

Effect of nitric oxide on H^+ -efflux in presence of various nutrients in *Candida albicans*

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In the present study tentative link has been established between H^+ -efflux and effect of NO in presence of various nutrients (glucose, 2-deoxy-D-glucose, xylose, proline, glutamic acid and lysine) in *C. albicans* using sodium nitroprusside (SNP) as a potent source of NO. It was observed that there was a decreasing trend in pH with time, in control, while SNP treated cells showed an initial decline in pH for 10-15 min, followed by an increase in pH up to 30 min. In presence of glucose there was an enhancement in H^+ -efflux by 9-fold whereas proline, glutamic acid and lysine showed enhancement by 3, 6 and 1.5-fold respectively. Similar trends in increase in pH after 15 min in SNP treated cells of *Candida* was observed in presence of all nutrients used. It was demonstrated for the first time that H^+ -ATPase of *C. albicans* was affected by NO.

Keywords: *Candida albicans*, H^+ -efflux, H^+ -ATPase, Nitric oxide, Nutrients, Sodium nitroprusside

Candida albicans is a common opportunistic pathogen in immuno-compromised patients and its dimorphic property is generally considered to be a virulent factor¹. Phagocytosis by macrophages linked with release of nitric oxide (NO) is probably the most important mechanism in protecting the non-compromised host against candidiasis². It has recently been identified that NO acts as a potent effector molecule³, released mainly by phagocytes performing vital roles in vascular cell signalling and immune system⁴. It may also contribute to the morbidity of infection by acting as a cytotoxic mediator. NO related antimicrobial activity has been demonstrated against a remarkably broad range of pathogenic microorganisms including viruses, bacteria, fungi and parasites⁵. It has been reported that prolonged exposure to NO inhibits activity of a number of enzymes such as aconitase and cytochrome *c* oxidase by triggering the production of peroxynitrite (ONOO⁻) in mitochondria leading to the depletion in ATP concentration^{6,7}. However, little data is available about the susceptibility of *Candida albicans* to NO *in vitro* in the absence of macrophages⁸.

It is well-known that *C. albicans* and other yeasts possess H^+ -ATPase that nurtures intracellular pH and generates an electrochemical gradient of protons necessary for secondary transport systems. It uses the

free energy of ATP hydrolysis to translocate protons from the cell interior to the medium. Earlier we have reported that *C. albicans* exhibited a strong stimulation of H^+ -efflux in presence of nutrients⁹ (glucose, proline, and glutamic acid). Therefore, we have tried to establish a link between H^+ -efflux and effect of NO in presence of various nutrients in *C. albicans*. We have used sodium nitroprusside (SNP) as a potent source of NO¹⁰, a compound widely used by several workers for *in vitro* studies.

Materials and Methods

Chemicals—All biochemicals and sodium nitroprusside were obtained from Sigma-Aldrich, USA and all inorganic chemicals were from Merck (India).

Candida albicans (ATCC 10261) was obtained from Dr. Rajendra Prasad, Jawaharlal Nehru University, New Delhi. Stock cultures were maintained on slants of nutrient agar (yeast extract 1%, peptone 2%, D-glucose 2% and 2.5% agar) at 4°C. To initiate growth for experimental purposes, one loop full of cells from an agar culture were inoculated into a 25 ml of YEPD nutrient medium and incubated at 30°C for 24 hr i.e., up to stationary phase (primary culture). Primary culture (500 µl; 10⁸ cells ml⁻¹) were re-inoculated into 100 ml fresh YEPD medium and grown for 8-10 hr i.e., up to mid-log phase (secondary culture), which contains 10⁶ *Candida* cells ml⁻¹.

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The nutrients, glucose, 2-deoxy-D-glucose, xylose, proline, glutamic acid and lysine, were chosen based on the fact that H⁺ gradient is crucial for transport of neutral and charged compounds¹¹. Amino acids especially proline and glutamic acid have a special role in inducing dimorphism in *C. albicans*. Serrano¹² and others⁹ have reported earlier that glucose shows a strong stimulation of H⁺-efflux. Two structural analogues of glucose were also used to cross check the effect of glucose on H⁺-efflux.

Proton efflux measurements—Mid-log phase cells were harvested from YEPD medium by centrifuging the culture at 5000 rpm for 10 mins at 4°C. Cells were then washed twice with doubly distilled water and 200 mg cells (wet wt) were suspended in 10 ml solution containing 0.1 M KCl and 0.1 mM CaCl₂. The suspension was kept in a double-jacketed glass container with constant stirring. The container was connected to a water circulator at 25°C. The pH was monitored using a pH-meter for 30 min. Proton efflux rate was calculated from the volume of 0.01 N NaOH consumed in automatic titration in pH-stat mode of Autotitrimer (Radiometer ETS 822, Copenhagen) over a period of 10 min¹³. Increments and rate of delivery of titrant was adjusted according to demands of the experiments and were 100 µl and 40 ml min⁻¹ respectively. Initial pH was adjusted to 7.0 using 0.01 N HCl/NaOH. Sodium nitroprusside (20 mM)¹⁴ and different nutrients (5 mM)⁹ were added to the cell suspension after adjusting the pH to 7 and then readings were noted every minute. Controls were also run simultaneously.

Results

The pH changes in absence or presence of SNP and nutrients were monitored every minute for half an hour and H⁺-efflux rates were calculated using pH stat assay as described earlier¹³. In control, a decreasing trend in pH was observed with time, whereas the mixture of cells and SNP showed an initial decline in pH for 10-15 min, followed by an increase in pH in later half i.e. upto 30 min. In Fig 1, two controls (A and B) have been shown because various sets of experiments were conducted with two different cell harvests. In the case of cells and SNP mixture, there was no significant change in relative rate of H⁺-efflux from 0 to 15 min whereas for the same mixture in contrast to control there was a decrease in acidity with almost same rate from 15 to 30 min (Table 1, Fig. 1).

H⁺-efflux in presence of sugars and SNP—The change in pH in presence or absence of sugars

(glucose and its analogues, 2-deoxy-D-glucose and xylose) and SNP is presented in Fig. 2. It was observed that glucose enhanced H⁺-efflux rate by 9 fold compared to control. The initial decrease in pH in glucose treated cells in presence or absence of SNP was similar for 15 min. Interestingly, there was an

Table 1—Effect of SNP and different nutrients on the rate of H⁺-efflux in *Candida* cells. Different sets of experiments with two controls A and B are shown here. In the controls (A and B) *Candida* cells were present in 0.1 mM CaCl₂ and 100 mM KCl at pH 7.0 and 25°C

Treatment/Concentration (mM)	Relative H ⁺ efflux rate (min)	
	0-15	15-30
Control (A)	1*	1**
Cells + SNP (20)	0.93	-0.95
Cells + glucose (5)	9.10	27.15
Cells + glucose (5) + SNP (20)	8.80	-19.15
Cells + Lys (5)	1.42	2.05
Cells + Lys (5) + SNP (20)	1.62	-2.30
Cells + Glu (5)	6.10	12.10
Cells + Glu (5) + SNP (20)	7.10	-15.10
Control (B)	1 [#]	1 [@]
Cells + SNP (20)	1.04	-1.05
Cells + xylose (5)	1.90	4.29
Cells + xylose (5) + SNP (20)	1.68	-2.29
Cells + 2-deoxy-D-glucose (5)	3.48	20.05
Cells + 2-deoxy-D-glucose (5) + SNP (20)	3.03	-3.46
Cells + Pro (5)	3.26	4.58
Cells + Pro (5) + SNP (20)	2.96	-5.42

*0.058 nmole min⁻¹ mg⁻¹ yeast cells; ** 0.009 nmole min⁻¹ mg⁻¹ yeast cells

[#]0.028 nmole min⁻¹ mg⁻¹ yeast cells; [@] 0.007 nmole min⁻¹ mg⁻¹ yeast cells

(-) indicates decrease in acidity. Values in brackets are concentration used

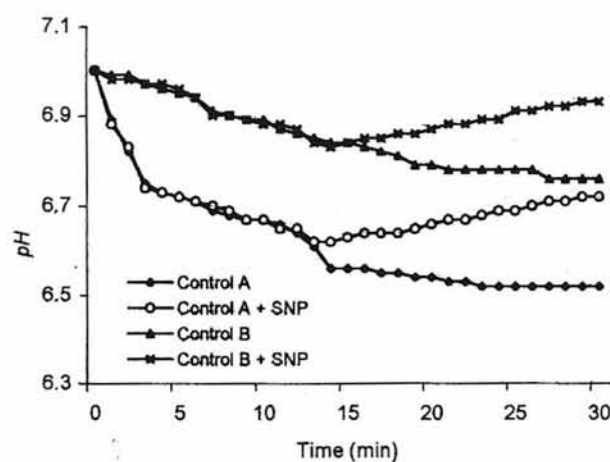


Fig. 1—Effect of SNP (20 mM) on H⁺-efflux in *Candida albicans*. In the controls (A and B), *Candida* cells were present in 0.1 mM CaCl₂ and 100 mM KCl respectively at pH 7.0 and 25°C

increase in pH in SNP treated cells in later half of the experiment (Fig. 2a). Similar trends of pH change were seen with 2-deoxy-D-glucose and xylose. It was observed that magnitude of increase or decrease in acidity was pronounced many fold in presence of glucose as compared to both of its analogues used (Table 1). In case of glucose, pH dropped from 7.0 to 5.8 in first half whereas for 2-deoxy-D-glucose and xylose, pH decreased only to 6.4 and 6.6, respectively (Fig. 2b, c). For SNP treated cells, in presence of glucose, pH increased from 5.8 to 6.0 whereas the increase in pH for 2-deoxy-D-glucose and xylose were 6.6 and 6.7 from 6.4 and 6.6, respectively.

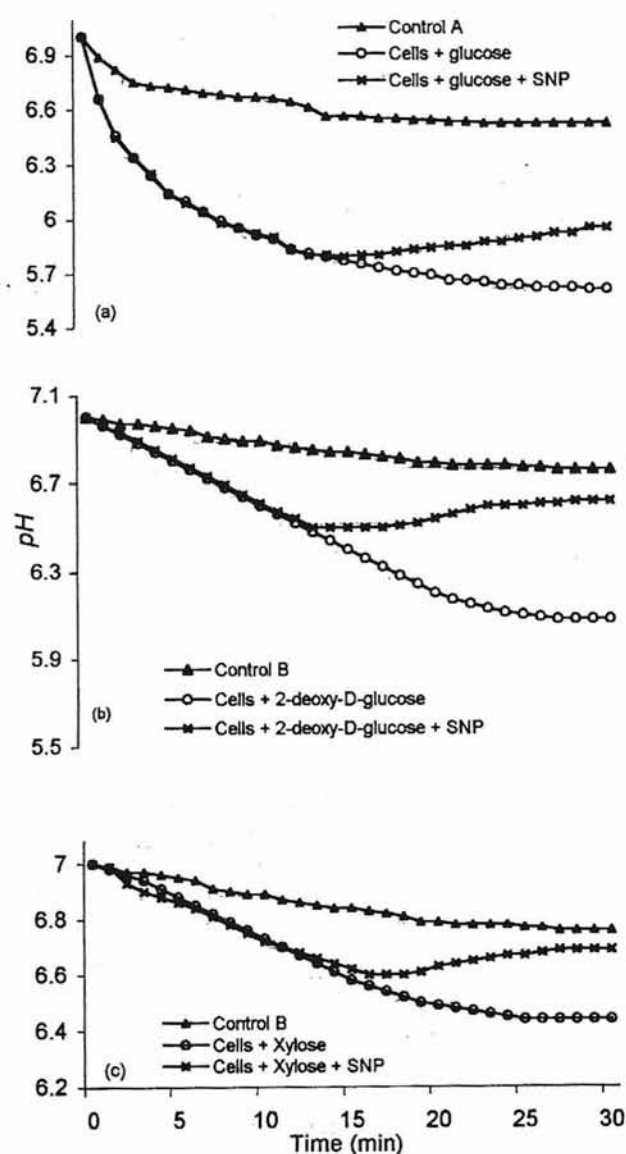


Fig. 2—Effect of SNP and sugars (5 mM) on H^+ -efflux in *Candida albicans*. *Candida* cells were present in 0.1 mM $CaCl_2$; 100 mM KCl; and (a) glucose, (b) 2-deoxy-D-glucose, and (c) xylose at pH 7.0 and 25°C.

H^+ -efflux in presence of amino acids and SNP—Amino acids being important molecules of cellular machinery, we have also tried to study the effect of some of them on H^+ -efflux in *C. albicans*. One neutral (proline), one acidic (glutamic acid) and one basic (lysine) amino acids were selected to study the H^+ -efflux in presence or absence of SNP. It was observed that proline showed similar trend in pH change like sugars while the other two (glutamic acid and lysine)

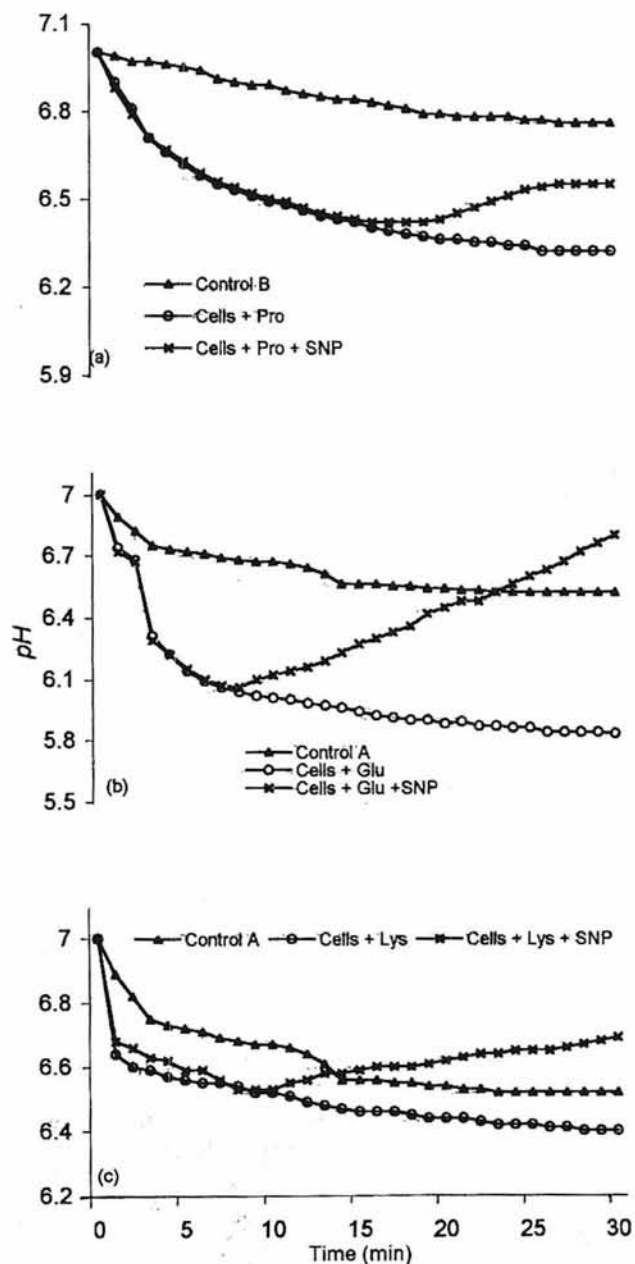


Fig. 3—Effect of SNP and sugars (5 mM) on H^+ -efflux in *Candida albicans*. *Candida* cells were present in 0.1 mM $CaCl_2$; 100 mM KCl; and (a) proline, (b) glutamic acid, and (c) lysine at pH 7.0 and 25°C.

showed slightly different trends of pH change in first 15 min of the study. Proline showed a drop in pH from 7.0 to 6.4 in first half in the presence or absence of SNP, while in SNP treated cells the pH increased to 6.6 (Fig 3a). Unlike proline, in the presence of glutamic acid, a sudden decrease in pH was observed and it reduced from 7.0 to 6.1 in 8 min. Similar trends of pH were observed up to 8 min in presence or absence of SNP with glutamic acid, followed by a sharp increase in pH in SNP treated cells upto 30 min from 6.1 to 6.8 (Fig 3b, Table 1). It was observed that similar to proline, H⁺-efflux rate was enhanced with lysine. In presence or absence of SNP with lysine, pH decreased from 7.0 to 6.5 in first 10 min. It was noticed that when cells were exposed to SNP and lysine, pH increased from 6.5 to 6.7 (Fig 3c). H⁺-efflux rate in both the halves were greater than the control (Table 1).

Discussion

NO production has been proposed as one of the major antimicrobial mechanism of murine macrophages¹⁵, which are active against different kind of pathogens, such as viruses, bacteria, fungi, protozoa and helminths. It is a very labile molecule and can pass easily through the cell membrane. Role of NO in anti-*Candida* activities is reported to be well-established¹⁶, but its effect on H⁺-efflux, necessary for secondary transport is not known. In view of this, we conducted this study to establish a link between H⁺-efflux and NO in presence of various nutrients in *C. albicans*. It is well known that for secondary transport, *C. albicans* generates proton gradient across its plasma membrane. *Candida* cells extrude protons as a natural process leading to increase in acidity in the medium. In the present study, we observed that in control there was a decrease in pH, but in presence of SNP (NO donor), initially there was decrease in pH followed by reversal of the trend i.e., increase in pH in later half of the experiment was seen. Similar trends were observed in presence of nutrients and SNP. The observation that H⁺-efflux was highest in presence of glucose was in agreement with earlier report of Serrano¹². Furthermore, greater stimulation with glucose as compared to charged molecules at the same concentration could be explained on the basis of the net negative charge on the surface of yeast and in the Donnan free space¹⁷. The H⁺-efflux rate in presence of glucose was found to be 9-fold of control as compared to 3 fold for 2-deoxy-D-glucose and 2

fold for xylose. Our observation for 2-deoxy-D-glucose may be explained in the light of the fact that it is a structural non-metabolic analogue of glucose, which cannot be utilized by the cells and hence leading to a reduced H⁺-efflux rate. Similar reason may account for decreased efflux in presence of xylose, which is also a structural analogue of glucose. Glutamic acid and proline have a special role in inducing dimorphism in *C. albicans*. The enhancement in H⁺-efflux rate in presence of proline, glutamic acid and lysine was more as compared to control, however the rates were different. Proline being a neutral amino acid is possibly transported easily than glutamic acid and lysine¹¹, causing greater H⁺-efflux i.e. 3-fold over control. The sudden decrease in pH in presence of glutamic acid may be due to its acidic nature. Even lysine showed a reduced H⁺-efflux rate as compared to proline, because lysine is a charged amino acid.

Interestingly, SNP treated cells in presence or absence of nutrients had a very pronounced effect around 10 to 15 min in pH change resulting in decrease in acidity observed throughout the study. Decrease in acidity in later half of the experiment may be attributed to the effect of NO generated by SNP as reported earlier¹⁰. Increase in pH in SNP treated cells in later half of the experiment may be due to the effect of NO on membrane permeability and disruption in electron transport system of mitochondria, resulting in depletion of ATP concentration. Due to this, H⁺-ATPase of *Candida* will function at slower rate because it requires ATP for efflux of proton. Our observation may be correlated to the fact that NO disrupts mitochondrial function by reversibly inactivating cytochrome *c* oxidase, thus stimulating superoxide anion production by the respiratory chain¹⁸. The resulting superoxide anion through formation of ONOO⁻, may be responsible for irreversible inhibition of complex I and III of mitochondrial electron transport chain¹⁹. It is also known that ONOO⁻ causes lipid peroxidation of membrane leading to cell injury²⁰. NO, through its intermediates affects permeability transition of mitochondrial membrane²¹ and also disturbs the transmembrane electrochemical proton motive force of mammalian cells³. Increase in pH in later half in our study may be due to the disturbance in permeability of cell membrane of *Candida* leading to movement of protons from the medium to inside the cells according to concentration gradient. Effect of NO is reported to be pH dependent and it is more

pronounced in acidic range than neutral^{10,22}. Increase in pH after 10-15 min may be due to an increase in acidity in the medium generated by *Candida* cells, which is favourable for the action of NO, in disturbing membrane permeability and damaging cellular machinery including H⁺-ATPase. It is quite possible that NO might be affecting H⁺-ATPase directly by binding with its tryrosyl residues, because it has been reported earlier that ONOO⁻ (an intermediate of NO) affects both free and protein bound tyrosine with subsequent alterations of protein phosphorylation or perturbation of protein tertiary structure²³.

Our study indicated that NO had a significant effect on H⁺-efflux by interfering with the H⁺-ATPase present in the plasma membrane of *Candida albicans*. The nutrient transport across the cell membrane of *Candida* was also found to be affected by NO.

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