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Effects of 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) on in situ mitochondria of *Trypanosoma cruzi*

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Results obtained with in situ mitochondria of *Trypanosoma cruzi* showed that this protozoon had only two energy coupling sites, sites II and III that correspond to higher eukaryote mitochondria. Rotenone did not inhibit the oxygen uptake of the parasite. These results suggest that the NADH-ubiquinone segment of the respiratory chain has no activity. Studies with in situ mitochondria confirmed that BHA, an antioxidant food additive, blocks the mitochondrial electron transport chain at the succinate-cytochrome *b* segment being the molecular basis of this trypanocidal action.

Oxygen uptake; 2(3)-*tert*-Butyl-4-hydroxyanisole (BHA); Rhodamine 123; Mitochondrial respiratory chain; *Trypanosoma cruzi*

1. INTRODUCTION

Trypanosoma cruzi, the American trypanosomiasis protozoan agent, has biochemical peculiarities in its bioenergic processes: glucose aerobic fermentation [1], glycolytic enzymes localized in a microbody-like organelle named 'glycosome' [2], the presence of a single and unique mitochondrion [3] and two terminal oxidases, cytochrome $a + a_3$ and cytochrome *o* [4].

The discovery and identification of unique metabolic pathways of *T. cruzi* and its differences with those present in man and other mammal hosts is the basis for a rational approach of Chagas disease chemotherapy. Trypanocidal effects of 2(3)-*tert*-butyl-4-hydroxyanisole (BHA), a phenolic antioxidant, has been demonstrated [5]. This compound acted by inhibiting the oxygen uptake and produced a redox change of NAD(P) and cytochrome *b* on intact *T. cruzi* [5].

This report shows the study of in situ mitochondrial oxidative phosphorylation of digitonin-permeabilized *T. cruzi* epimastigotes. The presence of only two energy coupling sites, sites II and III that correspond to higher eurakyote mitochondria is described. Also, the inhibition by BHA on in situ mitochondria is exhibited, being the data similar to previous works on intact parasites [5,6].

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2. MATERIALS AND METHODS

2.1. *Materials*

Fetal calf serum, *t*-butyl-4-hydroxyanisole (BHA), sucrose, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), digitonin, Rhodamine 123 and all other chemicals were obtained from Sigma Chemical Co.

2.2. *Isolation of* T. cruzi *epimastigotes in situ mitochondria*

T. cruzi epimastigotes (clone Dm28c) were grown and harvested according to Aldunate et al. [5]. The pellet was resuspended in 200 mM sucrose, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM potassium phosphate buffer, pH 7.2. Cells were preincubated with digitonin 75 μg/mg of protein for 5 min.

2.3. *In situ mitochondria oxygen uptake*

Oxygen uptake measurements were carried out polarographically with a Clark electrode no. 5331 (Yellow Springs Instruments) in a Gilson 5/6 oxygraph. Digitonin-permeabilized epimastigotes (3.0 mg of protein/ml) were suspended in 107 mM NaCl, 52 mM potassium phosphate buffer, pH 7.5 (final volume 2.0 ml).

2.4. *Visualization of* T. cruzi *in situ mitochondria with Rhodamine 123*

Permeabilized parasites were washed twice and stained with Rhodamine 123 for 1 h at 20 μg/ml at 28°C. Stained permeabilized parasites were dried and fixed for 10 min with methanol and observed with a Leitz Ortholux microscope using excitation filters of 510–560 nm.

3. RESULTS AND DISCUSSION

T. cruzi contains a single mitochondrion distributed all along the parasite. The isolation of functional mitochondria by subcellular fractionation has not been able by the present time. The use of digitonin, a detergent compound, to permeabilize the parasite's plasma membrane has been used to study the Ca^{2+} transport on in situ mitochondria [7]. In the present paper, this tech-

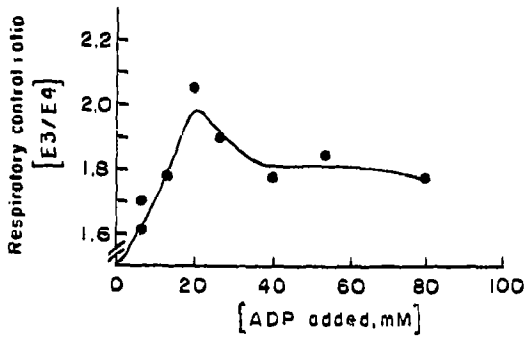

Fig. 1. Effect of ADP on the respiratory control ratio of *T. cruzi* epimastigotes in situ mitochondria. Oxygen uptake measurements were made on digitonin-permeabilized parasites in 5 mM succinate suspension medium adding different concentrations of ADP (see section 2).

nique was utilized to study the actions of BHA on the oxidative phosphorylation of *T. cruzi* in situ mitochondria.

3.1. *Oxidative phosphorylation in* T. cruzi *in situ mitochondria*

Table I summarizes the oxidation pattern of different substrates of *T. cruzi* in situ mitochondria. Additions of succinate, L-glutamate + L-ascorbate + TMPD stimulated the oxygen consumption of mitochondria (state 4).

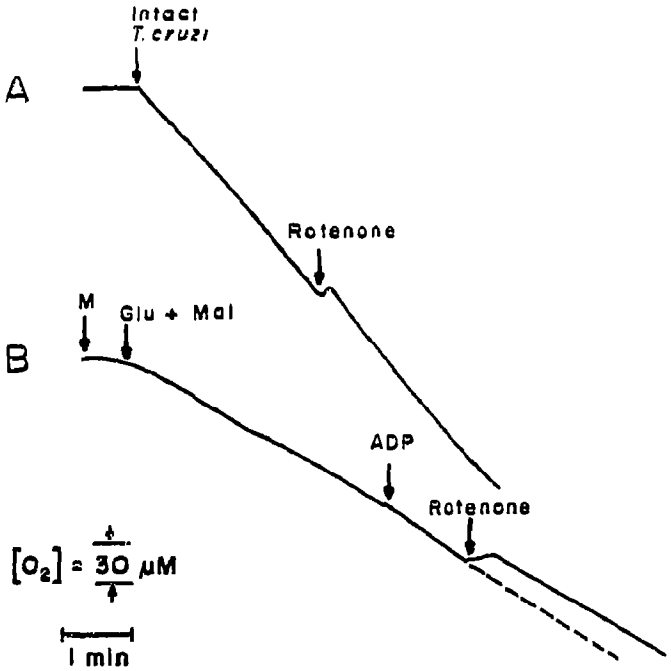

Fig. 2. Effect of rotenone on oxygen uptake of intact (A) and in situ mitochondria (B) of *T. cruzi* epimastigotes. At the point indicated by the arrow 25 μM rotenone was added to the suspension medium. Substrates added to M (mitochondria) were: Glu + Mal (10 mM glutamate + 10 mM malate), and 20 mM ADP was used (see section 2).

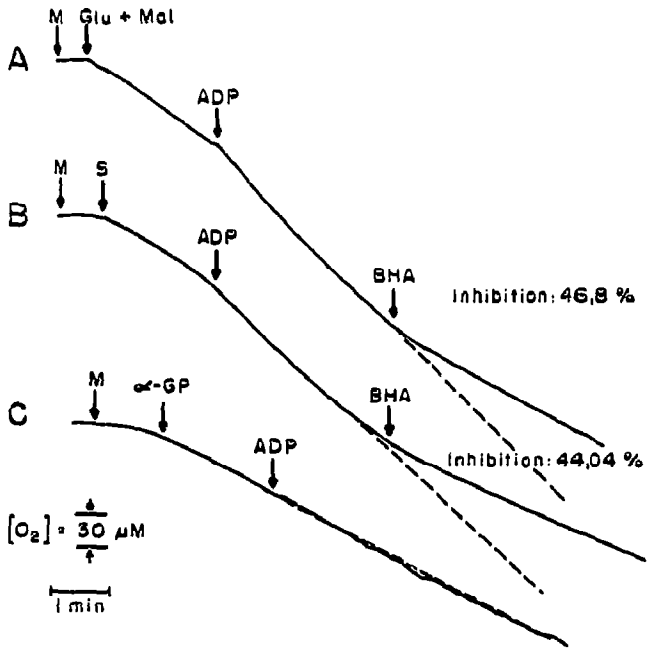

Fig. 3. Effect of BHA on oxygen uptake of *T. cruzi* epimastigotes in situ mitochondria. Substrates added to M (in situ mitochondria) were: Glu + Mal (glutamate 10 mM + malate 10 mM); S (succinate 5 mM); α-GP (α-glycerophosphate, 10 mM), ADP 20 mM and BHA 1 mM (see section 2).

If ADP was used as a phosphate acceptor the oxygen uptake of the mitochondria was increased (state 3).

The best respiratory control ratio (RCR) was obtained with an ADP concentration range between 20 mM and 80 mM. Thereby, in situ mitochondria have electron transport coupled with ATP synthesis (Fig. 1).

Succinate and glutamate + malate ADP/O index were 1.99 and 1.95, and respiratory control values were 1.9 and 1.6, respectively (Table I). For ascorbate + TMPD, the values for the same parameters were 1.15 and 1.3, respectively.

The ADP/O results suggested that in *T. cruzi* in situ mitochondria only two phosphorylation sites were functional, sites II and III of higher eukaryotes. This conclusion was based on: (a) The ADP/O ratio obtained with glutamate + malate (1.95) could be explained by the fact

Table I

Oxidable substrates stimulation of oxygen consumption of in situ mitochondria of *T. cruzi* epimastigotes*

Substrate	State 3	State 4	RCR	ADP/O
Succinate	35.0 ± 0.4	19.2 ± 2.2	1.9 ± 0.04	1.99 ± 0.04
Glutamate+ Malate	35.7 ± 1.1	22.4 ± 0.9	1.6 ± 0.1	1.95 ± 0.12
Ascorbate+ TMPD	176.7 ± 8.7	133.7 ± 7.9	1.3 ± 0.04	1.15 ± 0.06

*Data are expressed ± S.D.; n=6. See section 2 for further details.

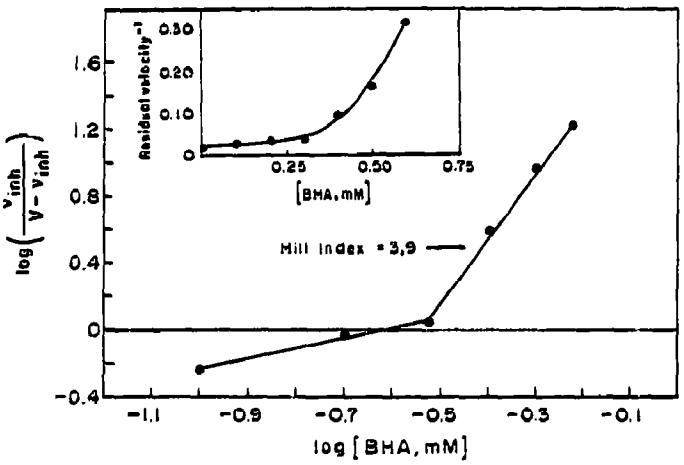

Fig. 4. Hill plot of the BHA inhibition of the oxygen uptake of *T. cruzi* epimastigotes in situ mitochondria. V corresponds to non-inhibited oxygen uptake rate; v_{inh} to inhibited respiration rate. All linear regression coefficients are greater than 0.99. In the insert same data are shown in a Dixon plot (see section 2).

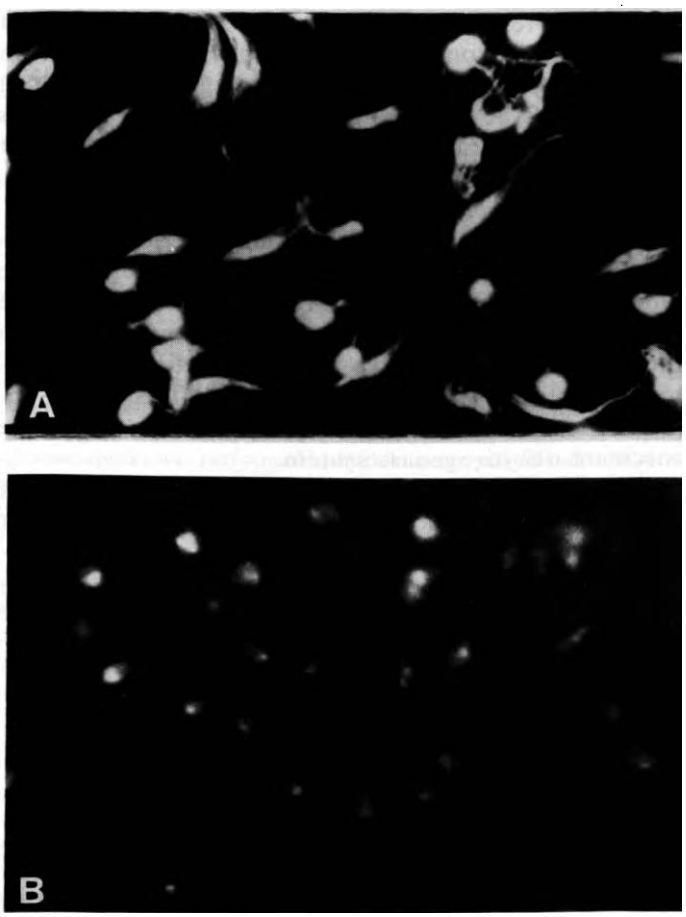

Fig. 5. (A) Rhodamine 123-stained *T. cruzi* epimastigotes in situ mitochondria and (B) treated with BHA. Permeabilized cells were incubated for 1 h with 20 μg/ml of Rhodamine 123. BHA-treated mitochondria were incubated with 0.5 mM of the drug per 2 mg of protein for 5 min before staining with the dye. Photomicrographs were taken at 710× magnification (see section 2).

that site I of phosphorylation could not be active in this protozoum. Supporting this observation, Fig. 2 showed that rotenone did not affect the oxygen consumption of intact *T. cruzi* (Fig. 2A), nor that in in situ mitochondria after the addition of glutamate + malate (Fig. 2B). Also, rotenone had no effect on the oxygen uptake when succinate or ascorbate + TMPD were used as oxidizable substrates (data not shown). All the data shown confirmed the idea that the NADH-ubiquinone segment in the respiratory chain of *T. cruzi* epimastigotes was not active. (b) The oxygen uptake velocity in state 4, state 3 and the ADP/O ratio using succinate and glutamate + malate were not significantly different. Thus it could be proposed that only two sites were active when any of these substrates were used. (c) The ADP/O ratio obtained when ascorbate + TMPD were used as substrates corroborated the phosphorylation activity of site III. So a new question to resolve is, which mechanism is involved in the NADH reoxidation?

3.2. *BHA effects on* T. cruzi *epimastigotes in situ mitochondria*

When BHA was added to the suspension the oxidative phosphorylation dependent of glutamate + malate or succinate (Fig. 3A and 3B) was inhibited by approx. 45% on each occasion. This event could be counterbalanced when ascorbate + TMPD was used (data not shown). These results confirmed the hypothesis that oxygen consumption inhibition by BHA is done by blockage of the step preceding cytochrome oxidase in the oxidative chain of *T. cruzi*. α-Glycerophosphate does not stimulate the oxidative phosphorylation in mitochondria of this parasite (Fig. 3C).

Fig. 4 shows the inhibition kinetics of BHA on the oxidative phosphorylation of in situ mitochondria, when succinate was used as an oxidizable substrate. The drug inhibition followed a bimodal pattern as shown in the Hill and Dixon plot (insert). Low concentrations of BHA produced a Michaelian type inhibition and higher concentrations followed a positive-cooperativity inhibition model with a Hill index value of 3.9. Fig. 5A shows in situ mitochondria of *T. cruzi* epimastigotes stained with Rhodamine 123. This compound is a fluorescent dye that is preferentially taken up by mitochondria, probably because the dye is cationic and the organelle maintains a high transmembrane potential. *T. cruzi* epimastigotes were permeabilized with digitonin and maintained in a succinate ADP medium. It can be observed that Rhodamine 123 stained the mitochondrion of permeabilized cells just as in intact parasites [8]. The dye incorporation diminished when the in situ mitochondria were treated with BHA before the incubation with Rhodamine 123 (Fig. 5B). This result can be explained by the fact that BHA blocks the oxidative phosphorylation, the necessary condition for the incorporation of Rhodamine 123 in *T. cruzi* in situ mitochondria.

All these results confirm that the molecular basis of the BHA inhibition of growth and oxygen consumption of intact *T. cruzi* occurs by the blockage of the electron transport of the mitochondrial respiratory chain. This blockage occurs at the succinate-cytochrome *b* segment as postulated in previous papers [5,6]. Similar data were obtained in *Trypanosoma brucei* and tumoral cells [6].

Also, the BHA effects were studied in rat liver mitochondria and the primary action of the drug was the inhibition of NAD^+- and FAD-linked dehydrogenase systems. The inhibition of both dehydrogenases led to the cessation of oxidative phosphorylation and cytotoxic effects [9]. In the case of *T. cruzi*, this work supports the conclusion that BHA primarily affects the succinate dehydrogenase system.

Studies to determine the molecular target(s) of BHA action location in the parasite are now realized in our laboratory.

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