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Research Article

Trichomonas vaginalis degrades nitric oxide and expresses a flavorubredoxin-like protein: a new pathogenic mechanism?

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Abstract. Besides possessing many physiological roles, nitric oxide (NO) produced by the immune system in infectious diseases has antimicrobial effects. Trichomoniasis, the most widespread non-viral sexually transmitted disease caused by the microaerophilic protist *Trichomonas vaginalis*, often evolves into a chronic infection, with the parasite able to survive in the microaerobic, NO-enriched vaginal environment. We relate this property to the finding that *T. vaginalis* degrades NO under anaerobic conditions, as assessed amperometrically. This activity, which is maximal (133 ± 41 nmol NO/10^8 cells per minute at 20 °C) at low NO concentrations (≤ 1.2 μM), was found to be: (i) NADH dependent, (ii) cyanide insensitive and (iii) inhibited by O_2. These features are consistent with those of the *Escherichia coli* A-type flavoprotein (ATF), recently discovered to be endowed with NO reductase activity. Using antibodies against the ATF from *E. coli*, a protein band was immunodetected in the parasite grown in a standard medium. If confirmed, the expression of an ATF in eukaryotes suggests that the genes coding for ATFs were transferred during evolution from anaerobic Prokarya to pathogenic protists, to increase their fitness for the microaerobic, parasitic life style. Thus the demonstration of an ATF in *T. vaginalis* would appear relevant to both pathology and evolutionary biology. Interestingly, genomic analysis has recently demonstrated that *Giardia intestinalis* and other pathogenic protists have genes coding for ATFs.

Key words. Nitric oxide; *Trichomonas vaginalis*; A-type flavoprotein; lateral gene transfer; evolution; protist; human pathology.

The flagellated microaerophilic parasite *Trichomonas vaginalis* is the causative agent of trichomoniasis, the most widespread non-viral sexually transmitted disease, estimated to affect more than 200 million people worldwide [1], with three to four million new cases per year in the US alone. Trichomoniasis affects mostly women, and its clinical manifestations range from an asymptomatic carrier state to severe vaginitis, with extensive damage of the vaginal epithelium. The disease is associated with severe complications, such as a significantly increased risk of developing invasive cervical cancer [2], a six-fold higher probability of infection by HIV [3] and adverse pregnancy outcome [4]. Although humoral, secretory and cellular immune responses are elicited in infected individuals [5], trichomoniasis often evolves into a chronic infection, with the parasite able to survive in the microaerophilic environment of the vagina, escaping the hosts killing mechanisms.

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To date, the mechanisms by which *T. vaginalis* evades the hosts immune response remain largely unknown.

Nitric oxide (NO) plays a key role in the immune response of humans [6]. Production of NO by inducible NO-synthase in macrophages activated by interferon IFN-α or IFN-β is known to mediate a host-protective response in a number of microbial infections, including some protozoan diseases. The antimicrobial role of NO in intracellular (i.e. malaria, toxoplasmosis and leishmaniasis) and extracellular (giardiasis and cryptosporidiosis) parasitosis is documented [7]. The cytotoxic effects of NO seem to be partly due to the production of peroxynitrite $ONOO^-$ [8], a potent oxidizing agent causing deamination of nucleotides, protein tyrosine nitration and irreversible inhibition of metalloenzymes. In addition, NO plays a key role in the mechanisms of innate immunity, because it enables natural killer cells to respond to interleukin (IL)-12 and IFN-α and IFN-β stimuli at the onset of infection, regulating the production of IFN-γ [6, 9]. It is therefore not surprising that microbial parasites have developed protective mechanisms against NO, to inhibit NO synthesis by macrophages and/or scavenge host derived NO.

Several enzymes able to metabolize NO have been described. These include the haem b_3-containing nitric oxide reductase (NOR [10]), which catalyses in denitrifying and pathogenic bacteria the reaction:

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$$

The NORs are typically expressed in denitrifying bacteria, where NO is an intermediate of the denitrification pathway, but the same reaction is catalysed by the cytochrome P450nor in fungi [10]. NO can be also metabolized aerobically by flavohaemoglobin [11, 12], a ubiquitous enzyme catalysing the reaction:

$$2NO + 2O_2 + NAD(P)H \rightarrow 2NO_3^- + NAD(P)^+ + H^+$$

The A-type flavoproteins (ATFs) have also been reported to be characterised by NO reductase activity [13–16]. ATFs are NADH-dependent enzymes containing a non-haem di-iron active site [17], and are preferentially expressed (e. g. in *Escherichia coli*) under microaerobic conditions and in response to exposure to NO or NO-related species [18, 19]. ATFs are believed to be restricted to Prokarya and Archaea [20] although Andersson et al. [21] have predicted the existence of an orthologous enzyme in pathogenic protists (*Giardia intestinalis, Spironucleus barkhanus* and *Entamoeba histolytica*), based on genomic analysis.

In this paper, we report that *T. vaginalis* is able to metabolize NO under close to anaerobic conditions. We propose a correlation between this newly defined function and the expression of an active ATF immunologically detected in the cell lysate of the parasite.

Material and methods

Materials

Na/ascorbate, N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD), maltose and ascorbic acid oxidase were from Sigma (St. Louis, Mo.). All other reactants were of the highest purity available. NO stock solutions were prepared by equilibrating at room temperature degassed water with pure NO gas (Air Liquide, Paris, France) at 1 atm, yielding 2 mM NO in solution. Recombinant *E. coli* FlRd was purified according to Gomes et al. [14]. *T. vaginalis* isolates were obtained as vaginal specimens from women affected by trichomoniasis. Protozoa were cultured at 37 °C in Diamond's trypticase-yeast extract-maltose medium [22] supplemented with 10% bovine serum, under a 5% carbon dioxide atmosphere. Fresh isolates were stored in liquid nitrogen in the presence of 90% serum and 10% dimethylsulfoxide. All isolates originated in Italy and were characterized as free from *Mycoplasma* infection, and by phenotypic stability [23]. After axenization and before the experiments, using universal bacterial primers to detect any bacterial presence, protozoa were tested by PCR and found free of any contamination [24].

NO electrode measurements

Amperometric NO measurements were carried out at room temperature using a Clark-type NO electrode (ISO-NO; World Precision Instruments, Sarasota, FL.) interfaced with a personal computer. The instrument is equipped with a 2-mm probe inserted into a gas-tight chamber. The functional assays were performed at pH 7.3 in phosphate-buffered saline (PBS) containing 5 mM maltose and 20 µM ethylenediamine-tetracetic-acid sodium salt (EDTA). Such a medium was made O_2 free by extensive N_2 equilibration. When specified, ascorbate (10 mM) and ascorbic acid oxidase (0.13 mg/ml) were used to maintain anaerobiosis. As independently verified, ascorbic oxidase proved to be fully efficient in removing O_2 in the presence of up to 20 µM NO in the assay. TMPD (0.1 mM) was also occasionally used as a redox mediator. Prior to use, the viability of *T. vaginalis* cells was assessed by light microscopy, monitoring their mobility and their ability to exclude trypan blue. Cells were always > 90% viable in terms of trypan blue exclusion. Before the assay, *T. vaginalis* cells were washed twice in PBS containing 5 mM maltose. A slight mobility decrease of the protozoa was observed after washing compared to their mobility in the culture medium. In a typical experiment, aliquots of NO-equilibrated water were added to the reaction vessel under constant stirring up to a final NO concentration ranging from 0.6 to 14 µM. Afterwards, cells (typically $1.7–6 \times 10^6$) were added and the NO consumption rate monitored. Activity was calculated from the initial rate and corrected for the background NO

consumption observed before the addition of the cells. *T. vaginalis* cells were assayed for their NO consumption activity within 5 min after washing, since cells suspended in air-equilibrated maltose-supplemented PBS displayed a remarkable, spontaneous decrease in their NO-degrading activity within 30 min. Cell lysis was achieved by three cycles of freezing and thawing in the presence of 1 mM N-tosyl-L-lysine cloromethyl ketone protease inhibitor or the protease inhibitor cocktail for mammalian cell extracts (from Sigma, product number P8340, dilution 1:100).

Immunoblotting

Whole-cell lysates were prepared from *T. vaginalis* cultured in Diamond's trypticase-yeast extract-maltose medium; the equivalent of 4.4×10^5 cells was loaded. Proteins were separated by SDS-PAGE electrophoresis, blotted onto a nitrocellulose membrane and blocked using standard protocols. Blots were incubated with rat polyclonal antibodies raised against *E. coli* FlRd, followed by incubation with the anti-rat IgG-alkaline phosphatase conjugate (Sigma) and detected with 4-nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Sigma).

Results

***Trichomonas vaginalis* degrades NO**

The ability of *T. vaginalis* to metabolize NO was measured amperometrically under anaerobic conditions. As shown in figure 1 A, addition of viable intact *T. vaginalis* cells caused a prompt anaerobic degradation of NO in solution, which was repeated upon subsequent additions of NO. The latter finding supports the hypothesis that the observed NO consumption is due to a catalytic reaction, rather than to a non-specific, saturable NO-binding process. Under anaerobic conditions, at relatively low NO concentrations (0.6–3 µM), the activity estimated from the initial rate of NO consumption was 130 ± 33 nmol NO × 10^8 cells per minute (n = 6 independent experiments). The NO consumption rate was shown to be linearly dependent on cell density and addition of the cell-free culture medium had no effect, demonstrating that extracellular components were not responsible for the observed NO degradation. This NO scavenging function was insensitive to cyanide, as confirmed by preincubating *T. vaginalis* at 37 °C with 1 mM cyanide for 1 h (not shown). Similar results were obtained with the cell lysate incubated with cyanide at room temperature for 45 min, in the presence of protease inhibitors (inset to fig. 1, bottom panel). The activity was shown to be NADH-dependent; as illustrated in figure 1 B, lysed *T. vaginalis* cells had no catalytic NO-degrading activity, but this was substantially restored (up to ~ 50% compared to viable intact cells) after addition of NADH.

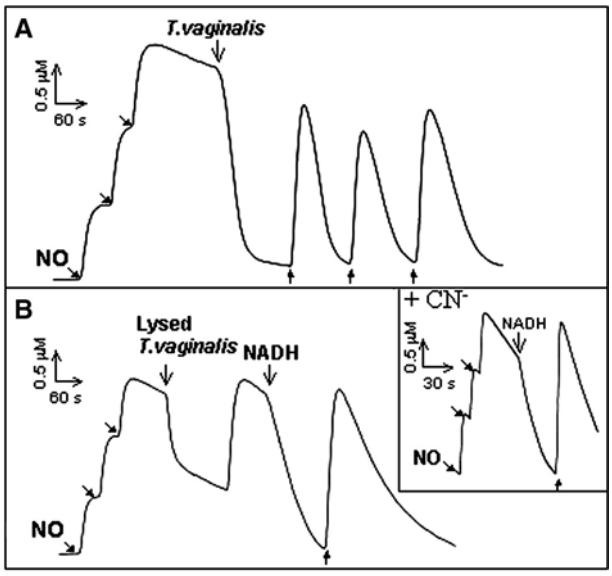

Figure 1. NO degradation by *T. vaginalis*. Reaction medium: PBS pH 7.3 containing 5 mM maltose, 20 µM EDTA. Deoxygenation of the reaction medium was achieved by exhaustive N_2-equilibration and by addition of 10 mM Na/ascorbate and 0.13 mg/ml ascorbic acid oxidase to scavenge contaminant O_2. V = 1 ml. Three aliquots of NO-saturated water were sequentially added yielding 3 µM NO in solution. Following the addition of 3×10^6 *T. vaginalis* cells (*A*), rapid NO consumption was observed. The activity estimated from the initial NO consumption rate was ~ 120 nmol NO/10^8 cells per minute. After NO exhaustion, further additions of NO in solution (black arrows) were followed by reactivation of the function. In contrast, the addition of 3×10^6 lysed cells (*B*) to 3 µM NO caused a fast and partial NO disappearance, but catalytic activity was not observed unless 1 mM NADH was added, causing reactivation of the function (~ 50% of the activity of intact viable cells). *Inset:* NO consumption sustained by addition of 1 mM NADH to 6×10^6 lysed cells pre-incubated with 1 mM cyanide for 45 min at room temperature. Assay performed in the presence of 1 mM cyanide.

The addition of NADH to intact cells had no effect on the activity. Consistent with an enzymatic function, the NO degradation was lost upon heat denaturation, as assessed with cell lysates in the presence of NADH (not shown).

The effect of NO and O_2 concentrations

The *T. vaginalis*-catalysed NO consumption was assessed over the NO concentration range 0.6–14.4 µM. As shown in table 1, at the highest concentrations (11.5–14.4 µM), the NO consumption rate dropped to about 30%. Therefore, at relatively high non-physiological concentrations, NO depresses the *T. vaginalis*-mediated NO degradation. At this stage, we cannot distinguish whether NO acts directly by inhibiting the molecular machinery responsible for the observed NO consumption or whether NO exerts its toxic effect more indirectly on a different molecular target, eventually causing NO to be degraded less efficiently.

O_2 was also found to inhibit the *T. vaginalis*-mediated NO consumption. In these measurements, O_2 removal from

Table 1. Effect of [NO] on the NO degradation rate.

[NO] (µM)	% activity	n
0.6–1.2	100 ± 31	4
3.5–4.6	83 ± 17	7
6.9–8.7	46 ± 18	2
11.5–14.4	35 ± 20	4

Experimental conditions as in figure 1. The maximal NO degradation activity, observed at the lowest NO concentration, was 133 ± 41 nmol NO/10^8 cells per minute, taken as 100%. These data suggest an inhibitory role of NO at relatively high concentrations. n, number of observations with different *T. vaginalis* cultures.

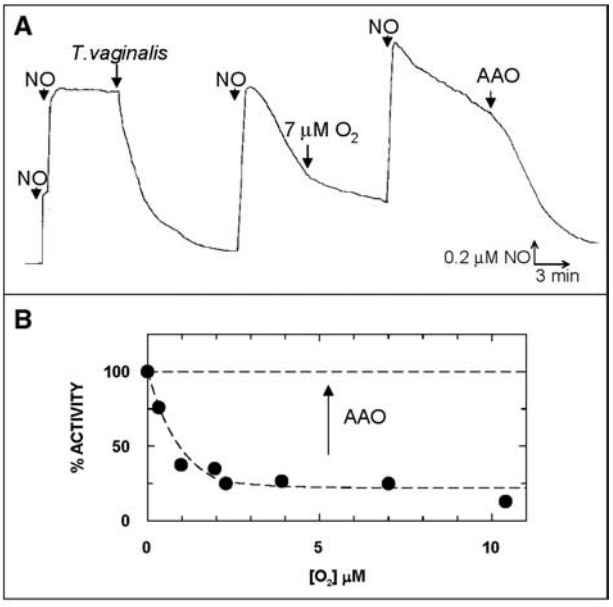

Figure 2. Effect of [O_2] on the NO degradation rate. Experimental conditions as in figure 1, with the following exceptions: (i) ascorbate and ascorbic acid oxidase were not added to scavenge contaminant O_2 in the reaction medium to start with and (ii) 100 µM TMPD was present. V = 2 ml. O_2 was added from a 1.3 mM stock-solution obtained by equilibrating water with pure O_2 at 1 atm at room temperature. (*A*) After injecting 5 × 10^6 *T. vaginalis* cells, 1.7 µM NO was promptly degraded. After addition of a second aliquot of NO, addition of 7 µM O_2 caused a significant (about 70%) decrease in the NO consumption rate. The inhibited rate was also seen after addition of a third aliquot of NO; this inhibition however was fully removed by complete de-oxygenation following addition of 0.13 mg/ml ascorbic acid oxidase (AAO). (*B*) NO consumption activity measured at different O_2 concentrations. The results of three independent experiments were combined and normalized to the activity measured under anaerobic conditions, taken as 100%.

the reaction medium for virtual anaerobicity was achieved by extensive N_2-equilibration, without adding ascorbate and ascorbic acid oxidase at the start of the assay. As shown in figure 2, in the absence of O_2, addition of *T. vaginalis* cells caused prompt consumption of NO in solution, with the rate significantly decreasing upon addition of O_2 (7 µM). Notably, this inhibitory effect of O_2 was fully reverted after complete deoxygenation obtained

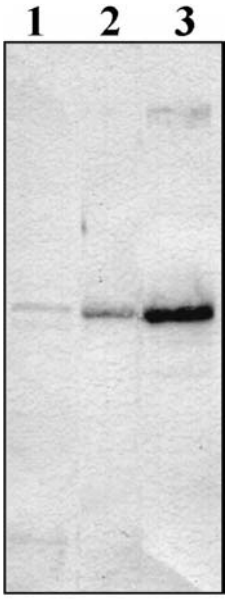

Figure 3. Immunoblot of *T. vaginalis* cell lysate. Lysate of 4.4 × 10^5 cells (lane 1), *E. coli* FlRd 2 ng (lane 2) and 10 ng (lane 3). Rat polyclonal antibodies raised against *E. coli* FlRd were used for the reaction. Details in Materials and methods.

by addition of ascorbic acid oxidase in the presence of excess ascorbate. From different experiments (fig. 2) relatively low O_2 concentrations (a few micromolar) seemed to induce a significant inhibition of the *T. vaginalis* NO consumption activity.

Immunodetection of an ATF

Whole-cell extracts of *T. vaginalis* were tested for the presence of an ATF by immunoblotting, using polyclonal antibodies raised against the *E. coli* recombinant FlRd enzyme [18]. As shown in figure 3, an immunoreactive protein with an apparent molecular weight (~ 60 kDa) very similar to that of *E. coli* FlRd is detected in *T. vaginalis*.

Discussion

NO, a key effector of the immune system [6], is produced by NO-synthase in several cell types, including macrophages. The antimicrobial effect of NO is documented in a number of infectious diseases [7] and appears to be due to a direct reaction of NO with a variety of targets and to the formation of peroxynitrite $ONOO^-$ [8] by reaction with $O_2^{\cdot-}$. In the presence of O_2, flavohaemoglobins are believed to efficiently scavenge NO by acting as NO-dioxygenases [12], although the in vivo significance of this reaction has been very recently challenged [25]. Under microaerobic conditions, an alternative NO-detoxifying role may be played by ATFs, NADH-dependent enzymes recently discovered to be endowed with a cyanide-insensitive NO-reductase activity [13–16]. In this paper,

we show that *T. vaginalis* is able to catalytically degrade NO and expresses a protein that can be recognized by antibodies specific for ATFs. We propose that this newly discovered function confers to this microaerophilic parasite the ability to evade the NO-based human immune response.

In 1997, Park et al. [26] reported that macrophage-produced NO is toxic for *T. vaginalis* under aerobic conditions. On the other hand, trichomoniasis often evolves into a chronic infection, implying that the parasite is able to survive in the NO-rich, microaerobic vaginal environment. This suggested to us that the parasite may have evolved the ability to cope with host-derived NO, a hypothesis supported by the finding presented here that *T. vaginalis* degrades NO under anaerobic conditions. We found that the NO consumption activity measured amperometrically is NADH dependent, cyanide insensitive and inhibited by O_2. These functional data rule out the possibility that the responsible enzyme is a flavohaemoglobin, but are consistent with the properties of an ATF, such as the FlRd from *E. coli* [13, 15]. Using polyclonal antibodies raised against *E. coli* FlRd, we immunodetected in *T. vaginalis* a protein band compatible with an ATF, a surprising result given that ATFs are typical of Prokarya and Archaea [20] and their expression has never been documented in Eukarya. The data support the proposal that in *T. vaginalis* an NO-detoxifying role is sustained by an ATF under anaerobic conditions, given that the vaginal environment is O_2 poor [27]. Moreover, consistent with data obtained with purified *E. coli* ATF (flavorubredoxin), *T. vaginalis* degrades low concentrations of NO more efficiently (≤ 1.2 μM, see table 1). This observation appears to correlate with the very recent finding that submicromolar NO enhances the expression of ATFs in *E. coli* [19].

In summary, we have shown that *T. vaginalis* is able to degrade NO efficiently and we envisage that this new function could be associated with an ATF which enables this parasite to evade the NO-based host immune response, conferring an advantage for survival. One may speculate that the impact of this observation may be more considerable if ATFs were also expressed in other pathogenic protists and, in fact, a genomic analysis has indicated that *G. intestinalis*, *S. barkhanus* and *E. histolytica* have genes coding for ATFs [21]. Furthermore, a BLAST search of the available genomic data on *T. vaginalis* (http://www.tigr.org) shows that at least three A-type flavodiiron proteins are encoded in this genome, all containing the two-domain core of this enzyme family, and the key residues to bind the di-iron catalytic centre, strongly corroborating our experimental observations. These genes would have been laterally transferred during evolution from anaerobic Prokarya to pathogenic protists to enhance their survival fitness for the microaerobic, parasitic life style [21, 28]. In this respect, the demonstration of an active ATF in *T. vaginalis* would be relevant to both human pathology and evolutionary biology.

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