Antioxidants promote establishment of trypanosome infections in tsetse

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SUMMARY

Efficient, cyclical transmission of trypanosomes through tsetse flies is central to maintenance of human sleeping sickness and nagana across sub-Saharan Africa. Infection rates in tsetse are normally very low as most parasites ingested with the fly bloodmeal die in the fly gut, displaying the characteristics of apoptotic cells. Here we show that a range of antioxidants (glutathione, cysteine, N-acetyl-cysteine, ascorbic acid and uric acid), when added to the insect bloodmeal, can dramatically inhibit cell death of *Trypanosoma brucei brucei* in tsetse. Both L- and D-cysteine invoked similar effects suggesting that inhibition of trypanosome death is not dependent on protein synthesis. The present work suggests that antioxidants reduce the midgut environment protecting trypanosomes from cell death induced by reactive oxygen species.

Key words: *Trypanosoma brucei brucei*, trypanosomiasis, tsetse, antioxidant, apoptosis.

INTRODUCTION

Trypanosomes of different species undergo cycles of development of varying complexity within the tsetse fly; few trypanosomes complete this cycle, the first hurdle being the fly midgut where incoming bloodstream-form trypanosomes ingested with the tsetse bloodmeal are subject to temperature shock (from 37 °C to 25 °C) and must survive in a medium of digesting blood over a period of 2–3 days. During this period trypanosomes are subject both to tsetse digestive enzymes and immune responses, providing the potential for the production of free radicals (Souza *et al*. 1997). There is a growing consensus that oxidative stress and the redox state of the cell play a central role in the regulation of apoptosis (Curtin *et al*. 2002) and evidence is accumulating to suggest that trypanosomatids and other unicellular organisms possess the machinery to carry out a form of programmed cell death (Ameisen, 2002; Chose *et al*. 2003; Debrabant *et al*. 2003; Hurd and Carter, 2004). African trypanosomes die in the fly midgut (Welburn *et al*. 1989) and *in vitro* display characteristics of apoptotic cells, and cellular markers of this process have been identified (Duszenko *et al*. 2006). Cell death in *Leishmania* can be induced by nitric oxide (Zangger *et al*. 2002) or hydrogen peroxide (Das *et al*. 2001) while reactive oxygen species (ROS) activate a cell death pathway in *Trypanosoma brucei brucei* (Ridgley *et al*. 1999). In mammalian cells, the redox state is controlled by thioredoxin and glutathione (GSH) systems which regulate cell growth and cell death by activation of transcription factors (Kwon *et al*. 2003). However, in kinetoplastids the glutathione-glutathione reductase system found in most other organisms is replaced by trypanothione and trypanothione reductase which provide the intracellular reducing environment (Fairlamb *et al*. 1985). Free radicals have been shown to limit the development of a range of parasites in their invertebrate hosts (Ascenzi and Gradoni, 2002); for example, nitric oxide (NO) limits development of malarial parasites in the mosquito *Anopheles stephensi* (Luckhart *et al*. 1998). Here we describe the effects of antioxidants on the survival or death of *T. b. brucei* in the midgut of tsetse flies.

MATERIALS AND METHODS

Fly infections

Glossina morsitans morsitans were infected on the day following the day of emergence from the puparium with bloodstream form *T. b. brucei* (stock Buteba 135) isolated from a cow in Uganda in 1990 and passaged in mice as required. Infective feeds were given *in vitro* using thawed stabilates of trypanosomes suspended in defibrinated ovine blood. Flies which did not feed were removed from the experiment. Following infection, flies were maintained at 25 °C and 70% relative humidity and fed on defibrinated ovine blood through an artificial membrane. Flies were dissected 10 days post-infection (or 10 days post-treatment with test compound) and midguts examined for the presence of trypanosomes by phase-contrast microscopy (X400).

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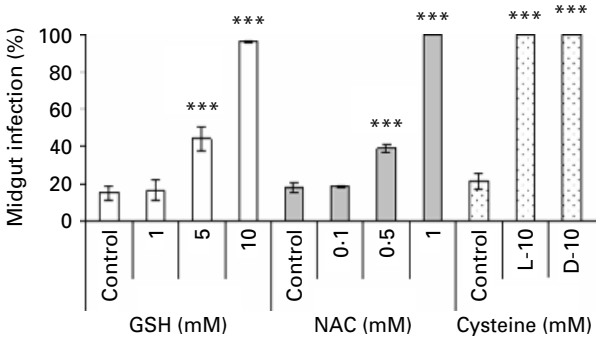

Fig. 1. Effect of GSH, NAC and L- or D-cysteine on midgut infections of *Trypanosoma brucei brucei* in male *Glossina morsitans morsitans*. Flies were infected at their first feed, the bloodmeal containing GSH, NAC or cysteine (L- or D-), dissected 10 days later and midguts examined for trypanosome presence by microscopy. Control flies were untreated. Data presented as the mean ± S.E.M. from 3 experiments. Significance: *** $P<0{\cdot}001$ versus the corresponding control values.

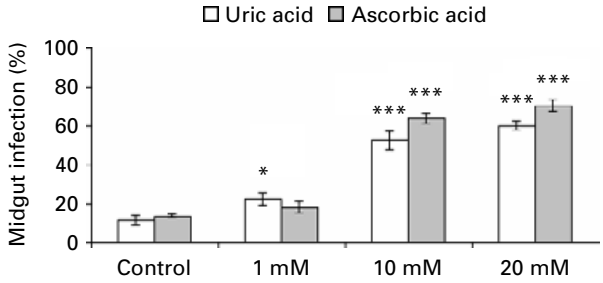

Fig. 2. Effect of uric and ascorbic acids on midgut infections of *Trypanosoma brucei brucei* in male *Glossina morsitans morsitans*. Flies were infected at their first feed, the bloodmeal containing uric acid or ascorbic acid, dissected 10 days later and midguts examined for trypanosome presence by microscopy. Control flies were untreated. Data presented as the mean ± S.E.M. from 3 experiments. Significance: *** $P<0{\cdot}001$; * $P<0{\cdot}05$ versus the corresponding control values.

Injection of test compounds

GSH equivalent to a 15 mM bloodmeal feed (150 μg/fly) was dissolved in saline and 2 μl were microinjected under the scutellum of newly emerged *G. m. morsitans* and flies were then infected 8 h later.

Test compounds

GSH, N-acetyl-cysteine (NAC), L- or D-cysteine, uric acid, ascorbic acid, superoxide dismutase, catalase, serotonin, ornithine, glutamate, cystine (all supplied by Sigma, UK) were made up in sterile saline and added in differing concentrations to infective bloodmeals as described above.

Statistical analysis

To examine if test compounds had a significant effect on midgut infection rates, generalised linear models with binomial errors were used on the proportion of flies infected, with the effects of replicates taken into account by entering replicates into the model first. Analyses were carried out in a two-stage process: first, overall differences between controls and treatments were considered and, if these were significant, then each compound was compared with its control in post-hoc testing. All analyses were carried out in R version 1.9.1 ((c) R project) and $P<0{\cdot}05$ was taken to indicate significance.

RESULTS

The effects of GSH, NAC and cysteine on trypanosome midgut infection rates in male *G. m. morsitans*, are compared in Fig. 1 which shows a clear dose response for each compound. Addition of GSH to the bloodmeal at 5 or 10 mM concentrations significantly ($P<0{\cdot}001$) increased midgut infection rates from 15% control (number of flies dissected, $n=88$) to 44% ($n=91$) and 97% ($n=92$) respectively. Addition of 0·5 or 1 mM NAC also significantly ($P<0{\cdot}001$) increased midgut infections from 17% control ($n=103$) to 39% ($n=98$) and 100% ($n=106$) respectively. Addition of 1 mM GSH or 0·1 mM NAC resulted in midgut infection rates of 16% ($n=92$; $P=0{\cdot}817$) and 18% ($n=98$; $P=0{\cdot}882$) respectively, not significantly different from controls. There were no significant differences in midgut infection rates between male and female *G. m. morsitans* fed GSH (data not shown). The effects of 10 mM L- or D-cysteine on trypanosome midgut infection rates in male *G. m. morsitans* are also shown in Fig. 1. Addition of either L- ($n=117$) or D-cysteine ($n=113$) to the infective feed significantly ($P<0{\cdot}001$) increased midgut infections from 21% control ($n=119$) to 100%.

The effects of uric and ascorbic acids on midgut infections of *T. b. brucei* in male *G. m. morsitans* are shown in Fig. 2 which shows a dose response for both compounds. The addition of 1 mM, 10 mM or 20 mM uric acid significantly increased (1 mM: $P=0{\cdot}043$; 10 mM and 20 mM: $P<0{\cdot}001$) midgut infection rates in *G. m. morsitans* from 12% control ($n=136$) to 22%, ($n=125$), 52% ($n=122$) and 61% ($n=119$) respectively. The addition of 10 mM or 20 mM ascorbic acid significantly increased ($P<0{\cdot}001$) midgut infection rates from 14% control ($n=95$) to 64% ($n=94$) and 71% ($n=89$) respectively. Addition of 1 mM ascorbic acid resulted in infection rates of 18% ($n=100$), not significantly greater ($P=0{\cdot}413$) than controls. Doses of uric acid above 20 mM could not be tested as flies refused to take an infective feed, while doses of 30 mM ascorbic acid were toxic to tsetse flies (data not shown).

Addition of 15 mM GSH to the bloodmeal 48 h post-infection significantly ($P<0{\cdot}001$) raised midgut infection rates from a control value of 6% ($n=106$) to 50% ($n=101$). There was no significant difference

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($P=0{\cdot}759$) in midgut infection rates when 15 mM GSH was added to the bloodmeal 72 h post-infection (control = 14% ($n=99$), treated = 16% ($n=102$)).

Injection of 150 μg of GSH into the haemolymph of *G. m. morsitans* (equivalent of a bloodmeal dose of 15 mM) had no significant ($P=0{\cdot}803$) effect on midgut infection rates which were 8% ($n=74$) compared to the control value of 11% ($n=71$).

Catalase, superoxide dismutase, glutamate, cystine, ornithine and serotonin had no significant effect on midgut infection rates (data not shown).

DISCUSSION

We have shown here that the addition of the antioxidants GSH, NAC, cysteine, ascorbic acid and uric acid to the bloodmeal significantly increased trypanosome midgut infection rates in *G. m. morsitans* suggesting that ROS promote trypanosome death in the fly midgut. Trypanothione, the main intracellular reducing agent in trypanosomes (Fairlamb *et al*. 1985), comprises 2 molecules of GSH (composed of amino acids: glutamic acid, L-cysteine and glycine) linked by 1 molecule of spermidine. Both L- and D-cysteine possess the same reducing power and in the present work both promoted 100% midgut infection rates in *G. m. morsitans* suggesting that thiols detoxify the tsetse midgut environment. Since L-cysteine but not D-cysteine is used in protein synthesis, it is unlikely that these effects result from the synthesis of trypanothione or other proteins by the trypanosome itself (Duszenko *et al*. 1992). NAC, which is able to cross cell membranes, was more effective in preventing trypanosome death in tsetse at lower concentrations than GSH, which is not cell permeable (Laragione *et al*. 2003), suggesting that protection of the trypanosome intracellular environment is important. GSH, when injected into the haemolymph of *G. m. morsitans*, failed to affect infection rates suggesting that haemolymph factors are not involved in clearance of trypanosomes from the gut.

Figarella *et al*. (2006) have recently shown that ROS are involved in programmed cell death induced by prostaglandins in bloodstream form *T. brucei* and found that the GSH or NAC inhibited this process. Prostaglandin synthesis is a common feature of insect midguts (Buyukguzel *et al*. 2002) but the ability of prostaglandins to induce cell death in bloodstream-form trypanosomes suggests a role in tsetse midgut immune responses.

Uric acid is the end product of nitrogen metabolism in most insects including tsetse (Moloo, 1978); *Drosophila* mutants unable to synthesize uric acid are more susceptible to oxidative stress than wild type flies (Hilliker *et al*. 1992). Trypanosomes cannot transport nor synthesize uric acid (De Koning and Diallinas, 2000) and it is doubtful that uric acid affects trypanosome survival in tsetse via direct trypanosome metabolism. Rather, it appears that the effect of uric acid is to detoxify free radicals within the tsetse gut thereby promoting trypanosome survival.

Ascorbic acid may act in a similar manner to uric acid and reduce oxidative stress in insects; ascorbic acid content in *Drosophila* is quite high, even when not provided in the diet (Massie *et al*. 1991). Mosquitoes in a chronic state of oxidative stress show increased refractoriness to malarial infection, exhibiting high levels of parasite melanization; however, pre-exposure of mosquitoes to ascorbic acid (by addition of ascorbic acid to larval and adults pre-infective bloodmeal) resulted in a 6-fold reduction in parasite melanotic encapsulation (Kumar *et al*. 2003). Significant levels of ascorbic acid have been detected in *T. cruzi* (Clark *et al*. 1994) and bloodstream-form *T. brucei* can synthesize and transport ascorbic acid (Wilkinson *et al*. 2005). In the current work higher doses of ascorbic acid (30 mM) were toxic to tsetse flies which could explain the failure to achieve 100% infection rates experimentally; this may also apply to results with flies fed uric acid amongst which higher doses had an anti-feedant effect.

Expression analysis has shown that a catalase gene is up-regulated in the posterior midgut of tsetse where bloodmeal digestion occurs while a superoxide dismutase gene is up-regulated in the midgut when compared to the flight muscle (Munks *et al*. 2005). During the current work neither catalase nor superoxide dismutase, when added to the infective bloodmeal, had any effect on trypanosome infection rates. This suggests that either these enzymes are not important in inhibition of trypanosome death, or that they are denatured in the tsetse midgut; one of the superoxide dismutases found by Munks *et al*. (2005) may be an extracellular enzyme and therefore should be adapted to function in the tsetse gut.

Previous work had shown that D-glucosamine and N-acetylglucosamine could promote trypanosome midgut infections in tsetse and it was hypothesized that the bloodmeal activated specific trypanocidal lectins that were inhibited by these sugars (Maudlin and Welburn, 1987). Recently it has been shown that glucosamine can scavenge ROS (Xing *et al*. 2005) suggesting an alternative explanation for the observed increases in midgut infection rates previously thought to be linked to inhibition of trypanocidal lectins by glucosamine (Maudlin and Welburn, 1987).

In conclusion, the work presented here shows that trypanosome cell death is normally induced in the fly midgut by ROS but trypanosomes can be protected from this process by the addition of antioxidants to the bloodmeal. Adding GSH to the bloodmeal 48 h post-infection resulted in only 50% midgut infection, suggesting that activation of the death process begins within 48 h of trypanosomes entering the fly. Since

trypanosomes cannot transport uric acid, most of the free radicals involved are likely to have been produced by the midgut environment although results obtained with cell permeable NAC point to some trypanosome intracellular involvement.

ROS have been implicated in mammalian immune responses to *T. brucei* spp. (Wang *et al*. 2002) and given the many similarities between vertebrate and invertebrate immunity (Hoffmann and Reichhart, 2002) it is not surprising that tsetse also possess defences based on ROS.

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