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MECHANISM OF NIFURTIMOX TOXICITY IN DIFFERENT FORMS OF *TRYPANOSOMA CRUZI*

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Abstract—Addition of nifurtimox (a nitrofuran derivative) to NAD(P)H-containing homogenates of *Trypanosoma cruzi* (epi-, trypo- or amastigote forms) determined the appearance of an e.p.r. spectrum that could be identified as corresponding to the nitroaromatic anion radical. The anion radical signal was observed after an induction period that depended on the oxygen concentration and the pyridine nucleotide in the incubation medium. Incubation of intact *T. cruzi* forms with nifurtimox also led to the appearance of the anion radical signal. The nitroaromatic anion radical, which is assumed to be the first product of nitroreductase activity, reacted with oxygen under aerobic conditions, as shown by the increased rate of superoxide anion and hydrogen peroxide production after addition of nifurtimox to homogenates of *T. cruzi* in the presence of NAD(P)H. The nifurtimox-induced peroxide production was higher with *T. cruzi* amastigotes than with epi- or trypomastigotes.

Nifurtimox§ is one of the most effective drugs used for the treatment of acute Chagas' disease [1, 2]. In accordance with observations of Docampo and Stoppani [3], pharmacological concentrations of nifurtimox are capable of inducing maximal stimulation of O_2^- production by microsomal and mitochondrial fractions of *Trypanosoma cruzi* epimastigotes and, also, of initiating diffusion of H_2O_2 outside intact *T. cruzi* cells. From these observations it was inferred that the trypanocidal action of nifurtimox is mediated by the products of partial reduction of oxygen, such as the superoxide anion (O_2^-) and hydrogen peroxide. In the present study we have confirmed and extended observations of Docampo and Stoppani [3] using blood (trypomastigote) and intracellular (amastigote) forms of the parasite. Moreover, formation of nifurtimox nitroaromatic free radical has been demonstrated in intact *T. cruzi* trypo-, epi- and amastigote forms and, also, in the homogenates obtained therefrom. The nitroaromatic anion radical is an essential intermediate in the mechanism previously postulated [3] for the induced electron transfer from reduced pyridine nucleotides [NAD(P)H] to oxygen.

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§ Abbreviations: nifurtimox: 3-methyl-4-(5′-nitrofurfurylidene-amino)-tetrahydro-4H-1,4-thiazone-1,1′-dioxide; nitrofurantoin, *N*-[5-nitro-2-furfurylidene]-1-aminohydantoin; and SOD, superoxide dismutase.

MATERIALS AND METHODS

Culture. T. cruzi (Y strain) was grown at 28° in the liquid medium described by Warren [4] except that 4% bovine serum was used instead of 10%. Six days after inoculation, cells were collected by centrifugation and washed with 0.15 M NaCl. The final concentration of epimastigotes was estimated as described before [5].

Intracellular and bloodstream forms. Isolation of intracellular [6] and bloodstream [7] forms was made from mice as described in the references. The final concentration of the cells was estimated by counting in a Neubauer chamber.

Homogenates. The cells were disrupted by freezing (at −20°) and thawing (at 2–4°), three times, or by grinding in a mortar as described before [7]. The homogenate preparation was completed by passing the suspensions three times through a hypodermic needle (24 gauge).

Reagents. Horseradish peroxidase (Type VI), L-epinephrine bitartrate, superoxide dismutase (Type I, from bovine blood), glucose-6-phosphate dehydrogenase (Type IX, from bakers yeast), NADH (Grade III), and NADPH (Type III) were obtained from the Sigma Chemical Co., St. Louis, MO. Nifurtimox was obtained from Bayer A.G., Leverrusen through the courtesy of Dr. A. Haberkorn. Nitrofurantoin was obtained from Eaton Vemaco Ltda., São Paulo, Brasil. Other reagents were of analytical grade.

Electron paramagnetic resonance spectroscopy. Observations were made at room temperature (about 24°), with a Varian E9 spectrometer at 100 kHz field modulation, using the conditions

described under Results. The incubation mixture (0.5 ml) contained 1 mM nifurtimox, 0.7 mM NADH or an NADPH-generating system (0.7 mM NADP, 10 mM glucose-6-phosphate and 1.3 units/ml of glucose-6-phosphate dehydrogenase), in 150 mM KCl, 20 mM $MgCl_2$, 20 mM Tris/HCl buffer, and 20 mM phosphate buffer (Na_2HPO_4-KH_2-PO_4), pH 7.4. When necessary, the medium was gassed for 15 min with N_2 that had been purified from traces of oxygen as described previously [8]. The cell homogenate was then added and, after further gassing, the mixture was placed in the aqueous flat cell as described before [9]. The determination of the g value was made by measurements of microwave frequency using a Systron Donner frequency meter and of the magnetic field intensity with a proton precission Systron Donner gaussmeter, which allow the g value to be calculated to the fourth decimal place. We used the relation $h \cdot \nu = g\beta H$, where h is Planck's constant; ν, the microwave frequency; β, the Bohr magneton; and H, the magnetic field intensity. Simulation of spectra was performed using a Varian 620-i on-line computer.

Determination of O_2^- *and* H_2O_2. Superoxide anion production was determined by the adrenochrome assay [10], measuring the absorption at 480–575 nm and using an absorption coefficient (ε) of 2.96 $mM^{-1} \cdot cm^{-1}$ [11]. The reaction mixture contained 1 mM epinephrine in 130 mM KCl and 20 mM phosphate buffer, pH 7.4. Hydrogen peroxide generation in homogenates was determined by the horseradish peroxidase assay [12], measuring the absorption at 417–402 nm (ε = 50 $mM^{-1} \cdot cm^{-1}$). The reaction mixture has been described before [12] and contained 0.3 to 0.8 μM horseradish peroxidase. An Aminco–Chance double beam spectrophotometer (American Instruments Co., Silver Spring, MD) was utilized. All determinations were made at 30°. Protein was determined by the method of Lowry *et al.* [13].

RESULTS

Incubation of amastigote homogenates with nifurtimox and pyridine nucleotides [NAD(P)H] determined the appearance of an e.p.r. spectrum (Fig. 1B) resembling the computer-simulated spectrum of the anion radical of the nitrofuran nifuroxime (Fig. 1A). The similarity of spectra presented in Fig. 1 affords good evidence for the assignment of Spectrum B to the nifurtimox nitroaromatic free radical.

It is generally agreed that nitroaromatic radicals cannot be detected in the presence of oxygen [15],

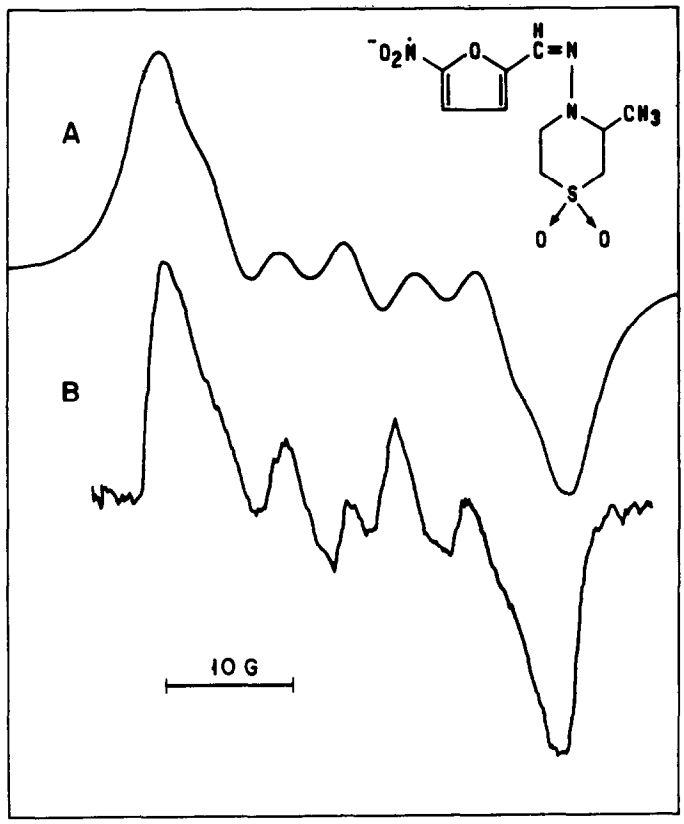

Fig. 1. Line A: Computer-simulated e.p.r. spectrum of the nifuroxime anion radical. The following coupling constants were used: $a^N_{NO_2}$ = 11.43 G; a^H_4 = 5.54 G; a^H_3 = 1.52 G; a^H_α = 0.89 G; a^N_{NOH} = 2.14 G; a^H_{NOH} = 0.24 G (Ref. 14); line width, 3.5 G. Line B: Electron paramagnetic resonance spectrum of the nifurtimox anion radical after anaerobic incubation of 1 mM nifurtimox with amastigote homogenate (5.6 mg/ml) and 0.7 mM NADH in an anaerobic medium containing 130 mM KCl and 20 mM phosphate buffer, pH 7.4. Signal g, 2.0044, Conditions for e.p.r. measurement were: microwave power, 50 mV; modulation amplitude, 2.0 G; frequency, 9.5 GHz; scan rate, 25 G/min; gain, 3.2 × 10^4. Other experimental conditions were as described under Materials and Methods. The structure above is that of nifurtimox anion radical.

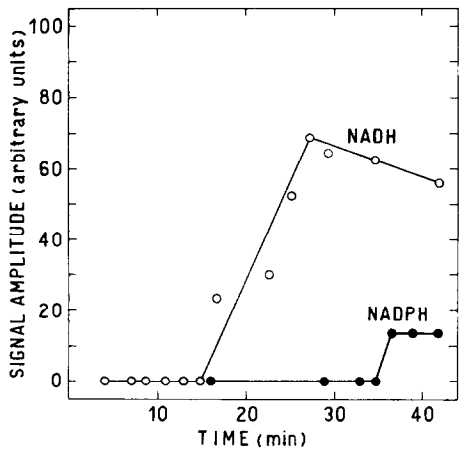

Fig. 2. Changes in the e.p.r. signal amplitude with time after mixing the amastigote homogenate (2.7 mg/ml) with nifurtimox and NADH (○) or an NADPH (●) generating system, in oxygen-saturated medium. Conditions for e.p.r. measurements were: microwave power, 50 mV; modulation amplitude, 4.0 G; frequency, 9.5 GHz; scan rate, 100 G/min; gain 6.3×10^4. Other conditions were as described in Fig. 1 and under Materials and Methods. The signal amplitude unit is defined as 0.01 of the amplitude of the maximum deflection in the recorder trace, obtained with an equivalent concentration of trypomastigote homogenate, in the presence of NADH.

because of Reaction 1 (Ref. 16).

$$ArNO^{\cdot} + O_2 \longrightarrow ArNO_2 + O_2^{\cdot-} \qquad (1)$$

When nifurtimox was added to aerated *T. cruzi* homogenates, the free radical signal was observed after an induction period that varied with the pyridine nucleotide used (Fig. 2). Following this induction period, which has also been observed when other nitro compounds were used [15], the free-radical signal reached a steady state. The induction period was shorter in the presence of higher protein concentration, or when using a de-aerated incubation medium, in accordance with the assumption that it reflects the depletion of oxygen in the reaction medium (Reaction 1). Radical formation as described in Fig. 1 depended on all three components of the system since omission of pyridine nucleotide (or nifurtimox), or thermal inactivation of the homogenate in a steam bath for 10 min, prevented the appearance of the free-radical signal. NADH was more effective than NADPH as a source of reducing equivalents. Similar results were obtained using epi- and trypomastigote homogenates (experimental results omitted).

Formation of nifurtimox anion radical in *T. cruzi* systems was also detected in intact cells. Figure 3 shows the time course of nifurtimox free-radical formation upon adding the nitrofuran to suspensions of *T. cruzi* epimastigotes (E) or trypomastigotes (T). Since no exogenous substrate was present, endogenous metabolites acted as electron donors for nifurtimox reduction. The induction period was shorter and the relative signal amplitude was higher in trypo- than in epimastigotes.

In good agreement with previous observations with epimastigote homogenates [3], the nifurtimox anion radical, the first intermediate of nitroreductase activity, was capable of transferring electrons to oxygen. The oxidation reaction was revealed by the increased rate of superoxide anion generation after nifurtimox addition to the amastigote homogenate, as well as by the effect of superoxide dismutase on adrenochrome formation (Fig. 4). In accordance with the mechanism of nifurtimox reduction already postulated [3], omission of NAD(P)H or homogenate from the incubation mixture prevented adrenochrome formation. Similar results were obtained with the trypomastigote homogenate (experimental data not shown). Production of hydrogen peroxide by *T. cruzi* homogenates was also stimulated by nifurtimox (Table 1), this latter effect

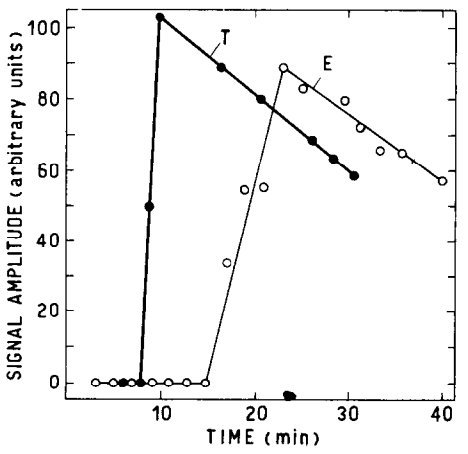

Fig. 3. Changes in the e.p.r. signal amplitude with time after mixing of 10^8/ml epimastigotes (E), or trypomastigotes (T), with an oxygen-saturated medium containing 1.0 mM nifurtimox. Other experimental conditions are described in the legend of Fig. 2 and under Materials and Methods.

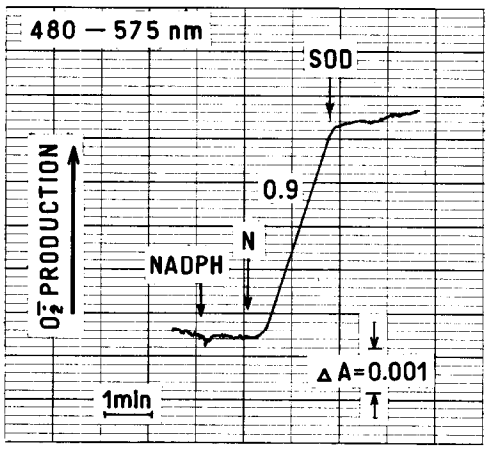

Fig. 4. Effect of nifurtimox on $O_2^{\cdot-}$ production by the amastigote homogenate. The reaction medium contained homogenate (0.4 mg protein/ml), 1 mM epinephrine, 130 mM KCl and 20 mM phosphate buffer, pH 7.4. Where indicated, 0.7 mM NADPH, 0.1 mM nifurtimox (N) and superoxide dismutase (SOD; 3.3 μg/ml) were added. The value against the trace indicates nmoles $O_2^{\cdot-} \cdot min^{-1} \cdot (mg\ protein)^{-1}$. Other experimental conditions are described under Materials and Methods.

Table 1. Effect of nifurtimox on hydrogen peroxide production by *Trypanosoma cruzi* homogenates*

Additions	*T. cruzi* form		
	Amastigote	Epimastigote	Trypomastigote
NADH	23.3 ± 5.2 (*a*)†	12.7 ± 2.9 (*e*)	6.3 ± 2.1 (*j*)
NADH + nifurtimox	38.3 ± 9.5 (*b*)	29.0 ± 8.2 (*f*)	25.7 ± 2.4 (*k*)
NADH + nitrofurantoin	ND‡	10.7 ± 3.2 (*g*)	ND
NADPH	14.0 ± 0.9 (*c*)	14.7 ± 5.7 (*h*)	4.2 ± 0.6 (*l*)
NADPH + nifurtimox	39.2 ± 9.6 (*d*)	19.3 ± 2.6 (*i*)	17.6 ± 3.0 (*m*)

* The complete incubation mixture contained 130 mM KCl, 20 mM phosphate buffer (pH 7.4), 0.3 mM NAD(P)H and 0.4 mg homogenate protein; total volume, 1.5 ml; temp. 30°. Where indicated, 0.1 mM nitrofuran was added. Other experimental conditions are described under Materials and Methods. The values indicate nmoles $H_2O_2 \cdot min^{-1} \cdot (mg\ protein)^{-1}$.

† Mean ± S.D. (three independent determinations). Analysis of variance: *a,b* and *j,k*, $0.02 > P > 0.01$; *c,d* and *e,f*, $0.01 > P > 0.001$; *h,i* and *e,g*, $0.3 > P > 0.2$; *l,m*, $0.05 > P > 0.02$.

‡ ND = not determined.

being greater with the amastigote form. The stimulation of H_2O_2 production by the epimastigote homogenate in the presence of NADPH was not significant. Nitrofurantoin, a nitrofuran derivative practically ineffective against *T. cruzi*, did not stimulate H_2O_2 generation (Table 1). The significant production of H_2O_2 by *T. cruzi* homogenates in the absence of nifurtimox (Table 1) seems to be at variance with the little or no H_2O_2 generation by intact epimastigotes [3]. It is possible, however, that cell disruption may expose peroxide-producing enzymes (e.g. flavoproteins) to oxygen, and so constitute an artificial mechanism for the formation of H_2O_2.

DISCUSSION

In this work we have attempted to characterize the initial steps in the metabolism of the trypanocidal agent nifurtimox in different forms of *T. cruzi*, with special emphasis on the nature of the drug radical anion initially produced. The e.p.r. measurements in Figs. 1–3 accord well with the reduction of the nitroaromatic compound to a free anion radical of the general form $ArNO_2^-$ (Reaction 2):

$$2\ ArNO_2^- + NAD(P)H + H^+ \longrightarrow 2\ ArNO_2 + NAD(P)^+ + 2\ H^+ \quad (2)$$

$$ArNO_2^- + O_2 \longrightarrow ArNO_2 + O_2^- \quad (3)$$

$$O_2^- + O_2^- + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (4)$$

$$O_2^- + H_2O_2 \xrightarrow{Fe^{3+}} OH + OH^- + O_2 \quad (5)$$

The initial reduction of the drugs appears to be initiated by flavin-linked nitroreductase [17]. The drug radical may react with molecular oxygen to generate the superoxide anion (Reaction 3; Fig. 4). This, in turn, will produce hydrogen peroxide, either spontaneously or by the action of the enzyme superoxide dismutase (Reaction 4; Table 1). The interaction of H_2O_2 and O_2^- may lead to the production of hydroxyl radical ($OH^\cdot$; Reaction 5) which has been postulated to be one of the most damaging low molecular weight radical species produced by ionizing radiation. Reactions 2–5 may also lead to extensive DNA damage [18] and lipid and organic peroxide formation [19]. Moreover, Reactions 2–5 occur in the different forms of *T. cruzi* (Table 1), which leads one to assume that they are responsible for the lethal effect of nifurtimox *in vivo*.

The mechanisms for detoxifying hydrogen peroxide in *T. cruzi* are not clear. Protective enzymes such as glutathione peroxidase or catalase, which act in mammalian tissues to destroy relatively low [20] or high [21] concentrations of hydrogen peroxide, respectively, appear to be of very little or no metabolic significance in *T. cruzi* [22, 23]. The organism is particularly sensitive to H_2O_2-generating agents, such as naphthoquinones [9, 24, 25]. This sensitivity may contribute to the susceptibility of *T. cruzi* to the cytotoxic effects of nifurtimox. In this connection, it seems pertinent to point out that the lethal effect resulting from the subversion of electron flow from the normal water-producing pathway to the superoxide anion pathway represents a promising approach to the chemotherapy of diseases caused by organisms poorly endowed with peroxide-detoxifying mechanisms [26]. Drugs to be used for that purpose must fulfill two conditions, namely, reduction to a free radical and autoxidation of the latter, with generation of superoxide anion and hydrogen peroxide [3, 9, 24, 25]. Other mechanisms of toxicity, however, cannot be excluded.

The enzymatic reduction of the nitro group is generally held to be required for the bactericidal action of nitrofurans. Nitroreduction is necessary for the mutation of bacteria and the breakage of bacterial or mammalian DNA, as well as for the covalent binding of nitrofuran reduction intermediates to protein and DNA. The reactive intermediate(s) responsible for these phenomena are still unknown but must have an oxidation state between that of the nitro-anion free radical and the amine [26]. Most workers believe that the hydroxylamine, which is the 4-electron reduction product, is the reactive metabolite responsible for most of the biological effects of the nitro compounds [26]. The present study does not rule out the potential involvement of such metabolites as mediators of hydrogen peroxide or superoxide anion radical production.

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