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Role of nitric oxide and superoxide in *Giardia lamblia* killing

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Abstract

Giardia lamblia trophozoites were incubated for 2 h with activated murine macrophages, nitric oxide (NO) donors or a superoxide anion generator (20 mU/ml xanthine oxidase plus 1 mM xanthine). Activated macrophages were cytotoxic to *Giardia* trophozoites (~60% dead trophozoites). This effect was inhibited (>90%) by an NO synthase inhibitor (200 µM) and unaffected by superoxide dismutase (SOD, 300 U/ml). *Giardia* trophozoites were killed by the NO donors, S-nitroso-acetyl-penicillamine (SNAP) and sodium nitroprusside (SNP) in a dose-dependent manner (LD_{50} 300 and 50 µM, respectively). A dual NO-superoxide anion donor, 3-morpholino-sydnonimine hydrochloride (SIN-1), did not have a killing effect in concentrations up to 1 mM. However, when SOD (300 U/ml) was added simultaneously with SIN-1 to *Giardia*, a significant trophozoite-killing effect was observed (~35% dead trophozoites at 1 mM). The mixture of SNAP or SNP with superoxide anion, which yields peroxynitrite, abolished the trophozoite killing induced by NO donors. Authentic peroxynitrite only killed trophozoites at very high concentrations (3 mM). These results indicate that NO accounts for *Giardia* trophozoite killing and this effect is not mediated by peroxynitrite.

Key words
- Nitric oxide
- Macrophage
- Peroxynitrite
- *Giardia lamblia*
- Cytotoxicity
- Superoxide

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Introduction

When macrophages are activated, they become competent to kill several pathogens and to lyse tumor cells. During the activation process, macrophages produce an array of biologically active molecules that includes tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and reactive oxygen intermediates such as superoxide and hydrogen peroxide (1).

Recent studies have shown that activated macrophages also produce large amounts of an unstable gas, nitric oxide (NO; Ref. 2). Nitric oxide is synthesized from L-arginine by the enzyme NO synthase (NOS), which occurs in three forms. The endothelial NOS (eNOS) is known to play a role in physiological vasodilatation whereas neuronal NOS (nNOS) is important for neurotransmission (2). The third NOS (inducible NOS; iNOS) is induced in macrophages and other cells by soluble stimuli such as bacterial lipopolysaccharide (LPS) and by particulate stimuli such as zymosan (3), both in the presence of cytokines such as interferon gamma (IFN-γ).

Nitric oxide produced by the inducible NOS has an important role in the cytotoxicity of activated macrophages against several parasites and tumor cells (2,4,5). Indeed, NO

has been shown to kill several pathogenic agents such as fungi (*Cryptococcus neoformans* (4); *Candida albicans* (6)), mycobacteria (7) and some intracellular protozoan parasites, such as *Toxoplasma gondii* (8), *Leishmania major* (9) and *Trypanosoma cruzi* (10). Cytotoxic effects of NO on an extracellular *Platyhelminth* parasite, *Schistosoma mansoni*, have also been reported (11). More recently, some reports indicated that peroxynitrite, the product of the reaction between NO and superoxide anion (12), may be the cytotoxic effector responsible for the killing of several targets (13,14).

The most prevalent cause of diarrhea in the world is the infection of the upper gastrointestinal tract by the protozoan *Giardia lamblia* (15). The infection can be life-threatening in undernourished children of Third World countries and in immunocompromised patients (15).

In the present report, we show that NO produced by either activated macrophages or NO donors is cytotoxic to *Giardia lamblia* trophozoites. This effect can be attributable to NO alone and does not require the participation of either reactive oxygen intermediates or peroxynitrite.

Material and Methods

Material

Murine recombinant interferon-γ was from Genzyme, (Cambridge, MA). LPS from *Salmonella typhosa* (0901) was obtained from Difco (Detroit, MI). S-Nitroso-acetyl-penicillamine (SNAP) and N-iminoethyl-L-ornithine (L-NIO) were kindly provided by Dr. S. Moncada, Wellcome Research Laboratories (Beckenham, UK). Zymosan, superoxide dismutase (SOD; from bovine erythrocytes), catalase and sodium nitroprusside (SNP) were from Sigma (St. Louis, MO). 3-Morpholino-sydnonimine hydrochloride (SIN-1) was kindly provided by Dr. R. Henning, Cassela AG, Frankfurt, Germany. Fetal calf serum (FCS) was from Fazenda Pigue (Rio de Janeiro, RJ, Brazil). All other tissue culture reagents were purchased from Sigma.

Macrophages

Murine peritoneal macrophages were harvested from C57/Black6 mice which had been injected *ip* 4 days before with 2 ml of sterile thioglycollate solution (3% w/v in water). Cells were plated onto 48-well plates at 10^6 cells/well, incubated for 2 h in 5% CO_2 at 37°C and washed with fresh medium to remove non-adherent cells. Assays were carried out in RPMI-1640 supplemented with 10% FCS, 20 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Giardia lamblia cultures

Trophozoites of the Portland I strain (P1, ATCC-30889) were kept in 15-ml plastic tubes with 14 ml TYI-S-33 medium supplemented with 0.1% bovine bile and 10% FCS (16). The medium was removed twice a week by aspiration and fresh medium was added to the tubes. For experiments, tubes containing the organisms were kept in ice-cold water for 20 min and centrifuged (250 *g*, 10 min) in the cold. With this procedure more than 95% of the parasites detached from the tube and could be recovered in the pellet.

Macrophage activation

Macrophage activation was induced by adding IFN-γ (20 U/ml) plus LPS (100 ng/ml; referred to as LI) or the same concentration of IFN-γ plus zymosan (80 µg/ml; referred to as ZI) to the macrophages. NO production was assessed by nitrite concentrations in the medium using the Griess reaction (17). Briefly, 100 µl Griess reagent (equal parts of 1% w/v sulfanilamide in 5% H_3PO_4 and 0.1% w/v naphthylethylene diamine dihydrochloride in water) was added to 100 µl of each supernatant in a 96-well plate. The

plates were read using a Tytertek MKI340P plate reader at 540 nm against a standard curve of sodium nitrite in culture medium.

Giardicidal assay

Peritoneal macrophages were activated as described above and 12 h later, at the peak of NO synthase activity (18), *Giardia* trophozoites were added at a ratio of 4:1 (effector:target). The plate was then further incubated for 2 h at 37°C in 5% CO_2 and transferred to a refrigerator (4°C) for 30 min to detach trophozoites. The suspension was centrifuged, supernatants were saved for nitrite assay and the pellet was resuspended in cold Trypan blue (0.1%) to count viable parasites. As there is a good correlation between dye uptake and parasite motility, we evaluated parasite viability by these two criteria, ranking as dead parasites only those that took up the dye and were not moving. When NO donors were used they were incubated at different concentrations with 1 x 10^6 *Giardia* trophozoites/ml in RPMI medium for 2 h at 37°C. The tubes were chilled and centrifuged and the pellet was resuspended in cold Trypan blue as described above. The results are reported as percent viable *Giardia* trophozoites in a total count of 200 parasites. Addition of NO donors did not change the pH of the incubation medium.

Peroxynitrite synthesis

Peroxynitrite was synthesized according to Beckman et al. (19). The concentrations of peroxynitrite used in the killing assays did not modify the pH of the medium. When tested as a bactericidal agent against *E. coli*, peroxynitrite prepared in our laboratory had an LD_{50} of about 250 µM, identical to the value obtained by Zhu et al. (14).

Statistical analysis

Results are reported as mean ± SD. Differences were considered to be significant when $P<0.05$, using analysis of variance followed by the Bonferroni *t*-test.

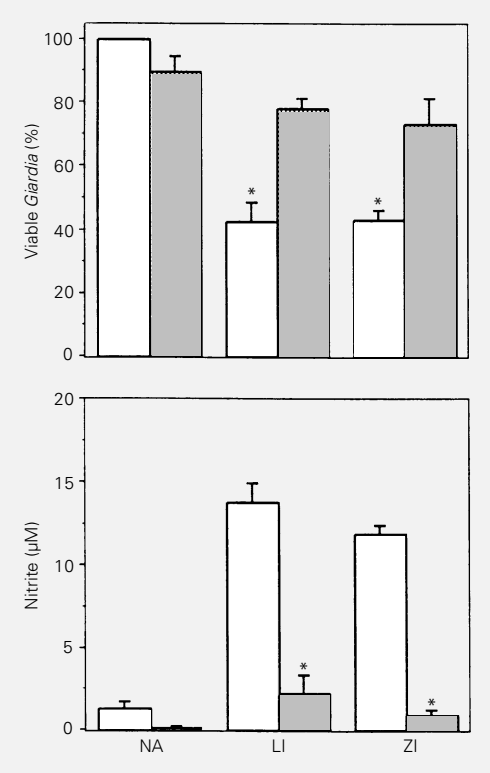

Figure 1 - Effect of macrophage activation on *Giardia* trophozoite killing (upper panel) and nitrite production (lower panel). Murine peritoneal macrophages were activated with IFN-γ (20 U/ml) plus LPS (100 ng/ml; LI) or with IFN-γ plus zymosan (80 µg/ml; ZI). Open and shaded bars are experiments performed in the absence or in the presence of L-NIO (200 µM), respectively. Twelve hours after macrophage activation, cells were washed with fresh medium, *Giardia* trophozoites were added at a ratio of 1:4 (target:effector; see Methods for details) and incubated for 2 h. Supernatants were removed for nitrite determination and the pellet was used for the *Giardia*-killing assay. Results are reported as means ± SD of triplicates. *P<0.05 when compared to non-activated cells (upper panel) or when activated cells in the absence of L-NIO were compared to cells in the presence of L-NIO (lower panel) (analysis of variance followed by the Bonferroni *t*-test). NA, Non-activated macrophages. The same results were obtained in two experiments.

Results

Activation of mouse peritoneal macrophages with ZI or LI induced NO synthase activity with production of NO as assessed by increased nitrite levels in the supernatants (Figure 1, open bars). It is important to point out that the nitrite concentrations shown in Figure 1 represent the accumulation over a period of 2 h of exposure to *Giardia* trophozoites. When measured 48 h after activation, nitrite concentrations were 3 ± 1 and 80 ± 5 µM (N = 3) in non-activated and activated macrophages, respectively. That NO was responsible for the killing of *Giardia* trophozoites by activated macrophages was confirmed by showing that the NO synthase inhibitor L-NIO (200 µM) inhibited both killing and nitrite accumulation (Figure 1, shaded bars). We also tested longer times of

exposure of trophozoites to activated macrophages. Although it seems that increases in exposure times did not cause higher trophozoite killing (data not shown), these experiments were biased due to the great increase in trophozoite mortality even in the absence of macrophages when kept in a culture medium other than TYI-S-33 for more than 3-4 h. Presumably, this is attributable to the high cysteine concentration in *Giardia* medium which is critical to ensure high trophozoite viability (15).

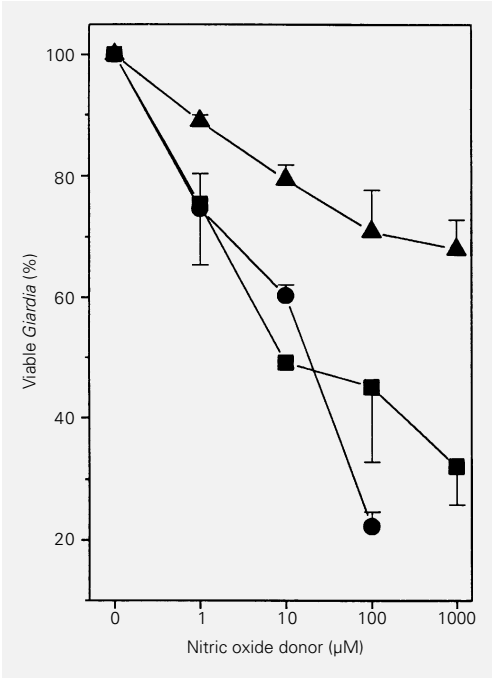

Figure 2 - Effects of increasing concentrations of NO donors on *Giardia* trophozoite killing. *Giardia* killing was measured by incubating trophozoites with SNP (C), SNAP (■) or SIN-1 (▲) at the indicated concentrations for 2 h at 37ºC. Results are reported as means ± SD of triplicates. The same results were obtained in three experiments.

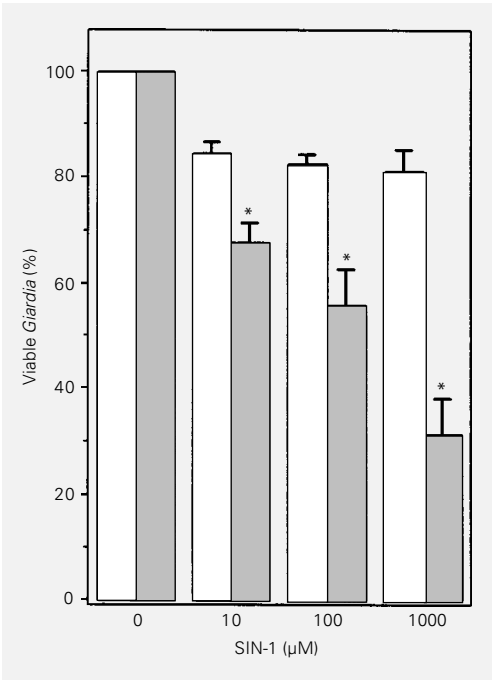

Figure 3 - Enhancement of the *Giardia* trophozoite-killing effects of SIN-1 by superoxide dismutase. Trophozoites were incubated with increasing concentrations of SIN-1 in the absence (open bars) or in the presence (shaded bars) of SOD (300 U/ml) for 2 h at 37ºC. Results are reported as means ± SD of triplicates. *P<0.05 when compared to groups without SOD (analysis of variance followed by the Bonferroni *t*-test). The same results were obtained in two experiments.

To confirm that NO was responsible for the killing we tested for the effects of NO generated by NO donors on *Giardia* trophozoites in a macrophage-free system. When added directly to *Giardia* trophozoites, SNP was the most potent cytotoxic agent (LD_{50} ~50 µM), followed by SNAP (LD_{50} ~300 µM) whereas SIN-1 was almost ineffective in this regard (Figure 2).

The presence of SOD did not change the killing effect of SNP or SNAP (data not shown). However, SOD greatly enhanced the effect of SIN-1. For instance, 100 µM SIN-1 killed 17.5% and 57% of trophozoites in the absence and in the presence of SOD, respectively (Figure 3). We also performed a set of experiments in which trophozoites were incubated in medium containing SIN-1, SOD and catalase (300 U/ml). The presence of catalase abolished the potentiation of the cytotoxic effect of SIN-1 produced by SOD (SIN-1 alone: 47.3 ± 0.7%; SIN-1 + SOD: 24.5 ± 3.3% and SIN-1 + SOD + catalase: 38.6 ± 0.9% of viable trophozoites, N = 3).

In order to test the possibility that peroxynitrite is the cytotoxic effector against *Giardia* trophozoites, we examined the effects of the simultaneous incubation of the NO donor SNAP and the superoxide-forming system (xanthine/xanthine oxidase). This procedure is likely to yield peroxynitrite. The xanthine/xanthine oxidase system killed (although not significantly) a small number of *Giardia* trophozoites. In contrast, SNAP (200 µM) killed 56.2% of the trophozoites (Figure 4, open bars). In the presence of superoxide, however, the cytotoxic effect of SNAP was completely abolished together with a decrease in nitrite levels (Figure 4, shaded bars). Similar results were obtained

when the NO donor was SNP (data not shown). Authentic peroxynitrite was cytotoxic to *Giardia* trophozoites but only at very high concentrations (Figure 5) when compared to SNP, SNAP or SIN-1 (the latter in the presence of SOD).

Discussion

The induction of NO production in peritoneal macrophages by either soluble (LI) or particulate (ZI) stimuli turned these cells into cytotoxic effectors against *Giardia* trophozoites. This effect was highly dependent on NO production as L-NIO, an NOS inhibitor, blocked both nitrite accumulation and trophozoite killing. Similar results have been reported for *Leishmania* killing by activated macrophages (20).

NO has been shown to be cytotoxic for a number of parasites (5), but in view of the demonstration that NO can react with superoxide anion to yield peroxynitrite (12), which is also cytotoxic to some organisms, the identity of the effector species remains controversial. As shown by the present results, NO alone accounts for cytotoxicity against *Giardia* trophozoites. First, the addition of an NO synthase inhibitor to activated macrophages completely inhibited trophozoite killing. Second, the NO donors SNAP and SNP reproduced the killing effect observed with activated macrophages, and this effect was not changed in the presence of SOD. Third, incubation of SNAP or SNP together with a superoxide anion producer (xanthine/xanthine oxidase), a procedure likely to yield peroxynitrite, dramatically reduced the cytotoxic effect of NO donors. Grisham and Miles (21) have shown that a maximal yield of peroxynitrite is obtained only when NO and superoxide anion react at equimolar concentrations. If one admits that all NO released by 200 µM of an NO donor reacts with superoxide, this would release 200 µM peroxynitrite. Consistent with the conclusion that peroxynitrite is less cytotoxic for *Giardia* than NO, we have found that authentic peroxynitrite exhibited some cytotoxic effects only at high concentrations (3 mM). Sensitivity to peroxynitrite seems to vary among microorganisms since peroxynitrite is an effective cytotoxic agent for *Escherichia coli* (14) and *Trypanosoma cruzi* (13), but not for *Leishmania* (20) or for *Giardia* (present study). It may be that in physiological situations, defense cells could use either NO or peroxynitrite as cytotoxic molecules, depending on the kinetics of production of superoxide anion and NO. For instance, human neutrophils stimulated with

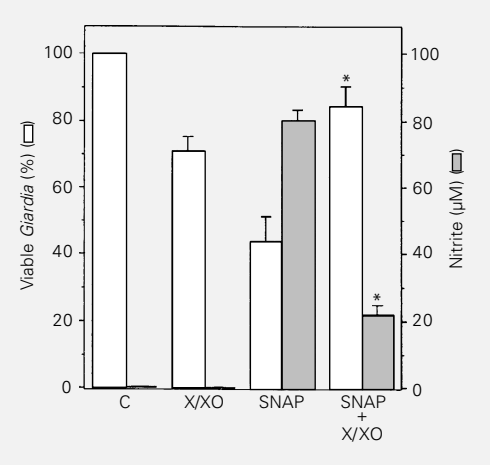

Figure 4 - Effects of SNAP, xanthine/xanthine oxidase (X/XO) system or both on *Giardia* trophozoite killing (open bars) and nitrite accumulation (shaded bars). Trophozoites were incubated either with SNAP (200 µM), xanthine (1mM)/xanthine oxidase (20 mU/ml) or both for 2 h at 37ºC. Results are reported as means ± SD of triplicates. *P<0.05 when compared to the effects of SNAP alone (analysis of variance followed by the Bonferroni *t*-test). The same results were obtained in two experiments. C, Control.

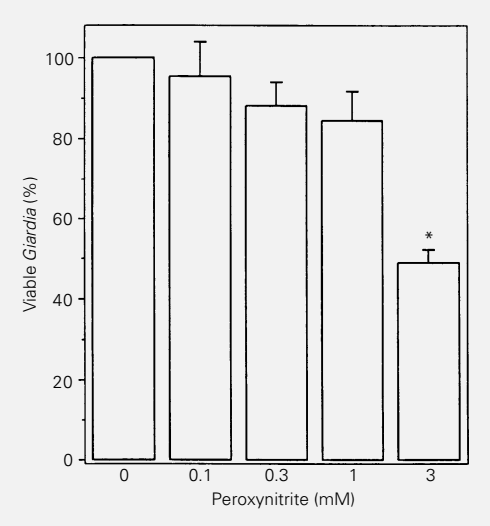

Figure 5 - Effect of increasing concentrations of authentic peroxynitrite on *Giardia* trophozoite killing. Peroxynitrite anion was obtained as described in the Methods section and incubated with trophozoites for 2 h at 37ºC. Addition of peroxynitrite did not change medium pH. Results are reported as means ± SD of triplicates. *P<0.05 when compared to the group without peroxynitrite (analysis of variance followed by the Bonferroni *t*-test). The same results were obtained in two experiments.

phorbol esters produce NO and superoxide with very similar kinetics, thus favoring peroxynitrite formation (22). On the other hand, in mouse macrophages activated by zymosan, superoxide is produced several hours earlier than NO, yet these cells potently killed *Leishmania*. In this system, peroxynitrite formation is unlikely to occur (20).

SIN-1, which is reported to release both NO and superoxide anion simultaneously (23) and therefore to yield peroxynitrite, exhibited a modest trophozoite-killing effect. However, superoxide removal (achieved by using SOD) greatly enhanced the cytotoxic effect of SIN-1. This enhancement, however, was blunted by catalase. It was reported that SOD enhancement of the SIN-1 cytotoxic effect on a human liver cell line was dependent on hydrogen peroxide resulting from dismutation of superoxide and not on increased NO release (24). These investigators showed that the cytotoxic species are not NO, peroxynitrite or hydroxyl radical (·OH). Indeed, this seems to be the case in our experiments with SIN-1, since catalase inhibited the SOD-enhancing trophozoite-killing effect of SIN-1. Until this matter is addressed in more detail, one should be careful in interpreting the effect of SIN-1 in the presence of SOD. This point, however, seems to be relevant only for SIN-1, as the addition of SOD and/or catalase did not have any effect on the ability of activated macrophages or SNAP to kill *Giardia* trophozoites (data not shown).

Reactive oxygen species seem to be important for the killing of some intracellular parasites. Although activated macrophages are able to produce toxic reactive oxygen species (during phagocytosis, for example), this seems not to be the case for *Giardia* killing, as the parasite is too big (15) to be ingested by the macrophage. Therefore, in an *in vivo* infection, activated macrophages should use an easily diffusible chemical species to exert their killing action, as the majority of the target will be extracellular. The following lines of evidence point to NO as this effector: i) the almost complete inhibition of trophozoite killing by activated macrophages when an NO synthase inhibitor was present in the medium (Figure 1); ii) superoxide anion does not easily cross membranes (25); iii) hydrogen peroxide does (25), and catalase does not interfere with the killing caused by activated macrophages or SNAP (data not shown); iv) peroxynitrite has a very short half-life and is inactivated by physiological concentrations of bicarbonate (>25 mM; Ref. 14). In summary, from the vast array of cytotoxic molecules potentially produced by defense cells, NO seems to account for the majority, if not all, of macrophage giardicidal effects.

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