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Research article

Leishmanicidal activity of peroxynitrite

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SUMMARY. **Nitric oxide reacts with superoxide to produce peroxynitrite which has been reported to be highly microbicidal to *Trypanosoma cruzi* in phosphate buffer but ineffective against *Leishmania major* in culture medium. This contradiction and the potential importance of peroxynitrite as a cytotoxic effector molecule of both macrophages and neutrophils led us to re-examine its leishmanicidal effects. Our results demonstrate that peroxynitrite inhibits growth of *Leishmania amazonensis* promastigotes in a concentration-dependent manner both in phosphate buffer and culture medium (DMEM containing 20% fetal calf serum). In the latter, 43% growth inhibition was observed with 4 mM peroxynitrite whereas in buffer a 70% inhibition was already observed with 0.5 mM peroxynitrite. Treated parasites presented reduced motility and became round in shape further confirming the leishmanicidal activity of peroxynitrite. The latter was attenuated by reduced glutathione supporting the view that peroxynitrite-mediated oxidation of critical thiol groups is a major mechanism accounting for its trypanocidal activity.**

INTRODUCTION

The molecular mechanisms by which macrophages exert their microbicidal activity have been extensively studied but remain to be fully understood since activation of murine macrophages by mediators such as interferon-γ leads to the production of several reactive intermediates.[1] Superoxide anion and hydrogen peroxide are formed through oxygen reduction by a membrane-bound NADPH oxidase.[2] Nitric oxide is produced through oxidation of L-arginine by an inducible nitric oxide synthase.[3] Furthermore, the simultaneously produced superoxide radical and nitric oxide can react with each other ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$)[4] producing the potent oxidant peroxynitrite anion.[5-10] Consequently, it has been difficult to assess the precise contribution of reactive oxygen- and nitrogen-derived species to the microbicidal activity of macrophages. An important step in this direction is to examine the cytotoxic potential of each of the formed species.

Cellular damage by superoxide anion has been extensively studied and it is generally attributed to the highly reactive hydroxyl radical produced by the transition metal ion-dependent Haber-Weiss mechanism.[11] Nitric oxide, in addition to its functions as a biological messenger,[3,12] has been implicated in cytotoxic processes due to its ability to interfere with cell energy metabolism,[13] protein[14] and DNA synthesis[15] and iron homeostasis.[16] The cytotoxic potential of the peroxynitrite anion has been less examined but the compound is an oxidant stronger than both nitric oxide and superoxide anion,[5-10] being able to oxidize proteins,[7,8,17] membrane lipids[9] and DNA,[18] in the absence of transition metal ions. Peroxynitrite anion has been shown to be highly microbicidal by killing both *Escherichia coli*[19] and *Trypanosoma cruzi*[20] in a dose-dependent manner. These properties are in contradiction with a recent report describing the ineffectiveness of peroxynitrite in the killing of another trypanosomatid, *Leishmania major*.[21] The complex reactivity of peroxynitrite anion under physiological conditions (Fig. 1)[5-10] and its potential importance as a cytotoxic effector molecule of both macrophages[22] and neutrophils[23] led us to re-examine

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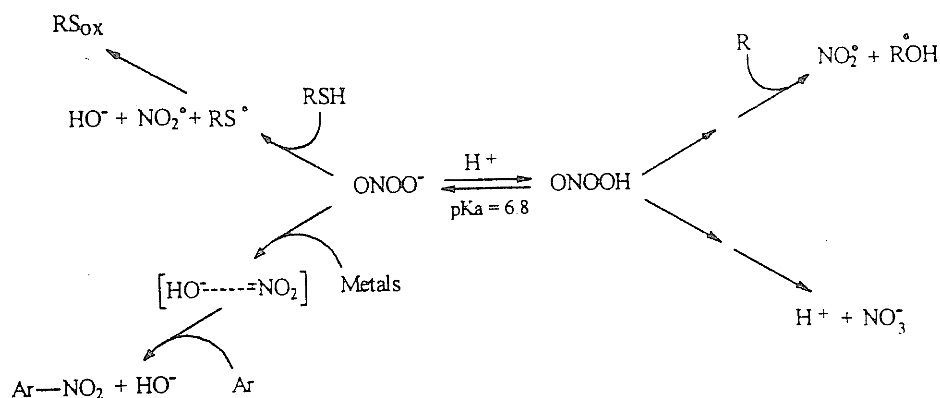

Fig. 1—Some possible routes of peroxynitrite decomposition. The anion can directly oxidize sulfhydryl groups (RSH), react with transition metal ions to form a nitronium-like species which nitrates phenolics (Ar), and can protonate to peroxynitrous acid which rearranges to form nitrate and oxidizes nearby molecules (R) by a hydroxyl radical-like mechanism (references 5–10; 17, 22, 27).

its leishmanicidal effects. Our results demonstrate that peroxynitrite is highly toxic to *Leishmania amazonensis*, confirming previous studies with *Trypanosoma cruzi*.[20]

MATERIALS AND METHODS

Chemicals and biochemicals

Reduced glutathione and Chelex-100 were from Sigma Chemical Company (St Louis, USA). DMEM was from Gibco (Paisley, UK). Fetal calf serum was from Cultilab (São Paulo, BR). [^{3}H] Thymidine was from Amersham (Buckinghamshire, UK). Peroxynitrite anion was synthesized, purified and kept frozen as previously described.[6,7] Stock solutions of peroxynitrite were prepared in 0.1 N NaOH and the concentrations determined by absorbance at 302 nm ($\varepsilon = 1670 \times M^{-1}.cm^{-1}$). All solutions were prepared using distilled water treated with a Millipore Milli-Q system. The phosphate buffer was pretreated with Chelex-100 to remove transition metal ion contamination.

Parasites

L. amazonensis (MHOM/BR/73/M2269) promastigotes were grown at 28°C in DMEM containing antibiotics and 10% FCS.[24] The parasites were harvested by centrifugation, washed twice in DMEM containing 20% FCS and resuspended in the incubation medium before exposure to peroxynitrite anion.

Leishmanicidal assay

Parasite killing was determined by the ability of surviving parasites to incorporate [^{3}H] thymidine. The parasites in 96-well flat-bottom plates (Costar Cambridge, USA) (4×10^6 cells/well) were treated with peroxynitrite in either 200 mM phosphate buffer, pH 7.0 (100 µl final volume) or DMEM supplemented with 20% FCS and HEPES buffer (7.5 mM) (200 µl final volume). Since the stock solutions of peroxynitrite were prepared in 0.1 N NaOH, the volume of NaOH corresponding to 4 mM peroxynitrite was added to all parasite suspensions, including the controls. The final pH of the incubations was routinely checked and did not exceed 7.5 unless stated. After 30 min incubation with peroxynitrite at room temperature the cultures were pulsed with [^{3}H] thymidine (1 µCi/well); in the case of parasites incubated with peroxynitrite in phosphate buffer, addition of 100 µl DMEM supplemented with 20% FCS and HEPES buffer (7.5 mM) preceded [^{3}H] thymidine addition. After 24 h, the parasites were harvested on glass fiber paper and [^{3}H] thymidine incorporation was measured in a β-scintillator counter (Beckman, USA).

Parallel 30 min incubations of parasites and peroxynitrite were performed as described above and examined by light microscopy (Nikon Microphoto FX). A 1000 ASA Kodak ektra film was used for the microphotographs.

All experiments were performed at least 3 times and the results are expressed as mean ± SEM.

RESULTS AND DISCUSSION

Peroxynitrite anion is stable at highly alkaline pH but rapidly decomposes at pH 7.5 with a half-life of about 1 s.[7,10] The decomposition pathway is a rather complex process influenced by the presence of different target molecules which can become oxidized, either attenuating or potentiating the cytotoxic potential of peroxynitrite (Fig. 1). Peroxynitrite-mediated killing of both *E. coli*[19]

and *T. cruzi*[20] was observed by treatment of the microorganisms with peroxynitrite in phosphate buffer, whereas peroxynitrite inactivity towards *L. major* was described in experiments performed in a multi-component culture medium, i.e., DMEM containing 20% FCS.[21] Although peroxynitrite reacts with amino acids[8] and serum components[17,25] it is unlikely that at the concentrations employed in the reported leishmanicidal assays, up to 8 mM peroxynitrite,[21] its microbicidal activity would be completely suppressed by DMEM containing 20% FCS. Indeed, re-examining the in vitro toxicity of peroxynitrite towards *L. amazonensis* promastigotes in the latter medium, we observed a concentration-dependent inhibition of parasite growth as monitored by [^{3}H] thymidine incorporation (Figure 2A). Peroxynitrite concentrations of 4 mM and 8 mM inhibited parasite growth by 43% and 90%, respectively. In the latter case, however, it was difficult to keep the final pH of the incubation mixture at the value of 7.5 due to the high pH of peroxynitrite stock solutions (in 0.1 N NaOH) and the obtained 90% inhibition at pH 9.0 was not included in Fig. 2A. The leishmanicidal activity of peroxynitrite was inhibited in the presence of 8 mM GSH (Fig. 2A) and was greatly increased in the absence of DMEM containing 20% FCS (Fig. 2B). Indeed, if treatment of the parasites with peroxynitrite is performed in phosphate buffer, a 70% growth inhibition is already observed with 0.5 mM peroxynitrite (Fig. 2B). Most importantly, when 4 mM peroxynitrite, which inhibits growth by more than 95%, was added to the buffer 5 min before parasite addition, to permit complete decomposition, no leishmanicidal effect was observed (Fig. 2B). This result demonstrates that the cytotoxic agent is peroxynitrite or a reactive intermediate derived from it rather than stable decomposition products such as nitrate (Fig. 1).

The cytotoxic properties of peroxynitrite towards *L. amazonensis* promastigotes were also evident by examination of the parasites by light microscopy. Untreated cells remained highly mobile and spindle shaped over the 30 min incubation time, whereas treated cells became nonmobile and round in shape (Fig. 3). Similar parasite alterations have been described for *L. amazonensis* promastigotes treated with lytic sera[26] and *T. cruzi* epimastigotes treated with peroxynitrite.[20]

Taken together, our results clearly demonstrate that peroxynitrite is cytotoxic to *L. amazonensis* promastigotes, also under conditions where it was reported to be ineffective against *L. major* promastigotes.[21] It is unlikely that the different *Leishmania* species used in this study and in the previous one are responsible for the observed differences since the effects described here (Fig. 2, Fig. 3) are quite similar to those described for peroxynitrite acting on another trypanosomatid, *T. cruzi*, in regard to both growth inhibition and morphological parasite alterations.[20] Consequently, our results further emphasize the importance of understanding the complex reactivity of peroxynitrite (Fig. 1),[5-10] particularly its short half-life under physiologically relevant conditions, to evaluate its cytotoxic potential. Accordingly, the leish-

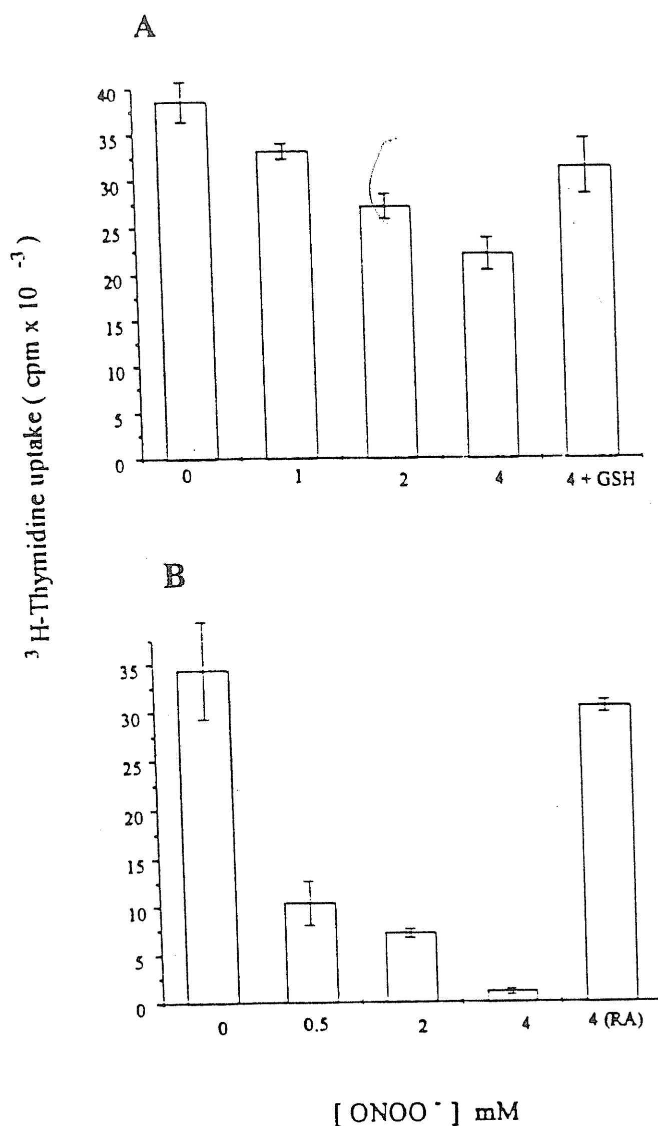

Fig. 2—Effect of peroxynitrite on [^{3}H] thymidine uptake by *L. amazonensis* promastigotes. Parasites were treated at room temperature and pH 7.5 with the specified concentrations of peroxynitrite in: (A) DMEM medium containing 20% FCS; (B) phosphate buffer (200 mM). In (A) the last column refers to incubations containing 4 mM peroxynitrite plus 8 mM GSH. In (B) the last column refers to reverse addition experiments, when 4 mM peroxynitrite was added to the buffer 5 min prior to the parasites. The experimental procedures and conditions are as decribed in Materials and Methods. The results represent the average of triplicate cultures ±SEM.

Fig. 3—Morphological changes observed after 30 min incubation of parasites in DMEM containing 20% FCS at room temperature. A light microscopy view of: (A) untreated *L. amazonensis* promastigotes; (B) *L. amazonensis* promastigotes treated with 4 mM peroxynitrite. Magnification, 1500 ×.

manicidal effect of peroxynitrite was completely inhibited when peroxynitrite was added to the buffer 5 min prior to the parasites to permit complete oxidant decomposition (Fig. 2B). Also, the cytotoxic effects of peroxynitrite were greatly attenuated by a multiple-component culture medium (Fig. 2) as could be anticipated from the known reactivity of the oxidant with amino acids[8] and sera constituents.[17,25]

The complex reactivity of peroxynitrite including direct oxidation of thiols,[7] metal-catalyzed nitration of protein tyrosines[22] and the metal-independent oxidation of biomolecules to free radical intermediates[17,27] (Fig. 1), suggests a multiplicity of possible cytotoxic mechanisms. The hypothesis that peroxynitrite-mediated oxidation of thiol groups critical for parasite survival is a major mechanism accounting for its trypanocidal activity[20,28] is supported by our results demonstrating a protective effect of GSH against its leishmanicidal effects (Fig. 2A).

Undoubtedly, the role of peroxynitrite as a critical effector molecule of neutrophils and macrophages in vivo remains to be established.[1,21] However, peroxynitrite formation by activated macrophages[22] and neutrophils,[23] its high reactivity expressed by multiple oxidative mechanisms[5–10] and its potent microbicidal activity in vitro[19,20,28, this work] are all evidence supporting the concept that peroxynitrite may be an important cytotoxic mediator in vivo.

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