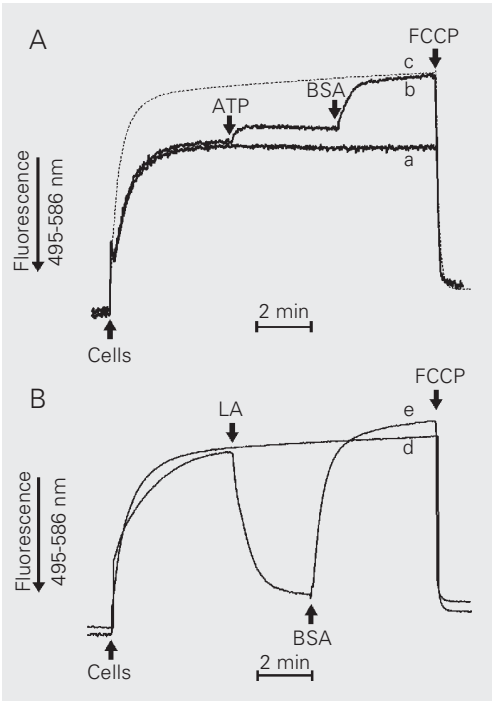

Figure 3. Effect of ATP and BSA (A) or LA (B) on mitochondrial $\Delta\psi$ of *Candida albicans* spheroplasts in the presence of 1 µM CAT. Spheroplasts (1 mg/ml) were added to standard medium containing 5 mM substrate cocktail (malate, glutamate, pyruvate, and α-ketoglutarate) and 5 µM safranine O. Additions of 5 mM ATP (line b), 0.5% BSA (lines b and e), 10 µM LA (line e), and 1 µM FCCP are indicated by the arrows. Lines *a* and *d* are controls without additions and line *c* is a control with ATP and BSA present from the beginning of the experiment. BSA = bovine serum albumin; CAT = carboxyatractyloside; $\Delta\psi$ = membrane potential; FCCP = carbonyl cyanide p-trifluoromethoxyphenylhydrazone; LA = linoleic acid.

ratio decreased from 2.4 to 1.1 in the presence of 2 µM linoleic acid, whereas state 4 respiration, observed after ADP phosphorylation, was stimulated by about 75% (Figure 4). These results mean that the linoleic acid-induced H^+ recycling can efficiently divert energy from oxidative phosphorylation in state 3 respiration even if the state 3 respiration rate is not modified, in agreement with Jarmuszkiewicz et al. (28). Therefore, the observed uncoupling of oxidase phosphorylation caused by linoleic acid can be attributed to CaUCP. Figure 5 shows that, in the presence of ATP and BSA, *C. albicans* mitochondria can efficiently phosphorylate ADP *in situ*, as indicated by the transient $\Delta\psi$ decrease induced by the addition of ADP.

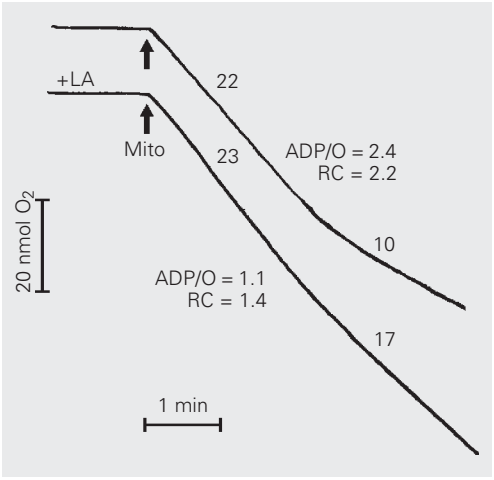

Figure 4. Effect of linoleic acid (LA) on oxidative phosphorylation. *Candida albicans* mitochondria (1 mg/ml) were added (as indicated by the arrow at "Mito") to 1.3 ml standard incubation medium (28ºC) with 5 mM substrate cocktail (malate, glutamate, pyruvate, and α-ketoglutarate) and 200 µM ADP in the absence or in the presence of 2 µM LA (+LA). The numbers on the traces indicate O_2 consumption rates in nmol O_2 min^{-1} mg $protein^{-1}$. RC = respiratory control; ADP/O = ADP/O ratio.

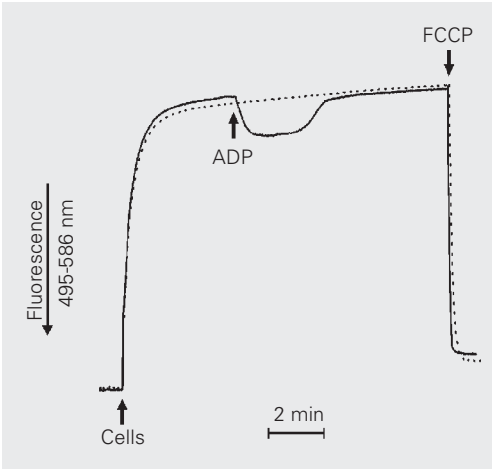

Figure 5. ADP phosphorylation by *Candida albicans* spheroplast mitochondria. Spheroplasts (1.0 mg/ml) were incubated in reaction medium containing 5 mM substrate cocktail (malate, glutamate, pyruvate, and α-ketoglutarate), 0.05% BSA, and 5 µM safranine O. Spheroplasts (cells), 200 µM ADP, and 1 µM FCCP were added where indicated by the arrows. Dotted line represents a control experiment without ADP addition. FCCP = carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

The presence of uncoupling protein in *Candida* ssp implies a role of this protein in yeast mitochondrial energy metabolism and raises the possibility of its involvement in cell protection against ROS overproduction. This protective role against ROS has been described for UCP2 (22) and UCP3 (25). Mitochondria from underexpressing mice had significantly higher levels of oxidative damage than wild-type controls (26). In plants, leaf discs of transgenic tobacco plants overexpressing AtPUMP1 showed an increase in the tolerance to oxidative stress promoted by exogenous hydrogen peroxide compared to wild-type control plants (24).

The presence in *C. albicans* of an alternative electron transfer chain (13) and alternative oxidase (39) absent in animal cells offers exceptional targets for the design of new chemotherapeutic agents. Blockage of these respiratory pathways and/or inhibition of the uncoupling protein (another target for drug design) could lead to mitochondrial dysfunction, increased production of ROS, and possibly to cell death.

Acknowledgments

The authors thank Prof. Dr. R. Docampo, University of Illinois at Urbana-Champaign, Urbana, IL, USA, for a critical reading of the manuscript.

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