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Leishmania amazonensis: Inhibition of 3′-nucleotidase activity by Cu^{2+} ions

Rafael Paletta-Silva, Rodrigo Vieira-Bernardo, Daniela Cosentino-Gomes, José Roberto Meyer-Fernandes *

Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, CCS, Bloco H, Cidade Universitária, Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil
Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem (INCTBEB), CCS, Bloco H, Cidade Universitária, Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil

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ABSTRACT

Free Cu^{2+} is toxic due to the capacity of free copper ions to catalyze the production of reactive oxygen species (ROS) that can modify the structure and/or function of biomolecules. In addition, non-specific binding to enzymes, which modifies their catalytic activities, can occur. In this work, the mechanisms underlying the ability of copper to inhibit 3′-nucleotidase from *Leishmania amazonensis* (*La*3′-nucleotidase) were investigated. To that end, *La*3′-nucleotidase activity was assayed with $CuCl_2$ in the presence of ascorbate or hydrogen peroxide to discriminate non-specific binding effects from pro-oxidant effects of copper. Copper inhibitory effects were greater at more acidic pH than at alkaline pH. The addition of enzyme substrate, adenosine 3′-monophosphate (3′AMP), prevented the inhibition of enzyme activity by copper. Thiol-containing compounds were able to protect the enzyme activity against inhibition due to copper. The specific copper chelating agent bathocuproine sulphonate (BCS) restored enzyme activity after pre-treatment of the enzyme with copper. *La*3′-nucleotidase activity was found to be resistant to ROS generated during oxidation reactions of ascorbate and hydrogen peroxide catalyzed by copper. Our results suggest that Cu^{2+} ions exert their inhibitory effects by binding to specific motifs of the 3′-nucleotidase protein and that the enzyme appears to be extremely resistant to ROS.

1. Introduction

Leishmaniasis is a clinically heterogeneous group of diseases caused by infection with protozoa of the genus *Leishmania* and affects 12 million people world-wide. The aggressiveness of individual species, their organ preference and the host immune status determine disease progression (Neuber, 2008). Protozoan parasites of the genus *Leishmania* undergo a life cycle in which they differentiate from non-infectious to infectious promastigotes in the midgut of sandflies and from promastigotes into amastigotes within mammalian macrophages (Cunningham, 2002). Infection by *Leishmania amazonensis* (*L. amazonensis*) causes the disfiguring mucocutaneous leishmaniasis and may also be responsible for the visceral form of leishmaniasis (Barral et al., 1991).

Because these parasites are unable to synthesize purine *de novo*, ecto-3′-nucleotidase/nuclease (3′NT/NU) plays a pivotal role in nutrition by generating free nucleosides via the hydrolysis of either exogenous 3′-nucleotides or nucleic acids (Cohn and Gottlieb, 1997). 3′-nucleotidase protein in parasite protozoan is exclusively located at the external surface of the plasma membrane (Dwyer and Gottlieb, 1984; Paletta-Silva et al., 2011), and this protein was not observed in mammalian cells (Dwyer and Gottlieb, 1984). Recently, our group demonstrated that the 3′-nucleotidase activity of *Leishmania chagasi* (*L. chagasi*) and *L. amazonensis* contributes to parasite–macrophage interaction (Vieira et al., 2011; Paletta-Silva et al., 2011). Despite the critical role of 3′-nucleotidase activity in parasite biology, the biochemistry of this enzyme remains poorly understood.

Copper is an essential trace element that plays a fundamental role in the biochemistry of all living organisms and is one of the most prevalent transition metals in biology, second only to iron (Malmström and Leckner, 1998). Copper is also a potent toxic agent as free Cu^{2+}, originating from the release of copper ions from proteins due to changes in their structure (Letelier et al., 2009). Free copper is one of the most powerful transition metals to catalyze oxidation reactions that form free radicals (Buettner and Jurkiewicz, 1996), such as hydrogen peroxide (Nelli et al., 2009; Shangari et al., 2007; Buettner and Jurkiewicz, 1996), superoxide anion radicals ($O_2^{\cdot}$) (Speisky et al., 2009) or hydroxyl radicals ($^{\cdot}OH$) (Arouma et al., 1991; Vidrio et al., 2008; Cao et al., 2009; Rice et al., 2009), as a consequence of Fenton or Haber–Weiss reactions. The toxicity of copper may also be due to the direct interaction with essential biomolecules that modifies their structures or enzyme activities (Kim et al., 2001; Jiménez et al., 2002; Letelier et al., 2005,2009). Specifically, copper interacts with the imidazole of histidine and the thiol group of cysteine.

* Corresponding author. Address: Laboratório de Bioquímica Celular, Instituto de Bioquímica Médica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, UFRJ, Cidade Universitária, Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil. Fax: +55 21 22708647.
E-mail address: meyer@bioqmed.ufrj.br (J.R. Meyer-Fernandes).

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In this work, we investigate and describe for the first time the mechanisms underlying *La*3′-nucleotidase inhibition by copper. To discriminate the pro-oxidant effects of copper from its capacity to non-specifically bind to protein, we use a system employing a combination of copper with ascorbate or hydrogen peroxide, which are conditions that generate substantial amounts of reactive oxygen species and alter the copper redox state.

2. Materials and methods

2.1. Materials

All reagents were purchased from E. Merck (São Paulo, Brazil) or Sigma–Aldrich (Sigma Co. St. Louis, MO). Deionized distilled water from a MilliQ resin system (Millipore Corp., Bedford, MA) was used in the preparation of all solutions including substrates and inhibitors. Hydrogen peroxide, ascorbate and cysteine solutions were prepared within minutes before performing 3′-nucleotidase activity assays.

2.2. Cell culture

The MHOM/BR/75/Josefa strain of *L. amazonensis* was used throughout this study. It was isolated from a human case of diffuse cutaneous leishmaniasis in Brazil by Dr. Cuba-Cuba (Universidade de Brasília, Brazil) and has been maintained within our laboratory in axenic culture as well as by hamster footpad inoculation. Promastigote populations were cultured in Warren's medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, at 22 °C and were then harvested at the stationary phase of growth (5 day after inoculation) by centrifugation and washing twice with a solution containing 116.0 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose and 50.0 mM HEPES buffer (pH 7.4).

2.3. Ecto-3′-nucleotidase activity measurements

Ecto-3′-nucleotidase activity was measured by the rate of phosphate release from 3′AMP hydrolysis. Intact promastigotes (1×10^7 cells mL^{-1}) were incubated for 1 h at 25 °C in a 0.5 mL reaction mixture containing 116.0 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 50.0 mM HEPES–Tris buffer (pH 7.4) and 5.0 mM 3′AMP. The reaction was initiated by the addition of cells and stopped by the addition of 1.0 mL of ice-cold 25% charcoal in 0.1 M HCl. This charcoal suspension was washed a minimum of 20 times with 0.1 M HCl before use to avoid Pi contamination (De Jesus et al., 2011). This procedure reduces background and removes non-hydrolyzed 3′AMP from the sample that otherwise would be spontaneously hydrolyzed in the presence of sulfuric acid in the Fiske–Subbarow reactive mixture used for final analysis (Fiske and Subbarow, 1925). Controls in which cells were added after interruption of the reaction were used as blanks. After the reaction, the tubes were centrifuged at 1500×*g* for 15 min at 4 °C, and 0.5 mL of clear supernatant was added to 0.5 mL of Fiske–Subbarow reactive mixture (Fiske and Subbarow, 1925). The ecto-3′-nucleotidase activity was calculated by subtracting the non-specific 3′AMP hydrolysis measured in the absence of cells. The concentration of Pi released in the reaction was determined using a standard curve of Pi for comparison.

2.4. Reversibility of the inhibition promoted by copper on 3′-nucleotidase activity

To verify the reversibility of the inhibition of 3′-nucleotidase activity promoted by copper, the enzyme activity was assayed in the presence of increasing concentrations of the specific copper chelator agent bathocuproine sulphonate (BCS). After pre-incubation of the cells with 200 μM $CuCl_2$ for 15 min at 25 °C, BCS was added to the reaction medium. After an additional 15 min, 5 mM 3′AMP was added to the reaction medium and ecto-3′-nucleotidase activity was measured as previously described.

2.5. Copper-catalyzed oxidation reaction

$CuCl_2$/ascorbate and $CuCl_2$/hydrogen peroxide reactions were monitored by the rate of oxygen consumption and oxygen generation, respectively. Oxygen consumption/generation rates were measured polarographically in an oximeter using a high-resolution respirometer (Oroboros Oxygraph-O2K). The electrode was calibrated between 0% and 100% saturation with atmospheric oxygen at 25 °C. The experiments were carried out in buffer with same composition used in the 3′-nucleotidase assays (Section 2.3). To analyze changes in 3′-nucleotidase activity resulting from copper-catalyzed oxidation of ascorbate reactions, *L. amazonensis* promastigotes were incubated in reaction medium containing 116 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 50.0 mM HEPES–Tris buffer, 5 mM 3′AMP pH 7.4, and several $CuCl_2$ concentrations (100 μM–1 mM), in the presence or absence of fixed concentrations of hydrogen peroxide (H_2O_2) (500 μM) or ascorbate (1 mM). The reaction was carried out as described in Section 2.3. All solutions were freshly prepared in MilliQ water and the reactions were carried out under aerobic conditions.

2.6. Statistical analysis

All experiments were performed in triplicate, with at least three independent experiments. The values shown for all experiments are the average ± SE. Kinetic parameters, apparent K_m and V_{max} values, were calculated using a nonlinear regression analysis of the data fitted to the Michaelis–Menten equation (Sigma Plot 2000-Jandel ScientiWc Software, 1986–2000, San Diego, CA, USA). The K_i value was calculated by fitting the experimental data to the equation $v_i/v_0 = K_i/(K_i + [CuCl_2])$ where v_i and v_0 are the velocities of 3′AMP hydrolysis in the presence and absence of $CuCl_2$, respectively. The data were analyzed statistically by means of the Student's *t*-test. *P* values of 0.05 or less were considered significant.

3. Results

To evaluate the inhibition profile of *La*3′-nucleotidase activity by copper, enzymatic assays were conducted in the presence of increasing concentrations of $CuCl_2$ and 1 or 5 mM 3′AMP. Inhibition promoted by copper occurs in a dose-dependent manner in both cases (Fig. 1). 3′AMP was able to protect *La*3′-nucleotidase activity; the K_i values obtained in the presence of 1 or 5 mM 3′AMP were 32 and 84 μM, respectively. Lineweaver–Burk plot analysis (inset Fig. 1) revealed that copper acts as a competitive inhibitor. The values of V_{max} and apparent K_m for 3′AMP were 898 ± 15 nmol Pi h^{-1} 10^{-7} cells and 0.070 ± 0.014 mM, respectively, in the absence of $CuCl_2$ (□) and 956 ± 70 nmol Pi h^{-1} 10^{-7} and 1.46 ± 0.30 mM, respectively, in the presence of 100 μM $CuCl_2$ (■).

It has been demonstrated that $ZnCl_2$ inhibits the 3′-nucleotidase activities of *Crithidia luciliae* and *Leishmania donovani* to a greater extent at alkaline pH than at acid pH (Neubert and Gottlieb, 1990; Gbenle and Dwyer, 1992). To determine if the inhibition of *La*3′-nucleotidase activity by copper would be pH-dependent, we investigated the dose-response of *La*3′-nucleotidase activity inhibition by copper at three different pH values. *La*3′-nucleotidase activity was more sensitive to $CuCl_2$ at acidic pH than at alkaline pH (Fig. 2); a difference in this profile could be observed at $CuCl_2$ concentrations less than 100 μM, as better visualized at a reduced

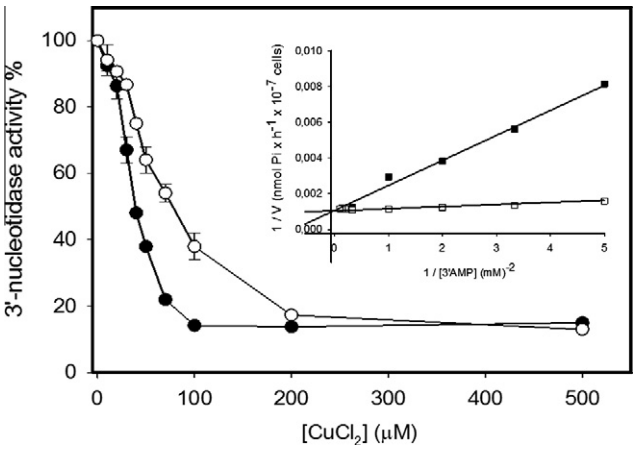

Fig. 1. Effect of increasing concentrations of $CuCl_2$ on *La*3′-nucleotidase activity and copper inhibition time course dependence. (A) Cell homogenate was assayed as described in materials and methods for 3′-nucleotidase activity with 1 mM 3′AMP (●) or 5 mM 3′AMP (○) in the presence of several copper concentrations. Control activities (914 ± 39 nmol Pi h^{-1} 10^{-7} cells for 1 mM 3′AMP and 882 ± 33 nmol Pi h^{-1} 10^{-7} cells) for 5 mM 3′AMP were taken 100% and the standard errors were calculated from the absolute activity values of three experiments with different cells suspensions and converted to percentage of the control value. Lineweaver–Burk plot of the inhibition of 3′AMP hydrolysis by 100 μM $CuCl_2$ (inset). The kinetic parameter values, apparent K_m and V_{max}, were calculated using a computerized nonlinear regression fit of the data to the Michaelis–Menten equation. At pH 7.4, the values of V_{max} and apparent K_m for 3′AMP were 898 ± 15 nmol Pi h^{-1} 10^{-7} cells and 0.070 ± 0.014 mM, respectively, in the absence of $CuCl_2$ (□) and 956 ± 70 nmol Pi h^{-1} 10^{-7} and 1.46 ± 0.30 mM, respectively, in the presence of 100 μM $CuCl_2$ (■). Data are means ± SE of three analyses using different cell suspensions.

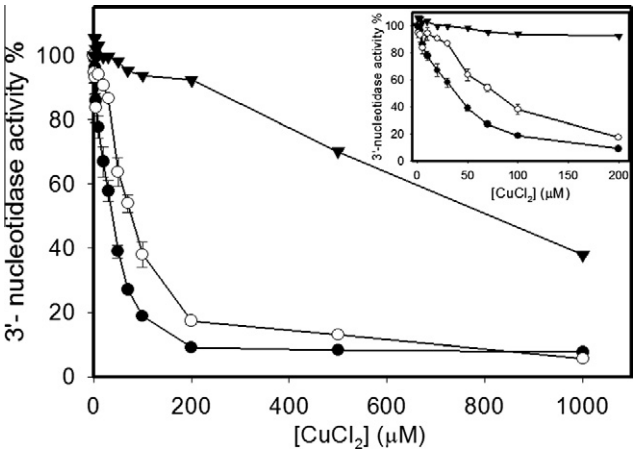

Fig. 2. Effect of pH on 3′-nucleotidase activity inhibition promoted by $CuCl_2$. *Leishmania amazonensis* promastigotes were assayed as described in materials and methods for 3′-nucleotidase activity in the presence of several $CuCl_2$ concentrations. Open circles (○) represent the assay at pH 6.5, closed circles (●) at pH 7.4 and triangles (▶) at pH 8.5, in HEPES buffer. *L. amazonensis* promastigotes were able to hydrolyze 5 mM extracellular 3′AMP at a rate of 707.17 ± 14.58 nmol Pi h^{-1} 10^{-7} cells at pH 6.5; 847.83 ± 51.23 nmol Pi h^{-1} 10^{-7} cells at pH 7.4; and 1303.67 ± 20.84 nmol Pi h^{-1} 10^{-7} cells at pH 8.5. Controls (no addition of copper) of each condition were set as 100% and the standard errors were calculated from the absolute activity values from four experiments using cell suspensions and converted to a percentage of the control value.

scale (inset Fig. 2). At pH 8.5 the enzyme activity was inhibited to a lesser extent with $CuCl_2$ inhibition only observed at concentrations higher than 200 μM.

To test the reversibility of copper inhibition, we tested whether the specific copper chelator bathocuproine sulfonate (BCS) would be able to restore *La*3′-nucleotidase activity after a 15 min pre-

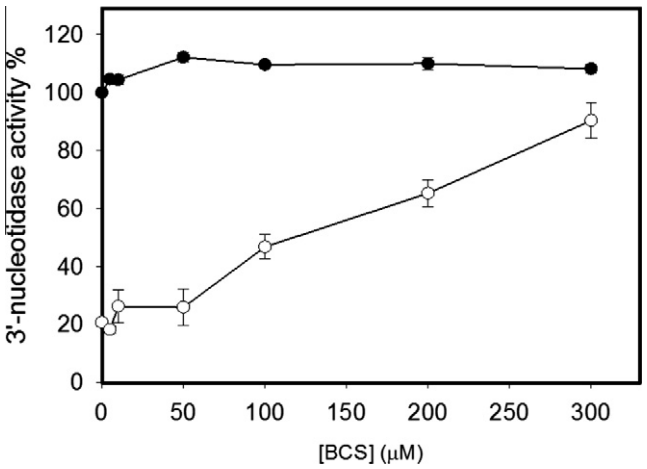

Fig. 3. Restoration of *La*3′-nucleotidase activity by BCS. *Leishmania amazonensis* promastigotes were assayed for 3′-nucleotidase activity at increasing concentrations of BCS in the absence (●) or presence (○) of 200 μM $CuCl_2$, in which cells were pre-incubated for 15 min with copper before the addition of BCS. 847.83 ± 51.23 nmol Pi h^{-1} 10^{-7} cells for 3′-nucleotidase activity was taken as 100% (control) and the standard errors were calculated from the absolute activity values of three experiments with cells suspensions and converted to percentage of the control value (no addition).

incubation of cells with 200 μM $CuCl_2$ (Fig. 3). BCS was able to restore *La*3′-nucleotidase activity in a dose-dependent manner (○). Further, we verified that BCS did not exert any stimulatory effect on enzyme activity (●).

Copper can bind to thiol or imidazole groups in proteins and free molecules containing such groups (Solomon et al., 2005; Jancsó et al., 2009; Kulon et al., 2008). It has been reported that thiol reagents like glutathione (GSH) and dithiothreitol (DTT), through their properties as antioxidants and/or metal-chelating agents (Stohs and Bagchi, 1994), can form adducts with Cu^{2+} ions as a cellular protection mechanism against copper overload (Harris, 2000; Kumar et al., 2002; Letelier et al., 2009). For this reason, we tested whether those molecules could prevent Cu^{2+}-induced inhibition of *La*3′-nucleotidase activity. β-mercaptoethanol, reduced glutathione (GSH), dithiothreitol (DTT) and cysteine (Cys) at 1 mM concentrations significantly inhibited *La*3′-nucleotidase activity (Fig. 4A) but were able to prevent the loss of enzyme activity when simultaneously added to the reaction medium with 1 mM of $CuCl_2$ (Fig. 4B). Cysteine was able to completely prevent the inhibitory effect of copper in a dose-dependent manner (Fig. 4C).

The results above suggest that the inhibitory effect of Cu^{2+} ions is due to the binding of the metal ions to specific residues of *La*3′-nucleotidase rather than from the oxidative property of Cu^{2+} ions. To further demonstrate this point, we tested whether *La*3′-nucleotidase activity could be modulated in a redox active environment in which copper catalyzes oxidation reactions to generate ROS. We used $CuCl_2$/ascorbate and $CuCl_2$/hydrogen peroxide systems to discriminate non-specific binding of copper ions from their pro-oxidant effects. First, oxygen consumption by the $CuCl_2$/ascorbate and oxygen generation by the $CuCl_2$/hydrogen peroxide systems were evaluated to ensure our experimental conditions generate ROS (Fig. 5A and Fig. 5B). The reactions between $CuCl_2$/ascorbate and $CuCl_2$/hydrogen peroxide occurred as expected demonstrating the existence of a ROS generating system.

It was observed that the presence of 500 μM hydrogen peroxide and increasing concentrations of $CuCl_2$ (○) in the reaction medium did not altered the dose-response inhibition profile of enzyme activity when compared to the assays in the absence of hydrogen peroxide (●) (Fig. 5C). Contrarily, the addition of 1 mM ascorbate

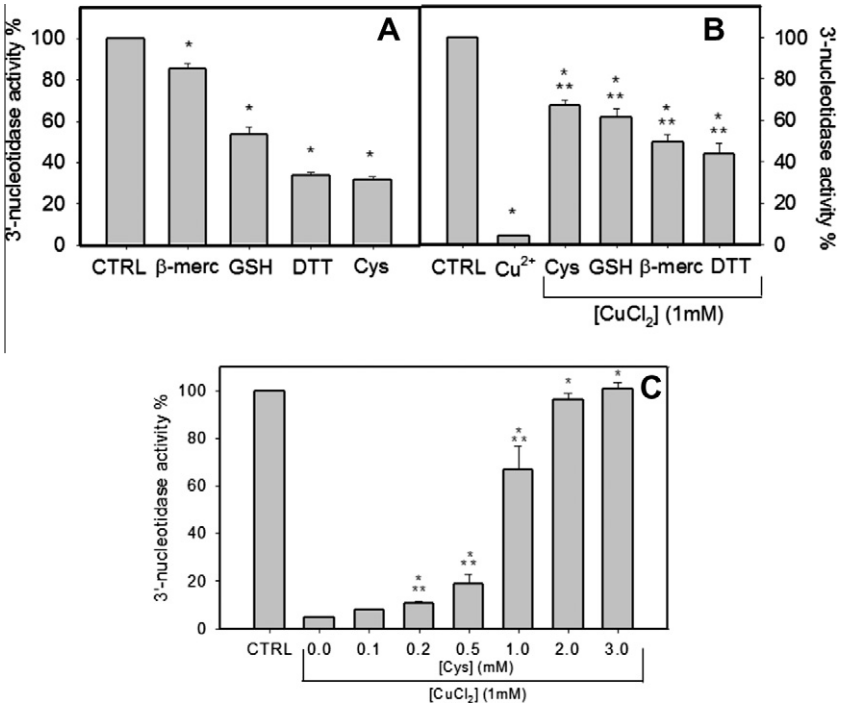

Fig. 4. Effect of reducting agents on *La*3′-nucleotidase activity. *Leishmania amazonensis* promastigotes were assayed as described in materials and methods in the presence of 1 mM reducing agents (A), 1 mM $CuCl_2$ and 1 mM thiol-containing reducing agents (B) or 1 mM $CuCl_2$ and increasing concentrations of cysteine (C). 847.83 ± 51.23 nmol Pi h^{-1} 10^{-7} cells for 3′-nucleotidase activity was taken as 100% (control) and the standard errors were calculated from the absolute activity values of three experiments with cells suspensions and converted to percentage of the control value (no addition). ∗Denotes a statistically significant difference ($p < 0.05$) compared to the control (no addition), and ∗∗denotes a statistical difference ($p < 0.05$) compared to 3′-nucleotidase activity in the presence of 1 mM $CuCl_2$.

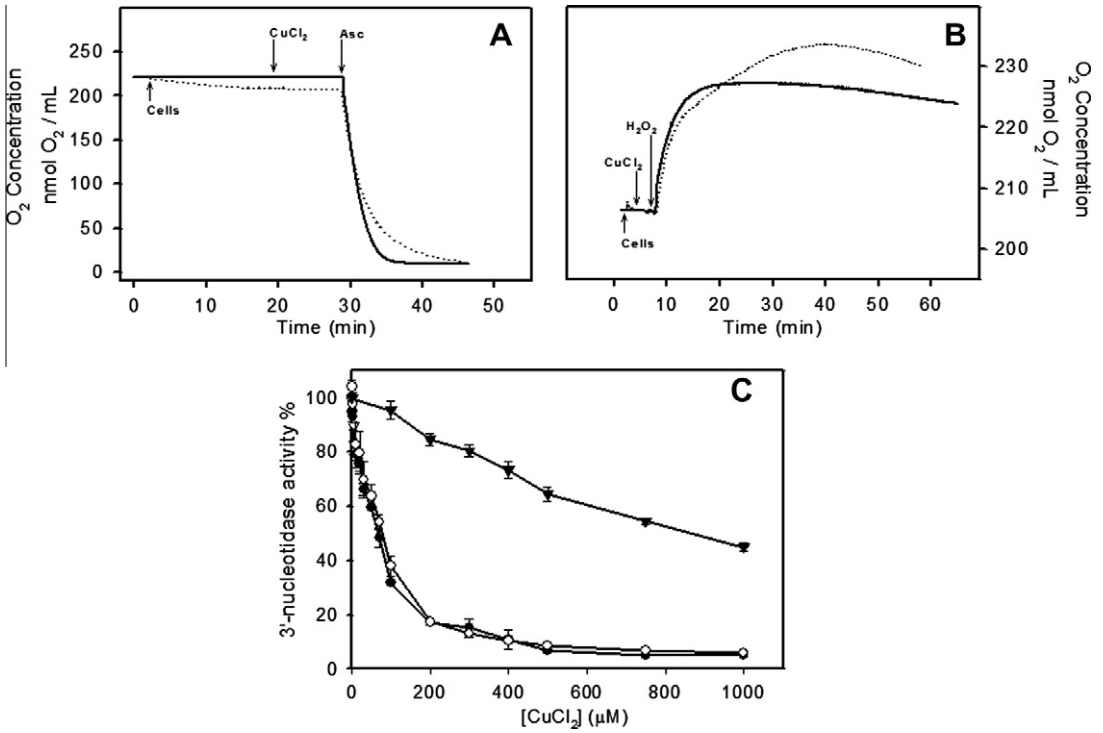

Fig. 5. Effects of $CuCl_2$/ascorbate and $CuCl_2$/hydrogen peroxide systems on *La*3′-nucleotidase activity. Oxygen measurements were performed as described in material and methods for $CuCl_2$/ascorbate (A) and $CuCl_2$/hydrogen peroxide (B) reactions. Dashed lines represent the assays in the presence of *Leishmania amazonensis* promatigotes and the solid lines in the absence of cells. (C) 3′AMP hydrolysis was assayed as described in Section 2.5 in the presence of $CuCl_2$ (●); $CuCl_2$ and hydrogen peroxide (○); and $CuCl_2$ and ascorbate (▶).847.83 ± 51.23 nmol Pi h^{-1} 10^{-7} cells for 3′-nucleotidase activity was taken to be 100% (control) and the standard errors were calculated from the absolute activity values of three experiments with cells suspensions and converted to percentage of the control value (no addition).

to the reaction medium prevented the inhibitory effect of $CuCl_2$ on 3′-nucleotidase activity (▼).

4. Discussion

The inhibition of *La*3′-nucleotidase observed in the presence of Cu^{2+} could be attributed to the following: conformational changes in its structure critical for its catalytic activity, and binding of metal ions to chemical groups present in residues directly involved in enzymatic catalysis. However, it is important to note the ability of transition metals to induce oxidative stress through the generation of hydroxyl radicals by the Fenton or Haber–Weiss reactions (Nishikawa et al., 1997; Letelier et al., 2005).

To investigate the pro-oxidative property of copper, 3′-nucleotidase activity was assayed in the presence of Cu^{2+}/ascorbate or Cu^{2+}/hydrogen peroxide systems, which are widely accepted in the literature for studies of this nature (Uchida and Kawakishi, 1993; Vidrio et al., 2008; Letelier et al., 2009,2010). The oxidation of ascorbate or hydrogen peroxide, catalyzed by copper, can generate hydroxyl radicals or hydrogen peroxide (Nishikawa et al., 1997; Vidrio et al., 2008; Nelli et al., 2009) and promote changes in the valence state of the metal by reducing it from Cu^{2+} to Cu^{1+}, as shown in Fig. 6 (Biaglow et al., 1997; Letelier et al., 2010; do Lago et al., 2011).

The presence of biological ROS substrates should increase the rate of oxygen consumption due to the consumption of ROS (Letelier et al., 2010). However, Fig. 5A shows that the presence of cells during the Cu^{2+}/ascorbate reaction leads to a slight inhibition of the rate of oxygen consumption. This could be due to a reduced availability of Cu^{2+}, which could be reacting with molecules other than ascorbate. At the same time, we can see by the high levels of oxygen consumption that there is intense ROS production as soon as ascorbate was added to the reaction medium. We further showed that the Cu^{2+}/H_2O_2 system induced ROS production, as observed by the production of low amounts of oxygen. Under this condition the presence of cells slightly increased oxygen production, which could be due to endogenous production of O_2^- in mitochondria fractions (Fig. 5B).

We ruled out that the inhibitory effects promoted by copper were caused by generation of ROS; the almost identical dose-response inhibition profiles for copper in the absence or presence of H_2O_2 clearly demonstrate that hydroxyl radicals did not interfere with enzyme activity. Moreover, the protection observed in the presence of ascorbate was likely due to the reduction of Cu^{2+}–Cu^{1+}, which in its monovalent form is unable to exert an inhibitory effect on *La*3′-nucleotidase.

Reduced thiol-containing compounds prevent oxidative damage promoted by transition metals in several enzymes (de Almeida-Amaral et al., 2006; Pinheiro et al., 2007; Cosentino-Gomes et al., 2009). It has also been demonstrated that GSH, the main non-enzymatic antioxidant in cells, and copper ions readily interact to form the complex Cu^{1+}–GSH (Speisky et al., 2009; Letelier et al., 2009) or Cu^{2+}–GSH (Kumar et al., 2002). These complexes were observed in studies to prevent copper damage in the cytochrome P450 enzymatic system (Letelier et al., 2009). Since no reducing agent with enough potential to reduce Cu^{2+}–Cu^{1+} was present in our experiments of Cu^{2+} binding to GSH, our data also suggests that the Cu^{2+}–GSH complex can occur. Additional evidence for copper binding effecting *La*3′-nucleotidase activity was the almost complete restoration of enzyme activity by the specific copper chelating agent BCS. Due to the high nuclear charge of Cu^{2+} ions we believe that a cupric-thiolate bond may occur (Solomon et al., 2005) and that this complex is responsible for protecting *La*3′-nucleotidase activity.

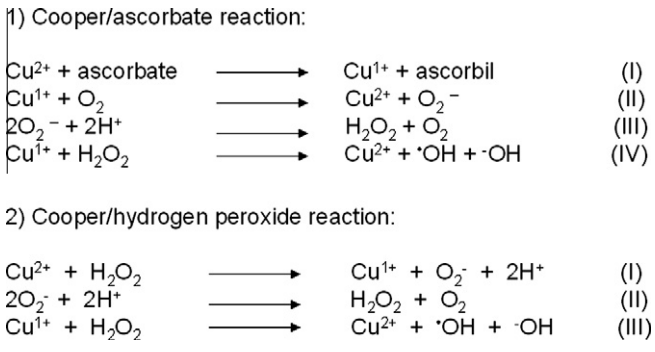

Fig. 6. Reactive oxygen species (ROS) generation by cooper/ascorbate or cooper/hydrogen peroxide reactions.

The inhibitory effect exerted by reducing agents on *La*3′-nucleotidase activity suggest a role of thiol groups on the enzyme catalysis/stability. The *La*3′-nucleotidase protein contains a unique cysteine residue in its C-terminal domain (Paletta-Silva et al., 2011). In *Ld*3′-nucleotidase the unique cysteine is located in its N-terminal domain and a similar inhibitory effect of reducing agents on enzyme activity was observed.

The observed kinetic inhibition profile of *La*3′-nucleoidase by copper and protection of enzyme activity by 3′AMP suggest that such phenomenon occur in a competitive manner. Copper may exert enzyme inhibitory effects by non-specific binding to protein. Structural features of the class I nuclease family allow us to consider this mechanism: amino acid sequences from several 3′-nucleotidase/nuclease proteins and other members of the class I nuclease family revel a highly conserved nine amino acid signature, including five histidine residues, that are responsible for Zn^{2+} co-factor coordination in the catalytic site (Volbeda et al., 1991; Debrabant et al., 1995; Lakhal-Naouar et al., 2008). Similar data were observed in the predicted amino acid sequence of *La*3′-nucleotidase (Paletta-Silva et al., 2011). Histidine residues are potential sites for Cu^{2+} binding in several proteins that are known to bind this transition metal (Kulon et al., 2008; Jancsó et al., 2009). We demonstrated that the inhibitory effect of copper was greater at acidic pH (pH 6.5) and enzyme activity was extremely resistant when assays were carried out at alkaline pHs. At acidic pHs, the structure of the active site in *La*3′-nucleotidase may be modified allowing copper to bind more efficiently.

For the P1 nuclease of *Penicillum citrinum* (*P. citrinum*), a member of the class I nuclease family, that includes 3′-nucleotidase, it has been demonstrated by electron paramagnetic resonance (EPR) that Cu^{2+} can replace the Zn^{2+} co-factor at the catalytic site, forming a Cu^{2+}-enzyme derivative that influenced the enzyme activity (Zheng et al., 2003). In another example, employing nuclear magnetic resonance (NMR) and examining the well-structured catalytic site of phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* (PC-PLC_{Bc}), which is related to P1 nuclease and 3′-nucleotidase, the same phenomenon occurs: Zn^{2+} are replaced by Cu^{2+} forming a Cu_3–PLC_{Bc} complex and when enzyme substrate is added a stable ES complex is formed without substrate catalysis (Epperson and Ming, 2001).

In the current work we provide new aspects of *La*3′-nucleotidase kinetics that may contribute to better understand of enzyme function. Since 3′-nucleotidase protein is extremely conserved among the genus *Leishmania* and possesses a pivotal role on parasite growth, development and virulence (Vieira et al., 2011; Paletta-Silva et al., 2011), the results reported in this work may be expanded to others *Leishmania* 3′-nucleotidase proteins. The understanding of enzyme kinetics is important to elucidate functional enzyme activity, providing tools, that may further contribute to new therapeutic approaches for the control of leishmaniasis.

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