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ORIGINAL ARTICLE

Trypanosoma cruzi dihydrolipoamide dehydrogenase as target of reactive metabolites generated by cytochrome c/hydrogen peroxide (or linoleic acid hydroperoxide)/phenol systems

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Abstract
This study determines that cytochrome c (cyt c) catalyses the oxidation of phenol compounds (Phen) in the presence of H_2O_2 or linoleic acid hydroperoxide (LOOH), generating Phen-derived free radicals or other reactive metabolites. These products irreversibly inactivated the dihydrolipoamide dehydrogenase from *Trypanosoma cruzi* (*T cruzi* LADH), depending on: the Phen structure, peroxide type, activated cyt c, incubation time and presence of an antioxidant. Nordihydroguaiaretic acid (NDGA) and caffeic acid (CAFF) with cyt c/H_2O_2 or cyt c/LOOH were the most effective inhibitors of *T cruzi* LADH. The comparison of inactivation values for *T cruzi* and mammalian heart enzymes demonstrated a greater sensitivity of *T cruzi* LADH to Phen. GSH, N-acetylcysteine, NAD(P)H, ascorbate and trolox, prevented *T cruzi* LADH inactivation by acetaminophen. The role of the Phen as potential trypanocidal systems is discussed.

Keywords: *Dihydrolipoamide dehydrogenase, Trypanosoma cruzi, cytochrome c, peroxides, phenol compounds*

Introduction

Trypanosoma cruzi (*T. cruzi*), a protozoa belonging to the order kinetoplastidae, is the causative agent of Chagas disease, a major public health problem affecting 18 endemic countries in the American Continent. It was recently estimated that the overall prevalence of human *T. cruzi* infection reaches 7 694 500 cases, that 100 million people are at risk of acquiring this infection and that the annual number of new cases is 55 585 [1].

At present, this disease has no effective treatment and, although significant advances have been reported on the control of both vectorial and transfusional transmission of the disease, the elimination of *T cruzi* from infected patients is essential to arrest the evolution of this condition [2]. In this sense, new approaches are being conducted with the aim of accomplishing the best specific treatment for Chagas disease. It has been previously demonstrated that peroxidases and other hemoproteins (Mb, Cyt. c) [3–6] catalysed the phenothiazines (PTZ) oxidation, the production of their free radicals ($PTZ^{+\cdot}$), which irreversibly inhibited the Trypanothione reductase (*T cruzi* TR) [4] and the Dihydrolipoamide dehydrogenase (*T cruzi*. LADH) [5,6]. Then, $PTZ^{+\cdot}$ cationic radicals may contribute to the trypanocidal action of these compounds [4–7].

LADH (a FAD disulphide oxido-reductase) is an essential component of 2-oxo acid dehydrogenase multi-enzymatic complexes, such as α oxoglutarate DH complex [8]. These complexes play an important role in *T cruzi* oxidative metabolism [6,7], through all evolutive cycles of the parasite. LADH reversibly catalyses the NADH-dependent reduction of lipoamide, $L(S)_2$ to dihydrolipoamide, $L(SH)_2$ (Reaction 1):

$$NADH + H^+ + L(S)_2 \leftrightarrow NAD^+ + L(SH)_2 \qquad (1)$$

The phenolic compounds (Phen) are good substrates of peroxidases some of them possess antimicrobial activity [9] and it has been shown that *T cruzi* LADH is inactivated by MPO/H_2O_2/Phen systems

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[6,7]. Furthermore, it has been suggested that PTZ and Phen, in combination treatment, could be strong trypanocidal systems looking for a synergistic inactivating effect on *T cruzi* LADH, aimed at reducing the PTZ concentration [7]. The potential role of the cytochrome c on $PTZ^{+\cdot}$ radicals generation and its anti-*T cruzi* LADH [5] effect lead us to extend this study with cyt c as a key component of peroxidative systems supplemented with phenols and peroxides.

Cyt c is a small globular monomeric hemeprotein which, apart from its functional role on mitochondrial respiration and electron transporter between the complexes III (ubiquinol cyt c reductase) and IV (cytochrome oxidase), acts as an initiating factor and regulator of apoptotic process [10], as antioxidant due to the superoxide removal capacity [11] and it is a potential pro-oxidant as ROS generator [12] and its peroxidase activity, which reacts with many substrates [5,13–17]. Cyt c has a heme group which is localized in a hydrophobic environment, near the N-terminal end of its polypeptidic chain which is linked by two thioether bonds with Cys^{14} and Cys^{17} forming with His^{18} and Met^{80} the fifth and sixth Fe's ligands, respectively [18]. The cyt c is placed in the mitochondrial inter-membranes space at high concentrations [19] and, although the H_2O_2 is normally found in low concentrations [20], it can reach excessive amounts under oxidative stress conditions. On the other hand, cyt c may induce the lipid peroxidation reacting with H_2O_2 [21] and lipid hydroperoxides [22], forming a peroxidase compound I-type intermediate [16,23]. Although for many peroxidases the H_2O_2 is an optimum substrate, other peroxidases are more active with lipid hydroperoxides. Likewise, the cyt c interaction with linoleic acid hydroperoxide (LOOH) was more effective than the respective H_2O_2 reaction [22]. Considering the reasons above mentioned, the following issues deserved special attention: (a) Cytochrome c as an oxidant of Phen (Figure 1) in the presence of H_2O_2 (cyt c/H_2O_2 system) or a generator of hydroperoxide linoleic acid (cyt c/LOOH system); (b) effect of both systems supplemented with Phen on *T. cruzi* LADH inactivation, as well as their dependence on the structure of Phen, the type of peroxide, inactivation time and the presence of antioxidants; and (c) comparative effect of cyt c/H_2O_2/Phen systems on *T cruzi* LADH and mammalian myocardium LADH, with the aim to support the suggestion to study this enzyme as a target for trypanocidal agents [24].

Methods

Enzymes and reagents

Recombinant *T cruzi* LADH was obtained by expression in *Escherichia coli* JRG 1342 cells as described [25]. *T cruzi* LADH stock and work solutions were prepared as indicated [6]. Enzyme stock solution remained stable for at least 1 year. Specific activity was 417 U/mg protein. Porcine heart LADH, bovine heart cytochrome c (cyt c), horse skeletal muscle myoglobin (Mb), soybean lipoxygenase type V (SLOX), H_2O_2, linoleic acid (9, 12-octadecadienoic acid), tyrosine (Tyr), acetaminophen (AAP), guaiacol (GUAI), catechol (CATE), caffeic acid (CAFF), dopamine (DPM), nordihydroguaiaretic acid (NDGA) and etoposide (ETOP) were obtained from Sigma (St. Louis, MO). SLOX specific activity ranged from 500 000–1 000 000 U/mg protein, as determined measuring the increase in A_{234} with linoleic acid substrate. Other reagents were the ones used previously [5,6].

Cytochrome c peroxidase activity assay

The following was determined: (1) With guaiacol (2-methoxy phenol) as hydrogen donor [26]. Unless stated otherwise, this cyt c activity was measured at 30°C in an assay mixture containing 13 mM guaiacol, 5 μM cyt c, 50 mM K-phosphate, pH 7.4 and 0.5 mM H_2O_2, as described [7]. The H_2O_2 and cyt c concentration effect on peroxidase activity was as studied previously [5]. (2) The catalytic activity of cyt c was also explored using tyrosine as substrate, measuring the fluorescence of the dityrosine formed by dimerization of tyrosyl radical-product of the peroxidation reaction, under conditions stated in the Results section. A similar study was made with AAP, which detected the dimerization of its phenoxyl radical to diacetaminophen by optical spectroscopy, measuring the absorbance at 320 nm. Likewise, the fluorescence emission spectra of products formed by AAP/H_2O_2/cyt c were obtained as indicated in the Results section. (3) Cyt c lipid hydroperoxidase activity: The reaction of cyt c with lipid hydroperoxide, hydroperoxyoctadecadienoic acid (below is LOOH) and phenols was also examined. Fluorescence measurements were performed in an Aminco SLM 8000 C spectrofluorophotometer, with 325 nm excitation and 380–480 nm (Tyr and AAP) or 430–530 nm (CAFF) emission. With these data, fluorescence spectra were traced. Lipid hydroperoxide generator system: LOOH was obtained incubating linoleic acid with soybean lipoxygenase for 3 min at 30°C as indicated in the respective figures. Prior to the experiments, the activity of linoleic acid peroxidation was estimated by the formation of conjugated diene, measuring the absorbance at 234 nm ($\varepsilon_{234} = 2.5 \times 10^4$ cm^{-1} M^{-1}) [27]. Immediately after confirming the LOOH production, its reaction with Cyt c was assessed by the depletion of conjugated diene, adding 5 μM cyt c to the reaction mixture. Control samples without SLOX or cyt c were determined in parallel [27].

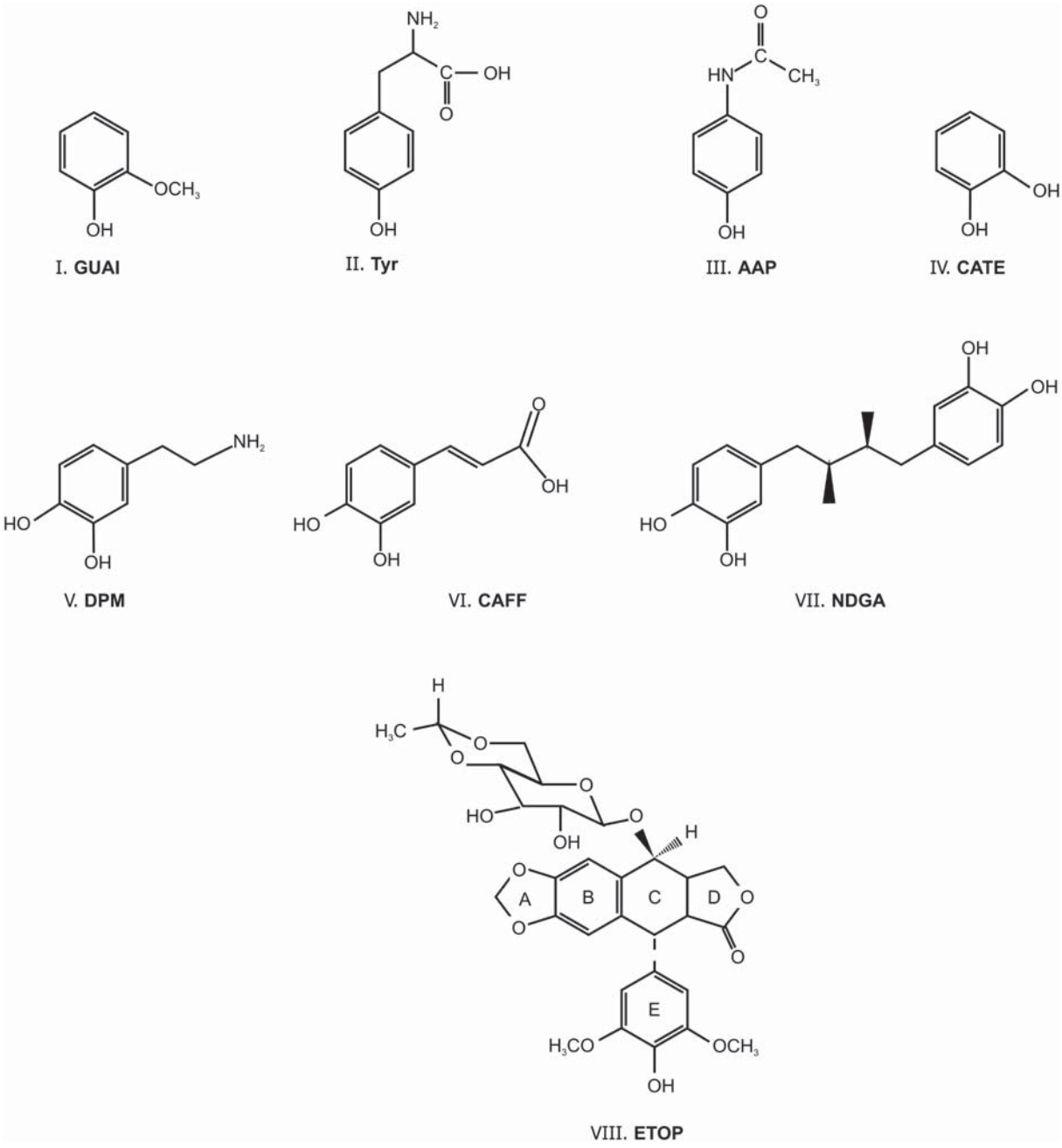

Figure 1. Structures of phenolic compounds used in this study: I. Guaiacol (GUAI); II. Tyrosine (Tyr); III Acetaminophen (AAP); IV. Catechol (CATE); V. Dopamine (DPM); VI. Caffeic acid (CAFF); VII. Nor-dihydroguaiaretic acid (NDGA); VIII. Etoposide (ETOP).

LADH inactivation

In a total volume of 0.1 ml, the LADH inactivation mixture contained 0.8 μM *T cruzi* LADH, 5 μM cyt c, 0.5 (or 0.25) mM H_2O_2 or LOOH generator system (60 nM SLOX plus 40 μM linoleic acid), 1 mM EDTA, 50 mM K-phosphate, pH 7.4 and phenolic compound as stated in the Results section. The mixture also contained 1.25 mM KCl, plus12.5 μM EDTA and 82 mM glycerol, resulting from dilution of the original LADH suspension. Control experiments (omitted) showed that glycerol failed to affect LADH or phenoxyl radical activity. Samples were incubated at 30°C for the respective time; 10 μl aliquots were added to the assay mixture and residual LADH activity was assessed as described below. Control samples without cyt c, H_2O_2, SLOX or linoleic acid or other constituents of the inactivation mixture were incubated in parallel.

LADH assay

LADH activity was measured by the rate of NADH oxidation using lipoamide as an electron acceptor [5,6]. The standard assay mixture contained 10 μl LADH inactivation sample as indicated above,

0.2 mM NADH, 1.0 mM lipoamide and 50 mM K-phosphate, pH 7.4; total volume, 3.0 ml. The initial velocity of LADH reaction was assessed at 340 nm by the slope of recorded tracings using a Perkin Elmer 550 UV/VIS spectrophotometer at 30°C.

Radical interceptors assay

Interceptor activity was estimated by its effect on phenoxyl radical production, through dimerization, by the cyt c/H_2O_2/AAP system. The reaction mixture contained 5 μM cyt c, 0.5 mM H_2O_2, 50 μM AAP, 1 mM EDTA, 50 mM K-phosphate, pH 7.4: final volume, 2.0 ml. Interceptors were added at the beginning of incubation as indicated in Figure 4. Production of diacetaminophen was monitored by measuring the fluorescence emission at 410 nm with excitation of 325 nm.

Expression of results

LADH specific activity values are presented as the percentage value of the control sample activity (100(%)). LADH inactivation (I(%)) by the Phen pro-oxidant systems was calculated as described [5,6]. Protection (*P*) by phenoxyl radical or reactive metabolites presumptive interceptors against the cyt c/H_2O_2/Phen systems was calculated from the equation $P(\%) = 100(I(\%) - ip(\%)/I(\%)$, where *P*, *I* and *ip* are relative radical interceptor activity (LADH protection), inhibition of LADH activity by the cyt c/H_2O_2/Phen system and inhibition of LADH activity by the latter system plus interceptor, respectively. Results are presented as mean ± SD values from at least three measurements for each sample. Statistical analyses were performed by Student's *t*-test for one or two samples.

Results

Peroxidase activity of cytochrome c

With H_2O_2. This study confirms that cytochrome c catalyses guaiacol oxidation in the presence of hydrogen peroxide [5] with an activity of 2.0 ± 0.15 nmoles H_2O_2 consumption/min/nmol cyt c. We observed that the cyt c displays low peroxidase activity compared to true peroxidases (e.g. HRP) and other non-peroxidase heme proteins (e.g. Mb). The peroxidase activity of heart Mb and HRP, determined under the same conditions, was 24 ± 1.9 nmoles H_2O_2/min/nmol Mb and 165 ± 6 μmoles H_2O_2 depleted/min/nmol HRP.

The native cyt c is hexacoordinated with no coordinated water molecule that can be displaced by H_2O_2 [28]. However, its sixth ligand is the methionine 80 that is readily disrupted by several factors leading to increased access to the heme iron and higher peroxidase activity. This effect has been related to partial unfolding of cyt c triggered by chemical or physicochemical processes [29–35]. The resulting heme pocket structural changes, or the β sheet structural conversion in cyt c [32] would to activate the peroxidatic effect with the formation of a compound I-type intermediate (Reaction 2) [16,28,36].

The kinetic of the cyt c peroxidase activity was also confirmed by measuring the tetraguaiacol as a product of the reaction, with an initial activation phase (a), a steady-state (b) and the curve level off (c), described in Gutiérrez-Correa and Stoppani [5], was also confirmed (data omitted). Figure 2 indicates that L-Tyrosine is also a substrate of the cyt c in the presence of H_2O_2, measuring tyrosyl radical (through of the dityrosine formation) as a product of its peroxidase activity. It is observed that the rate of dityrosine production depends on the H_2O_2 concentration (from 0.125 mM to 1.0 mM) and profiles of each curve resembles those of cyt c/H_2O_2/guaiacol systems which were previously described [5].

As can be observed in Figure 2, the curve with 0.5 mM H_2O_2 presents the following phases: activation (a), steady-state (b) and decreased activity (c). Tyrosine oxidation was also produced in a cyt c concentration-dependent manner (data not shown).

With lipid hydroperoxide. Formation of tyrosyl radical from cyt c/LOOH/Tyr was determined by measuring the fluorescence emission at 406 nm with 325 nm excitation. LOOH was obtained by a SLOX/linoleic acid system. Lipoxygenases are non-heme iron enzymes that catalyse the dioxygenation of 1,4-*cis*, *cis*-pentadiene containing fatty acids (e.g. linoleic acid,

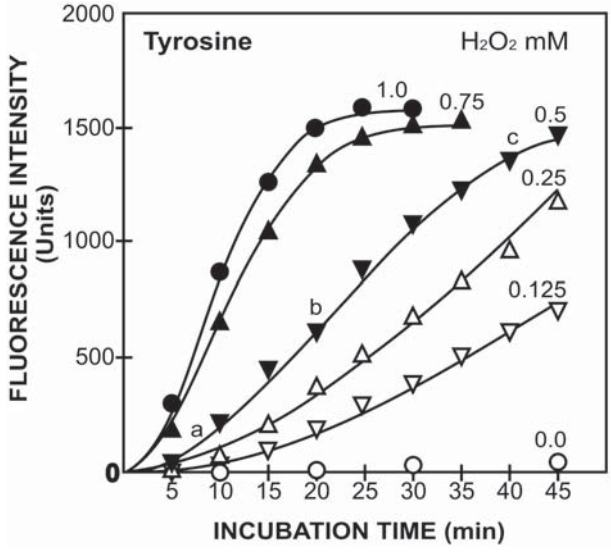

Figure 2. Cyt c peroxidase activity: H_2O_2 concentration-dependent. The reaction mixture contained 5 μM cyt c, 0.0, 0.125 to 1.0 mM H_2O_2 as indicated in the Figure, 25 μM Tyrosine, 50 mM K-phosphate, pH 7.4/1.0 mM DETAPAC. The product of the peroxidase reaction was measured as dityrosine by its fluorescence intensity at 408 nm (Exc = 325 nm). Curve 0.5 mM H_2O_2 shows a, b and c phases.

LA) to yield hydroperoxide lipid (e.g. LOOH) [37]. In this study, LOOH was obtained from a reaction mixture containing 75 nM SLOX, 0.25 mM linoleic acid (LA), 50 mM K-phosphate, pH 7.8 and 0.5 mM EDTA. After 3 min of incubation at room temperature, 0.2 mM Tyrosine and 5 μM cyt c were added. Then, a fluorescence emission spectrum was recorded at 15 min (Figure 3). The control spectra (Tyrosine/LOOH) obtained from a mixture without cyt c were registered at 0 min and 15 min. The initial and final control spectra were superimposed. Fluorescence emission spectra from the complete system presented a high peak at 406 nm corresponding to dityrosine, while a shoulder observed at 422 nm may correspond to other products of cyt c lipid hydroperoxide peroxidase activity, such as polymers of dityrosine. It is worth indicating that a typical peroxidase assay with 5 μM cit c, 150 μM tyrosine and 0.5 mM H_2O_2 gives a similar fluorescence spectrum (not shown).

Formation of reactive metabolites during the oxidation of acetaminophen by cytochrome c peroxidase activity

Results indicate that the cyt c/H_2O_2/AAP system generates the respective phenoxyl free radical detectable as a dimer due to its time-dependent increase of absorbance at 320 nm (data omitted). Production of AAP-AAP dimers was also demonstrated by fluorescence method (Figure 4). Such compounds presented a fluorescence emission peak at 410 nm (excitation 325 nm). The profile of the emission corresponding to dimer products shows a short initial activation step, followed by a steady state and decrease of the rate reaction phases (Figures 4A–C), similar to a peroxidase assay with cyt c/Tyr or GUAI (text, Figure 2, [5]). These observations support that the cyt c/H_2O_2 system could use AAP as an electron donor generating acetaminophen free radical. The latter could be reduced back to AAP by compounds such as GSH, NAD(P)H, ascorbate, trolox and others (Figure 4 and its legend).

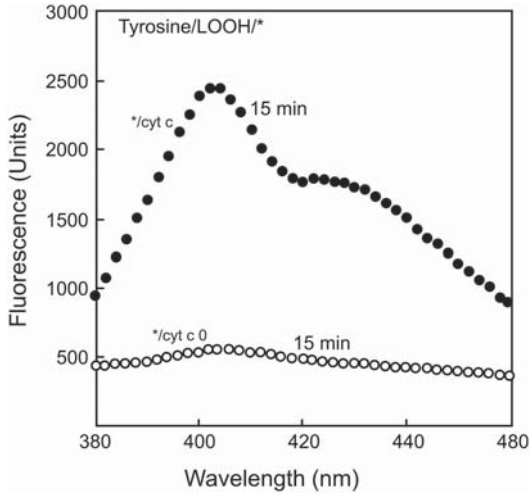

Figure 3. Fluorescence emission spectra of tyrosine/LOOH in the absence and presence of cytochrome c. Exc = 325 nm. Tyrosine at 0.2 mM concentration and 5 μM cyt c. The reaction medium also containing 75 nM SLOX, 0.25 mM linoleic acid, 50 mM K-phosphate pH 7.8 and 0.5 mM EDTA.

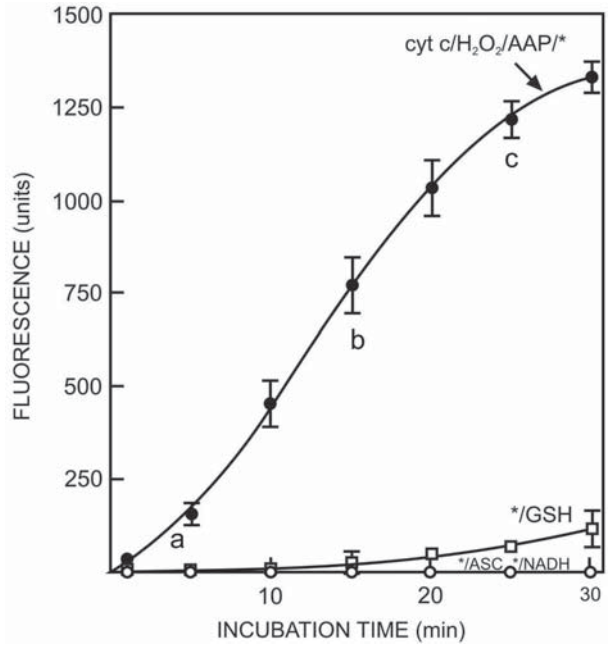

Figure 4. Production of diacetaminophen by the cyt c/H_2O_2/AAP system in the absence or presence of interceptor antioxidants (GSH, NADH, ASC). The reaction medium contained 5 μM cyt c, 0.5 mM H_2O_2, 50 μM AAP, 50 mM K-phosphate, pH 7.4/1 mM EDTA; and 0.2 mM GSH or Ascorbate, or 0.1 mM NADH was added as indicated in the Figure. Fluorescence emission at 410 nm (Exc = 325 nm) was measured. Fluorescence was not detected in complete systems with 0.2 mM NAC or Trolox as well as in control System cyt c/AAP (data not shown).

It is known that HRP [38,39] and others peroxidases [7,40–43] in the presence of H_2O_2 are able to oxidize AAP not only to dimers and polymers but also to N-acetyl-*p*-benzoquinone imine (NAPQI). This reactive metabolite exhibits readily detectable fluorescence emission spectra with a peak at 440 nm [44]. This study demonstrated the AAP oxidation by cyt c/0.5 mM H_2O_2 (Figure 5A) or cyt c/28 μM LOOH (Figure 5B) systems. Very similar emission spectra were obtained with both peroxides, with the highest emission at 436 nm. These results support the ability of cyt c to generate NAPQI from the AAP/peroxide systems.

Caffeic acid oxidation by the cytochrome c/H_2O_2 (or LOOH) systems

Oxidation of CAFF was assayed by absorption spectroscopy measuring its decay during the reaction with the cyt c/H_2O_2 system (Figure 6A). The decreasing of CAFF concentration depended on both cyt c and peroxide (Figure 6B inset, and its legend). These

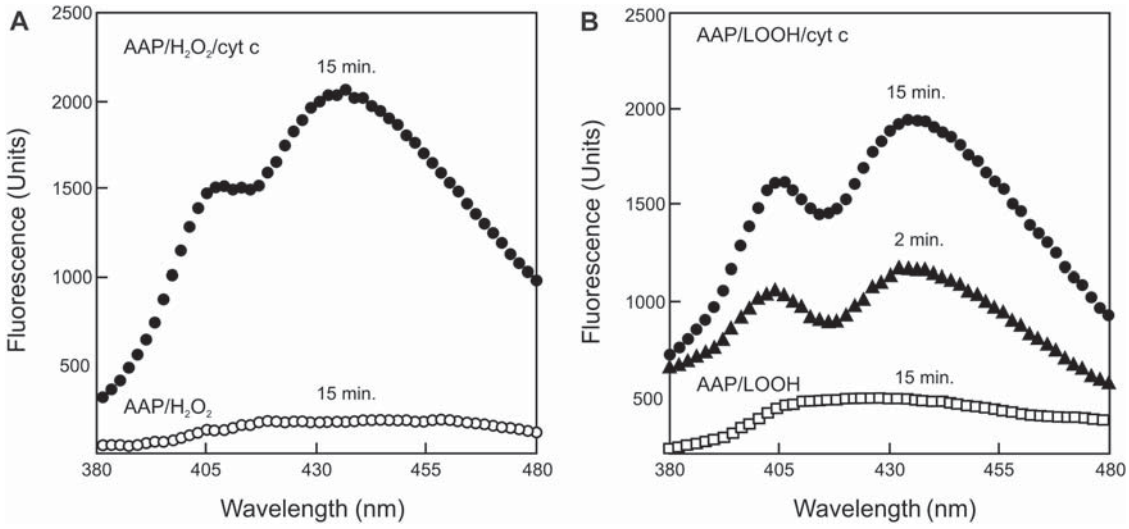

Figure 5. Fluorescence emission spectra of products formed by AAP/cyt c systems. (A) H_2O_2. The reaction medium contained 5 μM cyt c, 0.5 mM H_2O_2, 50 μM AAP, 50 mM K-Phosphate, pH 7.4/1 mM EDTA. The reaction was started by adding AAP. Fluorescence emission spectra, 380–480 nm (Exc = 325 nm) was recorded after 15 min incubation. Control sample (AAP/H_2O_2), lacking cyt c, recorded at initial time and after 15 min were superimposed (the latter only is shown). (B) LOOH. Initially, the reaction mixture contained tne generator system (30 nM SLOX, 40 μM Linoleic acid, 50 mM K-Phosphate, pH 7.8/1.0 mM EDTA) generating 28 μM LOOH after 3 min incubation. Then, the system was completed by adding 20 μM AAP and 5 μM cyt c as described in Material and methods. Control sample (AAP/LOOH). Fluorescence (Figure 5) emission spectra was recorded at 2 and 15 min incubation.

results suggest that *o*-semiquinone radicals and their quinones were products of the CAFF/cyt c/H_2O_2 reaction. The time course of CAFF decay resulted, at least, from the following reactions: (a) CAFF peroxidation; (b) caffeic acid *o*-semiquinones polymerization; and (c) *o*-semiquinones dismutation (Reaction 6) [45].

The cyt c/LOOH also resulted in decreased concentration of CAFF depending on the cyt c and LOOH generator system (spectra not shown).

CAFF oxidation by cyt c peroxidase activity was also monitored using the fluorescence method. There were changes in the fluorescence emission spectrum using H_2O_2 (Figure 7) or LOOH (spectra omitted). Spectral quantitative changes were observed at 470 nm with both peroxides. The spectra were very similar, including their dependence on the presence of cyt c and HOOH or LOOH. A significant change of the high peak, from 100% at 30 min to 30% at 45 min of incubation, was detected at 470 nm (Figure 7), which suggests further transformation of the relatively stable fluorescent intermediate to other products [45].

Effect of the cytochrome c/H_2O_2/phenolic compounds systems on T. cruzi LADH activity

T cruzi LADH was inactivated by cyt c/H_2O_2/Phenolic compounds systems, as a function of phenol structure and incubation time (Table I). The phenol concentrations were selected from previous experiments related to concentration-inhibition (omitted). After 15 min of incubation with cyt c/H_2O_2, NDGA and CAFF acid were the most effective phenols, despite being assayed with the lowest concentrations. In fact, 82% of LADH activity was inhibited at 15 min of incubation with 10 μM NDGA, but 25 μM CAFF was similarly active against LADH (88% inhibition). NDGA has two *o*-diphenol moieties linked to a C4 chain, whereas CAFF has one *o*-diphenol structure in conjugation with a vinyl side chain (Figure 1). LADH inactivation values (%) obtained after 30 min incubation of LADH with these phenol systems were 86% and 92%, respectively (Table I, Figures 8(1) and 9(1); and after 60 min of incubation they were 97% (NDGA) and 95% (CAFF) (Table I).

Catechol, one simple *o*-diphenol, and AAP, one monophenol *p*-substitued, assayed at 50 μM, produced less LADH inactivation within the first 30 min incubation (Table I and Figure 10). However, higher LADH inactivation values were observed at 60 min incubation: 85% for CATE and 93% for AAP (Table I).

Guaiacol, an *o*-methoxy substituted monophenol, was less active than AAP since, although 100 μM was used, LADH inactivation values (%) were 29, 55 and 72 at 15, 30 and 60 min of incubation, respectively (Table I). Etoposide, an anti-neoplastic agent used as an inhibitor of the topoisomerase II/DNA cleavable complex, which has a guaiacol-like group in its structure (Figure 1), was also studied as a substrate of the cyt c/H_2O_2 monitoring its effect on *T cruzi* LADH activity. This action was determined using 100 μM ETOP, the same concentration of the guaiacol assay, which allowed immediate comparison of effects of both phenolic compounds. Table I shows that

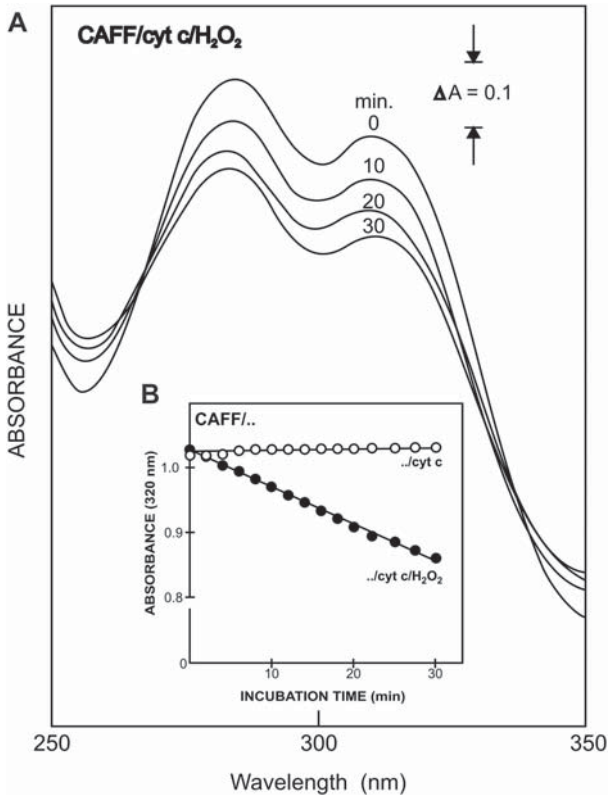

Figure 6. (A) Absorption spectra of caffeic acid under oxidation by cyt c/H_2O_2 system. The incubation medium contained 50 μM caffeic acid, 5 μM cyt c, 0.5 mM H_2O_2, 50 mM K-phosphate pH 7.4/0.2 mM DETAPAC. (B) Inset: Experimental conditions as in (A) experiment. Control sample (H_2O_2 omitted) is shown. In the absence of cyt c, absorbance was stable (0.97) during the experiment (data not shown). A decrease in A320 is associated with the caffeic acid oxidation cyt c/H_2O_2-dependent.

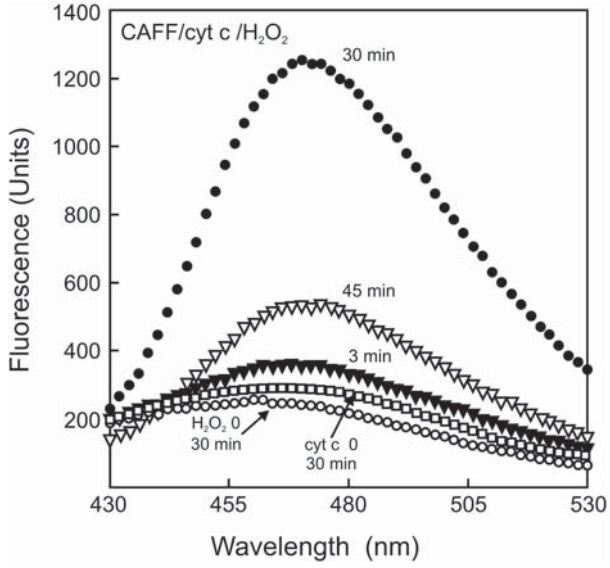

Figure 7. Fluorescence emission spectra of products formed by CAFF/H_2O_2/cyt c system. The reaction mixture contained 5 μM cyt c, 0.5 mM H_2O_2, 25 μM CAFF, 50 mM K-phosphate, pH 7.4/1 mM EDTA. Fluorescence emission spectra were recorded at 3, 30 and 45 min incubation. Control systems (H_2O_2 0 and cyt c 0) were recorded at 30 min incubation.

ETOP was more active against LADH than guaiacol, being the inactivation values (%) 45, 71 and 90 vs 29, 55 and 72 after 15, 30 and 60 min incubation, respectively ($p < 0.005$). Dopamine, a catechol *p*-ethyl-amine-substituted, was much less active and L-Tyrosine, a monophenol amino acid, was inactive (Table I).

Effect of the cytochrome c/LOOH/phenolic compounds systems on T cruzi LADH activity

This study previously confirmed that LA (40 μM) was oxidized by SLOX (60 nM) generating 28 ± 2 μM of hydroperoxyoctadecadienoic acid (LOOH), after 3 min of incubation. When cyt c and Phen were added to the produced reaction mixture containing LOOH, in absence of H_2O_2, the cyt c acted as a pseudo-lipohydroperoxidase monitored by its ability to inhibit *T cruzi* LADH. Table I and Figures 8–10(2) summarize the results of *T cruzi* LADH inactivation using different Phen (Figure 1) in the cyt c/LOOH systems. NDGA and CAFF/cyt c/LOOH (28 μM) systems were equally or more active than the corresponding cyt c/H_2O_2 (250 or 500 μM) systems. After 30 min of incubation with NDGA, inactivation values of 86% and 98% were obtained in cyt c/H_2O_2 or cyt c/LOOH, respectively. The results also indicate that LOOH systems attacked the enzyme faster. In fact, NDGA/cyt c/LOOH produced 90% *T cruzi* LADH inactivation within 1 min of incubation (Figure 8(2)), whereas the corresponding inactivation value was 27%, using H_2O_2 as an oxidizing agent of cyt c (Figure 8(1)). Similar effects were observed with other Phen (Table I, Figures 8–10(1) and (2)). Catechol/cyt c/LOOH was less active than CAFF, since that higher concentration of CATE produced similar levels of LADH inactivation within the same time (Table I). However, the reaction rate was faster with CAFF/cyt c/H_2O_2 than with the corresponding CATE system. The AAP/cyt c/LOOH system produced 65% of LADH inactivation within 30 min, but as well as other Phen/cyt c/LOOH, the reaction was faster leading to 60% of inactivation after 15 min incubation (Table I, Figure 10(2)). Comparison of experiments with dopamine shows significant difference in both peroxides, observing 58% loss of LADH activity in the LOOH system within 30 min. Table I also shows that the inactivating effect of ETOP is higher than GUAI on *T cruzi* LADH using LOOH, 90% and 60% after 30 min incubation, respectively.

Comparison between T cruzi LADH and mammalian LADH inactivation by cytochrome c/H_2O_2/phenolic compounds

The interactions among *T cruzi* LADH and porcine heart LADH with several Phen in cyt c/H_2O_2 systems

Table I. Inactivation of *T. cruzi* LADH by cytochrome c/H_2O_2 (or LOOH)/Phenolic compounds systems.

Phenolic compounds (μM)		LADH inactivation (%)				
		H_2O_2 systems incubation (min)			LOOH systems incubation (min)	
		15	30	60	15	30
NDGA	(10)	82 ± 1	86 ± 1	97 ± 2	97 ± 2	98 ± 0
CAFF	(25)	88 ± 1	92 ± 2	95 ± 1	85 ± 1	87 ± 1
AAP	(50)	30 ± 3	71 ± 2	93 ± 2	60 ± 1	65 ± 2
CATE	(50)	37 ± 1	65 ± 3	85 ± 2	82 ± 2	82 ± 1
GUAI	(100)	29 ± 1	55 ± 2	72 ± 2	35 ± 1	60 ± 2
ETOP	(100)	45 ± 3	71 ± 2	90 ± 2	60 ± 2	90 ± 2
DPM	(100)	7 ± 1*	16 ± 2*	29 ± 1	32 ± 1	58 ± 1
Tyr	(100)	3 ± 2*	4 ± 1*	4 ± 2*	nd	nd

The reaction mixture contained 0.8 μM *T. cruzi* LADH; 5 μM cytochrome c; 0.5 mM (except NDGA system, which contained 0.25 mM) H_2O_2 or 60 nM soybean lipoxygenase plus 40 μM linoleic acid (LOOH generator system); 10, 25, 50 or 100 μM phenolic compounds as indicated in parentheses; 1 mM EDTA and 50 mM K-phosphate, pH 7.4. Other conditions are described in the Methods section. Incubation time as indicated above; nd, not done. Values represent means ± SD. All control samples, except those from NDGA, showed 0–8% LADH inactivation. Control samples values H_2O_2/NDGA and LOOH/NDGA systems in Figures 8(1) and (2). Values represent means ± SD. Statistical one-sample *t*-test analysis was performed using 0 ± 5 (theoretical mean ± SD) (%) as control value. *$p < 0.001$ except values that were not significant.

were assayed. Table II shows a greater sensitivity of *T cruzi* LADH to the phenolic compounds systems, having AAP 5-fold higher inactivation of *T cruzi* LADH than mammalian LADH, under the same experimental conditions. However, under other conditions, 2 μM cyt c and 0.2 mM AAP, higher difference was observed within 30–60 min incubation (data omitted)

T cruzi LADH protection by interceptor antioxidants against reactive phenolic metabolites

Table III shows that thiol compounds, GSH and NAC, prevented *T cruzi* LADH inactivation by cyt c/H_2O_2/AAP system. Thiols were added to the reaction mixture before the peroxidative system. Both thiols efficiently protected LADH, ~ 90% at 60 min incubation. Likewise, under similar experimental conditions, LADH protection values (%), obtained after 60 min incubation with 0.2 mM GSH, cyt c/H_2O_2/phenol (phenol concentration as indicated in Table I) were: NDHG, 100; CAFF, 100; Catechol, 95; Guaiacol, 92 and Etoposide, 95. The consistent effects of GSH and NAC strongly supported the anti-reactive metabolites role of the thiol group.

On the other hand, the addition of 0.2 mM GSH to a LADH sample inactivated by the cyt c/H_2O_2/AAP system, as described in Table III, failed to restore LADH activity, thus supporting the irreversible character of the LADH inactivation mechanism dependent on reactive phenolic metabolites. Table III also shows the protective effect of other interceptor antioxidants on LADH inactivation by cyt c/H_2O_2/AAP. All of them also strongly protected LADH, from 87–98%, as observed after 60 min incubation.

Protection of *T cruzi* LADH by thiols and other antioxidants involved interception of reactive phenolic metabolites. In fact, the presence of GSH in the cyt c/H_2O_2/AAP reaction mixture quenched the fluorescence emission at 410 nm, which suggests the interception of phenoxyl radical derived from AAP. NADH, ascorbate, NAC and Trolox behaved similarly (Figure 4 and its legend).

Discussion

This study shows that the cytochrome c has peroxidase activity reacting with hydrogen peroxide (HOOH) or linoleic acid hydroperoxide (LOOH) and that phenolic compounds are substrates of those reactions. Peroxidases catalyse a one-electron oxidation of Phen substrates to phenoxyl radicals. Hence, Tyr and AAP oxidation were monitored by optical and fluorescence spectroscopy through their respective dimers. Then, the phenoxyl radicals dimerization supports the Tyr and AAP peroxidation by cyt c/H_2O_2 (Figures 2, 4 and 5A) and cyt c/LOOH (Figures 3 and 5B). In other words, cyt c catalyses the oxidation of structurally dissimilar Phen, primarily generating phenoxyl radicals. The reactions imply:

a) The intermediate compound I formation:

$$Cyt\,c - Fe^{III} + H_2O_2 \rightarrow Cyt^{+\bullet}c - Fe^{IV=0} + H_2O \quad (2)$$

$$Cyt\,c - Fe^{III} + LOOH \rightarrow Cyt^{+\bullet}c - Fe^{IV=0} + LOH \quad (3)$$

Cytochrome c activated by H_2O_2 (Reaction (2)) or LOOH [23] (Reaction (3)) generates an oxidant, oxo-ferryl cyt c [16], which contains a tyrosyl radical previously detected [28,36].

b) Generation of phenoxyl radicals by reactive cyt c species reacting with Phen:

$$Cyt^{+\bullet}c - Fe^{IV=0} + Phen - OH \rightarrow Cyt - C^{+}Fe^{III} + Phen - O^{\bullet} + OH^{-} \quad (4)$$

This study demonstrates the formation of phenoxyl radicals and other reactive metabolites by the reaction of H_2O_2 or LOOH with cyt c/ Phen (Figures 2–5A and B and 7). These reactive species can react, apparently by structural modification of the protein, with *T cruzi* LADH, causing its irreversible inactivation. The inhibitory role of these reactive metabolites was supported by: (a) Cyt c requirement for Phen activity. (Text; Figures 8–10(1) and (2)). Both peroxides with Phen excepting NDGA (Table I legend,

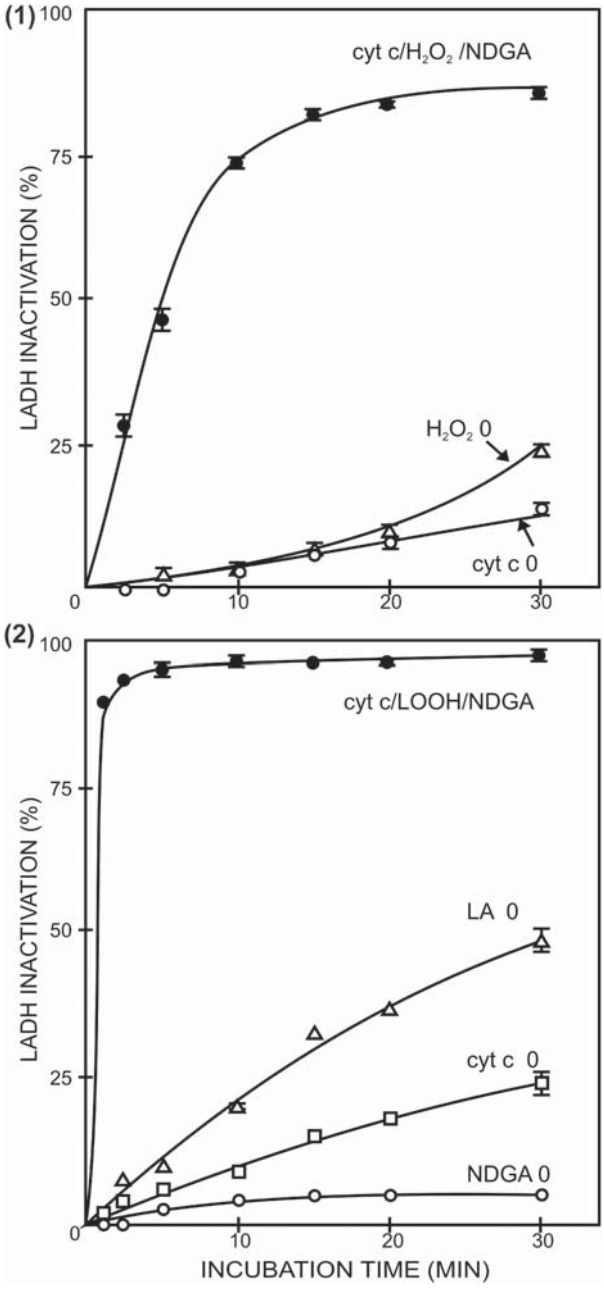

Figure 8. Time-course of *T cruzi* LADH inactivation by NDGA systems. (1) Cyt c/H_2O_2/NDGA and controls (H_2O_2 0, cyt c 0). (2) Cyt c/LOOH/NDGA and controls (LA 0, cyt c 0, NDGA 0). Conditions for experiments are given in the Table I legend and Methods section.

Figures 8(1) and (2)) failed to inhibit LADH to a significant degree; (b) Peroxide participation in the cyt c activation: The absence of H_2O_2 or lack of one or both components from LOOH generator system did not shown significant inhibitory effects (Figures 8–10); (c) Presence of Phen in the reaction mixture. Omission of Phen prevented *T cruzi* LADH inactivation (Table I, legend, Figures 8(2) and 9(2)), ruling out a direct action of the cyt c/peroxide system against this enzyme; (d) Time-dependent loss of activity with cyt c/peroxide/Phen systems (Figures 8–10); (e) Optical and fluorescence spectroscopy data (Figures 3–5); (f) Protective effect on *T cruzi* LADH of free radical interceptors (Table III, and text); and (g) Effect of LADH protectors on phenoxyl radical production determined by their dimerization (Text; Figure 4 and its legend).

T cruzi LADH inactivation by cyt c/LOOH/Phen systems deserves a special comment. In the present study, after the pre-incubation step, the mixture samples basically contained SLOX, most probably in the inactive form (ferrous enzyme), ~ 28 μM LOOH and

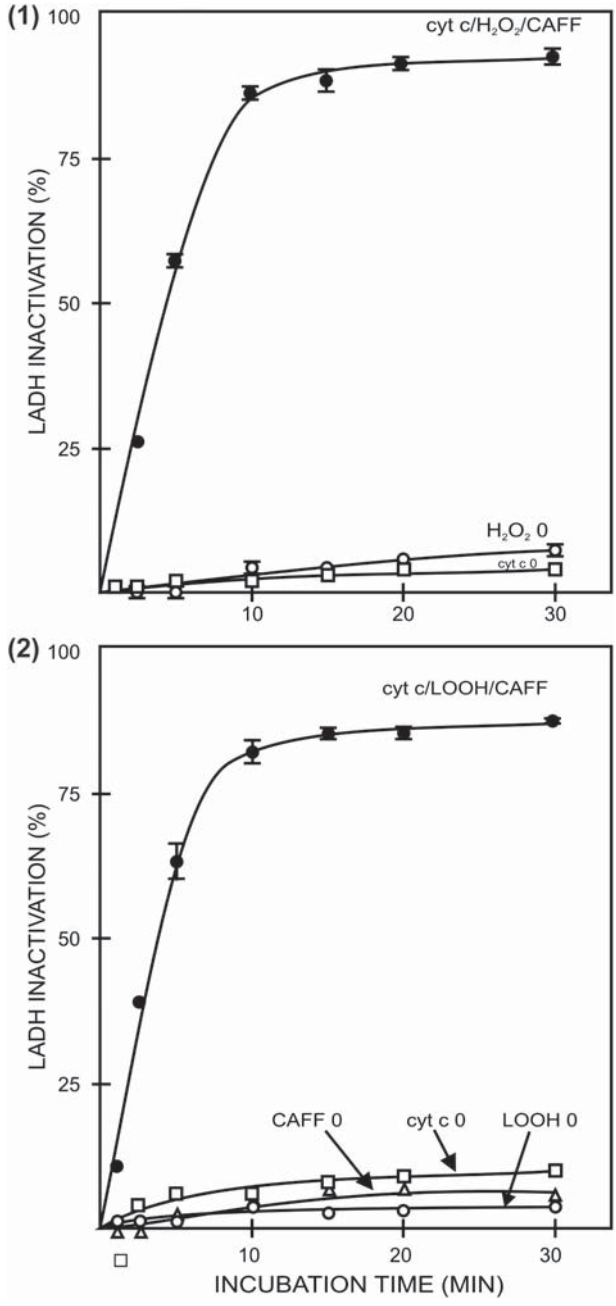

Figure 9. Time-course of *T cruzi* LADH inactivation by CAFF systems. (1) Cyt c/H_2O_2/CAFF and controls (H_2O_2 0, cyt c 0). (2) Cyt c/LOOH/CAFF and controls (LOOH 0, cyt c 0 and CAFF 0). Conditions for experiments are given in the Table I legend and Methods section.

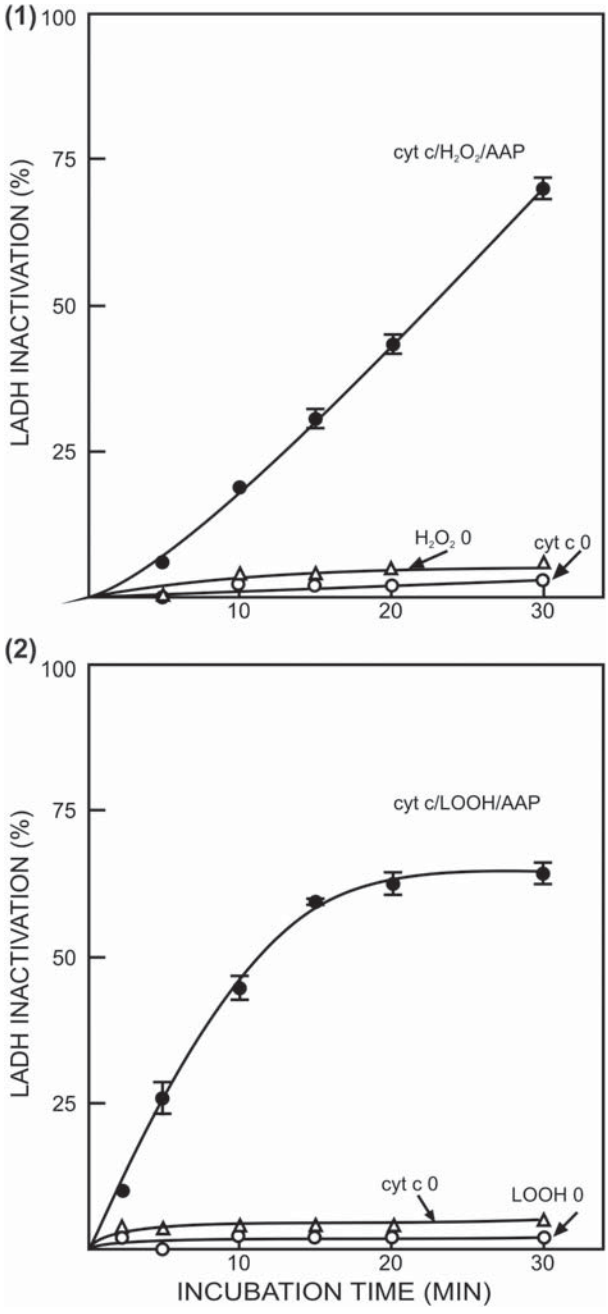

Figure 10. Time-course of *T cruzi* LADH inactivation by AAP systems. (1) Cyt c/H_2O_2/AAP and control in the absence of H_2O_2 or cyt c. (2) Cyt c/LOOH/AAP and control in the absence of LOOH or cyt c. Conditions for experiments are given in the Table I legend and Methods section.

residual linoleic acid (LA); plus cyt c and a phenol compound added to initiate each reaction. Production of ferryl cyt c from cyt c Fe^{3+} and LOOH (Reaction (3)) originates a catalytic cycle that involves cyt c peroxidase activity. Production of 'Reactive species' by cyt c/LOOH/Phen systems might explain *T cruzi* LADH inactivation since the strict requirement of cyt c rules out a pseudoperoxidase activity of SLOX (Figures 8–10(2); Table I, legend). Therefore, the cyt c-dependent inactivating effect does not support the SLOX function as a generator of reactive species in

Table II. *T cruzi* and heart LADH inactivation by Cyt c/H_2O_2/phenolic compounds systems.

Phenolic compounds (μM)		LADH inactivation (%)		LADH
		T cruzi	Heart	*T cruzi*/Heart
NDGA	(10)	82 ± 1	40 ± 2	2.0
CAFF	(25)	88 ± 1	43 ± 3	2.0
AAP	(50)	30 ± 3	6 ± 1	5.0
GUAI	(100)	29 ± 1	19 ± 1	1.5

The inactivation mixture contained 0.8 μM *T cruzi* LADH or 1.0 μM Heart LADH, 5 μM cyt c, 0.5 mM H_2O_2, except NDGA (0.25 mM), phenolic compounds as indicated in the Table, and 50 mM K-Phosphate/1 mM EDTA, pH 7.4. Incubation time 15 min. Other conditions as described in Methods. Values represent means ± SD. $p < 0.0003$ for all comparative assays.

the SLOX/LOOH/Phen system and as a primary cause of LADH inactivation. In agreement with this finding, it should be noted that SLOX/LOOH/Phen systems (i.e. in absence of cyt c) failed to generate Phen derivative products (Figure 5B and text). However, a control experiment with SLOX/cyt c/NDGA (i.e. LA absent) produced high LADH inactivation after 30 min incubation (Figure 8(2)), which may be explained by a higher auto-oxidation rate of NDGA at pH 7.8. The latter processes can generate semiquinone radical and then *ortho*-quinone, superoxide and H_2O_2 favouring the cyt c peroxidase activity, as suggested by the kinetics of this LADH inactivation reaction (Figure 8(2)). It is worth mentioning that NDGA is a lipoxygenase inhibitor [46] and potently inhibits human pancreatic and cervical cancer cells.

Cyt c/H_2O_2 or LOOH/Phen systems activity depended on the phenolic compound structure. The influence of *p* substituents on the inhibitory effect of Phen is important as it can be observed when comparing AAP and Tyr structures and the corresponding effect on *T cruzi* LADH (Table I). The lack of inactivating effect of tyrosine on *T cruzi* LADH could be explained by its poor pro-oxidant property due to the low oxidation rate by cytochrome c peroxidase activity and/or the high reactivity of the tyrosyl radical

Table III. Protection of *T cruzi* LADH by thiol compounds and other interceptors antioxidants against the cyt c/H_2O_2/AAP system.

Interceptor	*T cruzi* LADH inactivation (%)	
None	93 ± 2	
GSH	12 ± 1	(87)
NAC	6 ± 1	(94)
NADH	9 ± 2	(90)
NADPH	7 ± 2	(92)
Ascorbate	2 ± 0	(98)
Trolox	2 ± 0	(98)

The reaction medium contained 0.8 μM *T cruzi* LADH, 5 μM cyt c, 0.5 mM H_2O_2, 50 μM AAP, 50 mM K-Phosphate/1 mM EDTA, pH 7.4 and 0.2 mM interceptor. Incubation time 60 min. Other conditions as described in Methods. Values in parenthesis indicate protection (%). Statistical analysis was performed using the two-sample *t*-test method. $p < 0.0001$ for all protection values.

forming dityrosine free dimers or cross-linking with tyrosyl radical from activated cyt c [47]. Figures 5(A and B) indicate that the cyt c-dependent AAP oxidation occurs with H_2O_2 or LOOH as it is observed in the fluorescence emission spectra: a first peak at 405 nm, which appears to be compatible with the dimerization of its phenoxyl free radical (or N-acetyl-*p*-benzosemiquinoneimine (NAPSQI)); and a second peak at 436 nm corresponding to the respective quinone (NAPQI) [44]. NAPSQI anion radical is the first product formed by the one-electron oxidation of AAP through peroxidases and other hemoproteins [42,44,48–50], which leads not only to dimerization/polymerization, but also to dismutation yielding N-acetyl-*p*-benzoquinone imine and one original molecule, as in the HRP system, Reaction (5):

$$2NAPSQI \rightarrow NAPQI + AAP \qquad (5)$$

The role of AAP polymers on the *T cruzi* LADH inactivation is not studied yet, since the presence of phenol groups in their structures turns them into potential substrates of the cyt c peroxidase activity. The cytotoxicity of AAP, at least in part, appears depending on the NAPQI oxidative effects on cellular proteins. This metabolite acts as an electrophile and oxidant, causing GSH depletion and loss of thiol protein groups [44,50,51]. Then, NAPQI detected in AAP/cyt c system incubated with H_2O_2 or LOOH (Figures 5A and B, respectively) may be responsible, at least in part, for the *T cruzi* LADH inactivation due to oxidation of their cysteine residues, which could be reactive toward NAPQI and other reactive products derived from AAP. Some proteins targets of reactive metabolites of AAP have been identified *in vivo* such as glutamate dehydrogenase [52], and *in vitro* the *T cruzi* TR (unpublished experiment).

CAFF and NDGA (a mono *o*-diphenol and di-*o*-diphenol, respectively; Figure 1) were significantly more active than the monophenol compounds such as GUAI. (Table I, Figures 8 and 9(1) and (2)). It is also observed that the molecular mass and its complexity do not appear to be very important in the cyt c/peroxide interaction, since ETOP, a methoxy phenol complex, is more active than GUAI (Table I), having the former five cycles in its structure and being the latter a simple methoxy phenol (Figure 1).

Here CAFF is studied as a pro-oxidant against *T cruzi*. First, CAFF is a substrate of the cyt c/H_2O_2 system and its consumption, monitored by changes in its absorption spectra, shows linearity on time of incubation (Figures 6A and B(inset)). Similar changes were observed using LOOH instead of H_2O_2 (text). This effect, like in similar transformations catalysed by HRP or ferrylmyoglobin, seems to depend on the following reactions: (a) CAFF peroxidation generating *o*-semiquinone CAFF, (b) polymerization of *o*-semiquinone CAFF; and (c) *o*-semiquinone CAFF disproportionation to the original CAFF and its respective *o*-quinone [45,53] (Reaction 6):

$$2CAFF-O^{\bullet} \rightarrow CAFF-O^{-} + CAFF=O \qquad (6)$$

The latter product is probably the cytotoxic metabolite toward isolated hepatocytes, when CAFF is metabolically activated by cytochrome P450 [53]. It has previously been shown that *o*-quinone CAFF may produce *p*-quinone and dihydroesculetin. This latter product, with a *o*-diphenol in its structure, is also a peroxidase substrate which yields other cytotoxic *o*-quinone [53]. A characterization of the quinoid products resulting from CAFF/cyt c/peroxide systems remains to be done. It is known that CAFF by auto-oxidation generates $O_2^{-\cdot}$ and H_2O_2 [54]. However, the omission of H_2O_2 in the CAFF/cyt c system prevented *T cruzi* LADH inactivation, indicating the dependence on exogenous H_2O_2 for CAFF peroxidation by cyt c (Figure 9(1)). This reaction is also LOOH-dependent, since the absence of SLOX or linoleic acid prevented the LADH inactivation (Figure 9(2)). Likewise, the latter reaction was also cyt c-dependent, which indicates that the interaction between cyt c and LOOH is necessary for the peroxidative reaction (Table I, Figure 9(2)).

Protection of *T cruzi* LADH against AAP/H_2O_2/cyt c by thiol compounds is similar to other peroxidase/non-phenols systems [5–7]. GSH (and also NAC) decreased acetaminophen dimerization by the cyt c system (Figure 4 and its legend), apparently reacting with NAPSQI to form $GS^{\cdot}$ radical and AAP [39] or with NAPQI-generating RS-adducts [48]. This phenoxyl radical reaction supports the protective effect of GSH and provides further evidence about the role of phenoxyl radicals in LADH inactivation. However, this reaction generates a potential cytotoxic thiyl radical [6,55]. This protective effect is also explained by the competition between AAP (and other Phen) and GSH for the cyt c peroxidase activity, since the GSH is also oxidized by the oxyporphyrin ferryl group from the cyt c compound I [16] to thiyl radical. Then GSH, being an important target for phenoxyl radicals, could be a substrate of activated cyt c, resulting in both situations a thiyl radical, which acts as follows: (a) Reacts with GSH yielding glutathione disulphide anion radicals ($GSSG^{-\cdot}$). Electron transfer from this radical to O_2 produces superoxide anion [56], which undergoes other changes leading to a hydroxyl radical [55]; (b) Reacts rapidly with oxygen resulting in thiol peroxyl ($GSOO^{\cdot}$), which has oxidizing properties and is a source of singlet oxygen [56]; (c) Hydrogen abstraction from polyunsaturated fatty acids could play a role in the lipidperoxidation. This potential pro-oxidant effect of GSH could also occurs *in vivo* mediated by cyt c by its high concentration in the mitochondrial intermembranes space and possible activation by cardiolipin

[17]; and (d) The thiyl radical also reacts with NADH yielding non-radical glutathione and $NAD^{\cdot}$ radical, which readily reduces the molecular oxygen to superoxide anion. This last reactive species, spontaneously or enzymatically, dismutates to H_2O_2.

Protection of *T cruzi* LADH by NAD(P)H, Ascorbate and Trolox (water-soluble form of vitamin E) against AAP-$O^{\cdot}$ radicals (Table III) may be explained by the function of these compounds as interceptors of AAP-phenoxyl radicals. In the same way as GSH, if NADH is oxidized by AAP-$O^{\cdot}$, a non-radical AAP molecule and $NAD^{\cdot}$ radical (instead thiyl radical) is produced. Then, the nucleotide radical forms superoxide anion which dismutates to H_2O_2. Likewise, ascorbate and trolox react with a phenoxyl radical, resulting in a neutral molecule AAP and another free radical (ascorbyl and trolox radical). Then, the ascorbate and trolox are regenerated [7]. In agreement with this mechanism, NADH, ascorbate and trolox also suppressed the production of diacetaminophen, an indicator of the acetaminophen phenoxyl radical deactivation (Figure 4 and its legend).

Other possible mechanisms involved on *T cruzi* LADH protection is the NADH action as a competitive substrate for cyt c peroxidase activity, which prevents the peroxidation of AAP (or other phenolic compounds). In fact, it has been recently shown that GSH, NADH, Ascorbate and other substrates can be oxidized by cyt c compound I [16]. If in the Reaction 4, the pro-oxidant substrate *Phen-OH* is replaced by a potential competitive substrate (e.g. GSH, NADH or Ascorbate antioxidants), another free radical (thiyl, $NAD^{\cdot}$ or Ascorbyl, respectively) is obtained instead of phenoxyl radical. Then, the ascorbate as a substrate of the cyt c activated contribute to explain its *T cruzi* LADH protective action against AAP phenoxyl radical. A similar explanation should be suggested for the trolox [5–7].

This study also indicates a greater sensitivity of *T cruzi* LADH against Phen compared to mammalian heart lipoamide dehydrogenase (Table II). Ratio inactivation (%) *T cruzi* LADH/Heart LADH of 5 was found for AAP and the *T cruzi* LADH inactivation was 2-times higher than the mammalian Heart LADH inactivation for NDGA and CAFF. Findings on Phen action supported the hypothesis related to *T cruzi* LADH as a possible target for trypanocidal drugs [24] such as phenothiazines [5,6] or phenolic agents as is shown here. Alternatively, these drugs may be used as models to design new and more specific agents directed to *Trypanosoma cruzi*.

Phenolic compounds such as NDGA, AAP or ETOP can produce mitochondrial dysfunction [49,51,57], suggesting their location in these organelles, whose inner membranes bind to cyt c molecules.

A variety of factors such as HOCl, HOBr, HOI, peroxynitrite, nitrite/H_2O_2, nitric oxide/H_2O_2, polyunsaturated fatty acids and cardiolipin may increase the cyt c peroxidase activity [17,33–35]. This enhancement occurs because the sixth coordinated ligand, Fe Heme-Met^{80}, is usually displaced by oxidative modifications of Met^{80} leading to loss of cyt c's arrangement [32,33,35] and providing access to H_2O_2. This *in vivo* change may be accompanied by dysfunction of cyt c from the respiratory chain with a high production of ROS and mitochondrial membrane potential collapse. Under oxidative stress, the enhancement of cyt c peroxidase activity, in the presence of peroxides/Phen systems, produces phenoxyl radicals, semiquinone radicals, quinones and other reactive metabolites. All of them have the LADH and possibly other enzymes from *Trypanosoma cruzi* as a potential target. Phen peroxidation could be catalysed by cyt c from host cells and *T cruzi* if appropriate concentrations of H_2O_2 or LOOH are achieved. Cyt c [58,59] and the mitochondrial peroxidase activity [60] of Trypanosomatids have been studied and this activity could be attributed to the cyt c from parasites. Anti-*T. cruzi* effect may have a biological significance for Phen trypanocidal action, since the parasite is phagocytized by neutrophils, monocytes and macrophages [61,62] and internalized by other cells, including the heart muscle and reticular endothelial system cells. Phenoxyl and semi-quinone radicals interceptors-thiols, ascorbate, NAD(P)H and other compounds may prevent LADH modification by these free radicals. However, oxidative stress may also result from the generation of secondary radicals and extensive consumption of those free radicals interceptors, leading to cytotoxic activity. The findings presented here demonstrate that various phenolic compounds, particularly NDGA, CAFF, AAP and Catechol- with cytochrome c/Hydrogen peroxide (or LOOH)-, are potent and irreversible inhibitors of *T cruzi* LADH and, consequently, may have a potential effect as anti-Chagas' disease therapeutics, which deserves to be investigated.

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