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Effect of β-lapachone on hydrogen peroxide production in *Trypanosoma cruzi*

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Summary

β-Lapachone, an antimicrobial agent, markedly increase the generation of H_2O_2 in intact *Trypanosoma cruzi* epimastigotes (Y strain). Increase in H_2O_2 was determined by the horseradish peroxidase-H_2O_2 Compound II formation as well as by a cytochemical technique.

Key words: Trypanosoma cruzi; β-lapachone; H_2O_2 generation.

Introduction

β-Lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2-b]pyran-5,6-dione) (Fig. 1), an antimicrobial agent, originally obtained as a contaminant of lapachol preparations (Lima et al., 1962), causes ultrastructural and metabolic alterations in *Trypanosoma cruzi* epimastigotes (Docampo et al., 1977). *β*-Lapachone-supplemented epimastigotes showed alterations in nuclear chromatin as well as in the ultrastructure of the nuclear, cytoplasmic, mitochondrial and endoplasmic reticulum membranes (Docampo et al., 1977). Addition of *β*-lapachone to cell suspensions reduced the respiratory rate and inhibited glucose and pyruvate oxidation in epimastigotes. Apparently the metabolic effects of the drug paralleled ultrastructural alterations (Docampo et al., 1977).

Naphthoquinones and benzoquinones (Cadenas et al., 1977; Misra and Fridovich, 1972) usually increase the rate of H_2O_2 formation in biological

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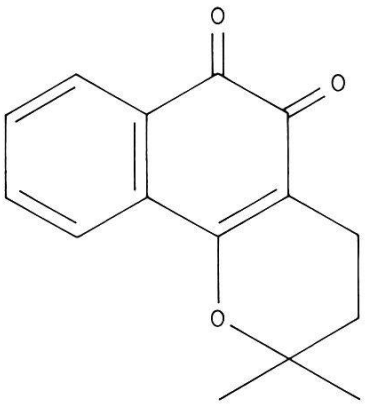

Fig. 1. Chemical structure of β-lapachone.

systems. In the present investigation, we show that the incubation of *T. cruzi* epimastigotes with β-lapachone results in a significant increase in the rate of H_2O_2 production. Both direct spectrophotometric and indirect cytochemical techniques have been used with consistent results.

Materials and methods

Microorganism. Trypanosoma cruzi (Y strain) was grown on Warren's liquid medium (Warren, 1960) at 28° C. Six days after inoculation, the cells were collected by centrifugation and washed with 0.154 M NaCl. The number of epimastigotes was estimated by counting the cells in a Neubauer chamber (Docampo et al., 1977).

Electron microscopy. Cells were preincubated with or without β-lapachone for 60 min, washed with 0.154 M NaCl and fixed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2) for I h. At 22–24° C. Controls lacked DAB or β-lapachone in the incubation medium or contained 2 mM KCN or 10 mM 3-amino-1,2,4-triazole in the incubation medium. When inhibitor was used, the glutaraldehyde-fixed epimastigotes were preincubated with it for 30 min at 37° C in 0.1 M Tris-HCl buffer pH 8,5 and then incubated in the test medium containing the inhibitor. The cytochemical localization of H_2O_2 was accomplished by using a modification of the diaminobenzidine (DAB) technique (Briggs et al., 1975; Graham and Karnovsky, 1966) designed originally for the localization of peroxidase. Glutaraldehyde-fixed epimastigotes were incubated for 60 min at 37° C with the DAB medium (prepared immediately before use and avoiding ultraviolet irradiation) containing 3 mg DAB, 0.1 M Tris-HCl buffer (pH 8.5) and 10 μg/ml β-lapachone (total volume: 5 ml). After incubation, cells were postfixed in 1.0% (w/v) osmium tetroxide in 0.1 M phosphate buffer in the cold. After dehydration with ethanol, the material was embedded in Epon, sections were cut with a LKB ultramicrotome, and observed in an AEI EM-6B electron microscope with a 50 μm objective aperture, operating at 60 Kv. In some cases the material was stained with lead citrate.

Fig. 2. *T. cruzi* epimastigotes (Y strain) treated with β-lapachone (10 μg/ml for 60 min) and exposed to diaminobenzidine for 60 min. Particle deposition is found either in chromatin patches in the nucleus, in the cytoplasm, around cytoplasmic vesicles and extracellularly. 25000 $\times$.

Fig. 3. *T. cruzi* epimastigotes (Y strain) treated with β-lapachone without DAB. General aspect of epimastigotes treated with β-lapachone. Major changes include: swelling of the mitochondrion, alteration of nuclear, mitochondrial, endoplasmic reticulum and cytoplasmic membranes (blebs) and the rearrangement of chromatin in patches. 17800 $\times$.

Abbreviations used: B: bleb; F: flagellum; K: kinetoplast; N: nucleus; V: vesicles.

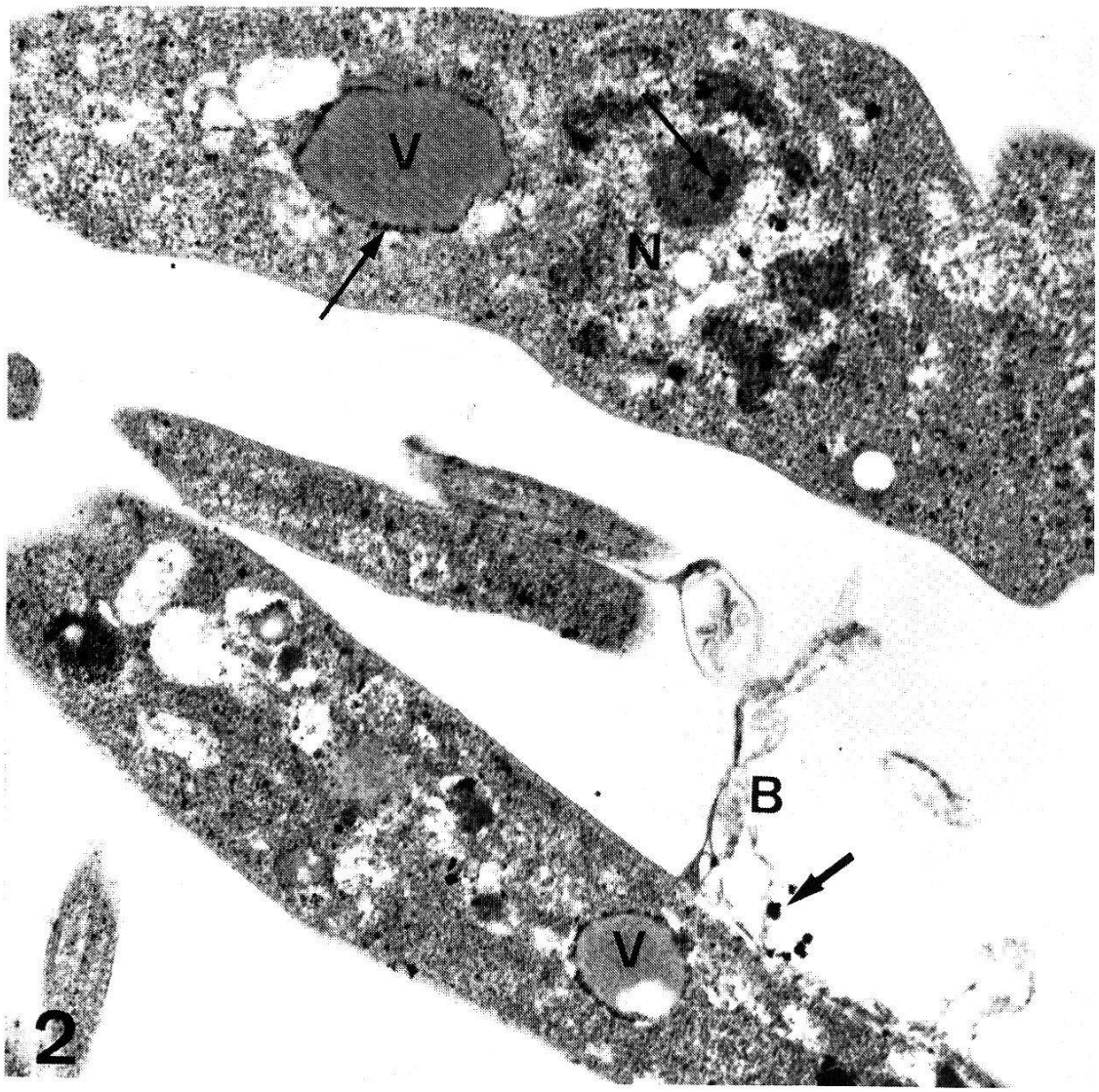

Fig. 2

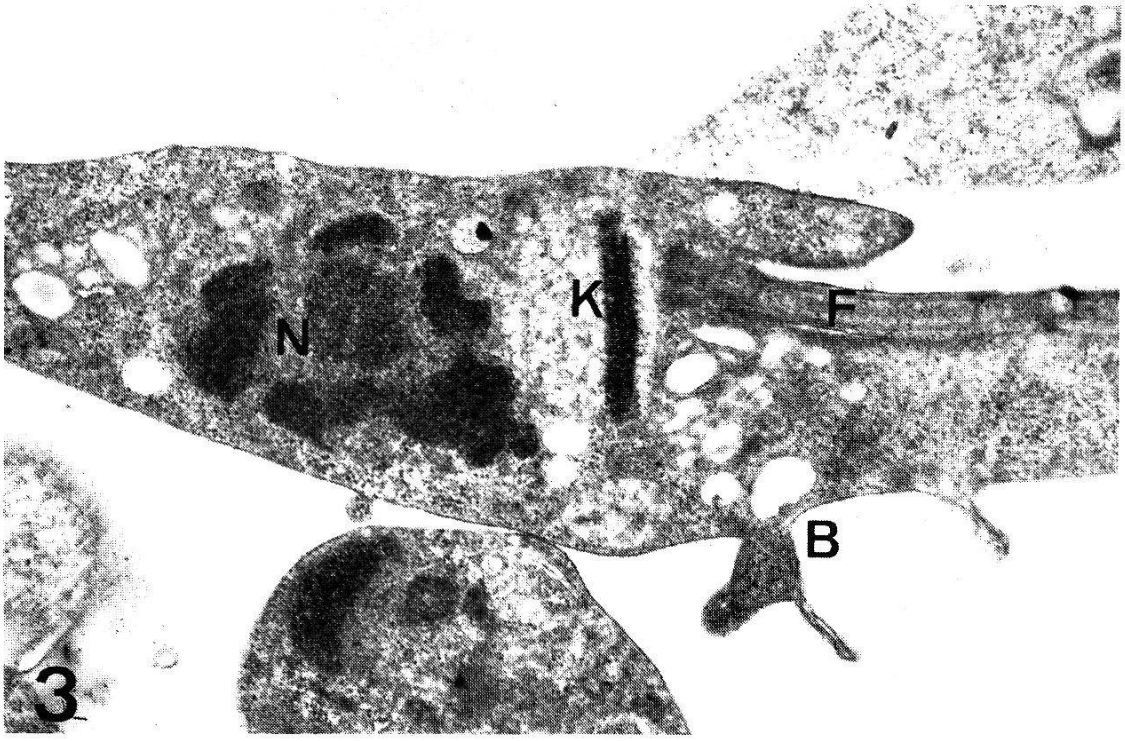

Fig. 3

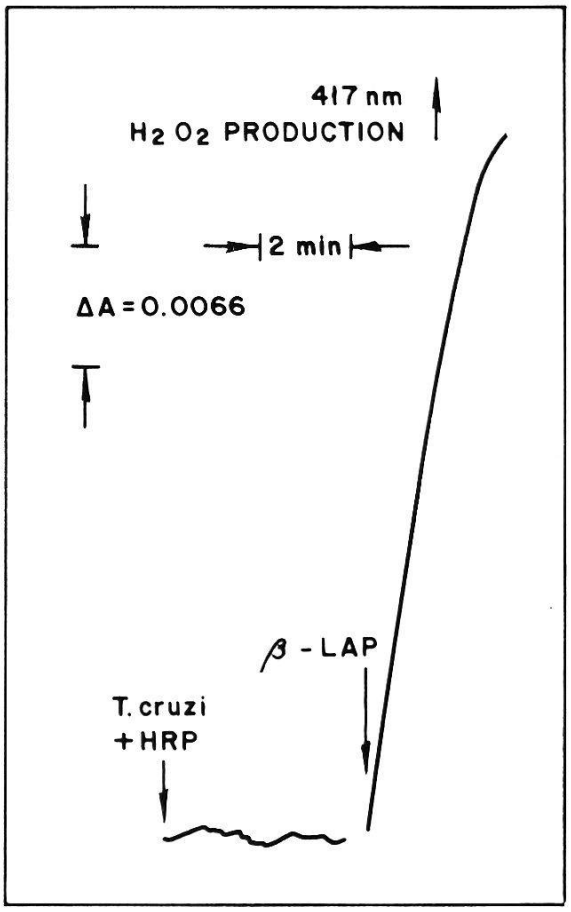

Fig. 4. H_2O_2 generation in intact *T. cruzi* epimastigotes resulting after addition of horseradish peroxidase (HRP) and β-lapachone (β-lap) as measured by HRP Compound II formation. *T. cruzi* epimastigotes (0.2 mg/ml protein) were suspended in a medium containing 0.120 M KCI, 20 mM potassium phosphate buffer at pH 7.2 and incubated at 30° C.

Generation of H_2O_2. This was determined by the rate of formation of horseradish peroxidase (HRP) Compound II, measuring the absorption at 417 nm and utilizing an absorption coefficient of 30 $mM^{-1} \cdot cm^{-1}$ (Boveris et al., 1977). Reaction mixtures contained 0.3–0.8 μM HRP. An Acta III spectrophotometer (Beckman Instruments, California, USA) was utilized. All determinations were made at 30° C.

Protein determination. These were done by the biuret assay (Gornall et al., 1949) in the presence of 0.1% sodium deoxycholate.

Chemicals. Analytical grade reagents were used throughout the experiments. HRP and DAB, were purchased from Sigma Chemical Co. β-Lapachone was obtained through a Program for the Synthesis of Antiparasite Drugs at the Federal University of Rio de Janeiro and was a gift of Drs. A. V. Pinto and B. Gilbert.

Results

The general structure of β-lapachone-treated *T. cruzi* epimastigotes was previously described (Docampo et al., 1977). The following alterations were observed: the chromatin was rearranged in patches, the nuclear membrane as well as the cell membrane showed blebs and the mitochondrion appeared swollen.

Examination of epimastigotes exposed to DAB and β-lapachone revealed the presence of an electron-dense reaction product in the nucleus and in the cytoplasm of cells (Fig. 2). In the nucleus the reaction product is preferentially located on the chromatin patches induced by β-lapachone. Oxidized DAB was also observed arround cytoplasmic vesicles (Fig. 2) which contained a material reacting with osmium tetroxide (De Carvalho and De Souza, unpublished). DAB-reaction product found extracellularly was associated with the cytoplasmic membrane blebs. It was absent in control preparations lacking DAB or β-lapachone (Fig. 3). Inhibitors of peroxidase activity, aminotriazole and cyanide, completely blocked the formation of intracellular and extracellular reaction products, thus indicating the need for this enzyme in this reaction.

The rate of formation of H_2O_2 in *T. cruzi* epimastigotes as detected by horseradish peroxidase Compound II Formation (3) is illustrated in Fig. 4. Addition of the cells to the incubation system did not result in any significant rate of H_2O_2 production. Further addition of β-lapachone (5 μg/ml), however, caused a marked stimulation of H_2O_2 production (0.28 nmol H_2O_2/min/mg protein).

Discussion

The cytochemical technique used in this investigation was originally designed for the ultrastructural localization of peroxidase (Briggs et al., 1975; Graham and Karnovsky, 1966). This enzyme in the presence of exogenous H_2O_2 oxidizes DAB to an insoluble, osmiophilic polymer, which can be visualized in the electron microscope. In the present case only DAB was supplied to the cells, and formation of the reaction product depended upon the presence of endogenous enzyme and H_2O_2. Peroxidase has been demonstrated in *T. cruzi* microbodies by cytochemical methods (Docampo et al., 1976), and peroxidase activity was measured in an epimastigote homogenate as well as in a particulate fraction obtained from the homogenate (Docampo et al., 1976). The fraction which did not sediment at 105 000 g showed a significant, although lower peroxidasespecific activity, which possibly reflected the release of enzyme from microbodies broken during cell fractionation (Docampo et al. 1976). β-Lapachone-treated epimastigotes showed alterations of cell membranes (Docampo et al., 1977), and it is possible that peroxidase in these microbodies was released into the cytoplasm as well as into the extracellular medium; the deposition of oxidized DAB reaction product could thus indicate the presence of endogenous H_2O_2. Pretreatment of epimastigotes with β-lapachone (before fixation) increased the deposition of oxidized DAB reaction products, this was possibly due to the release of peroxidase from the microbodies.

The present data suggest that β-lapachone causes a marked increase in the generation of H_2O_2 in intact cells of *T. cruzi* epimastigotes.

The emergence of O_2- and H_2O_2 as a result of reduction of molecular

oxygen with reduced β-lapachone (Docampo et al., 1977) was proposed as analogous to the already described ability of quinols to carry out these reaction (Misra and Fridovich, 1972). Superoxide anions are able to react non-enzymatically with hydrogen peroxide yielding hydroxyl radicals (HO·) which are powerful oxidants and therefore markedly toxic due to reactions with cellular components, particularly nucleic acids (Myers, 1973) and lipid (Tappel, 1973). The lack of catalase (Docampo et al., 1976) and the sensitivity of many *Trypanosoma sp.* to exogenous H_2O_2 (Fulton and Spooner, 1956) suggest that the toxicity of β-lapachone may depend on the generation of powerful oxidants resulting from the partial reduction of oxygen.

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