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Research Brief

Toxoplasma gondii: Effects of exogenous nitric oxide on egress of tachyzoites from infected macrophages

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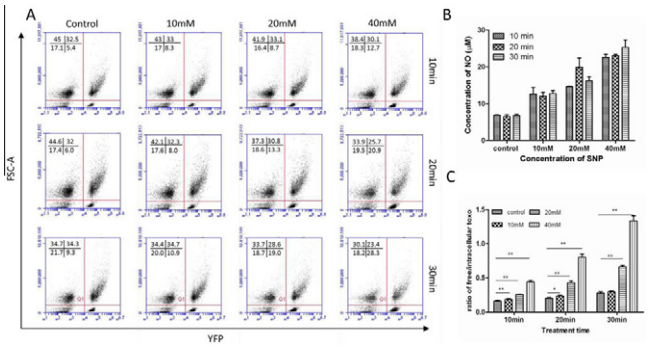
HIGHLIGHTS

- Nitric oxide released by SNP could induce *T. gondii* egress from infected macrophages.
- This early egress may depend on the parasite developmental stages in host cells.
- The target cells of NO are apoptotic macrophages.
- Our study provided a new angle to study interactions between *Toxoplasma* and its host.

GRAPHICAL ABSTRACT

NO released by SNP could trigger *Toxoplasma* egress from infected macrophages.



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ABSTRACT

Toxoplasma gondii is an obligate intracellular parasite that can infect any nucleated cells of warm-blood vertebrates. Invasion and egress by this protozoan parasite, both of which are crucial for its life cycle, are rapid events that are dependent upon parasite motility. A variety of chemicals and molecules have been utilized to induce *Toxoplasma* early egress from host cells. Here, we aimed to determine whether nitric oxide (NO) could induce egress of *T. gondii* tachyzoites from infected cells. Infected macrophages were collected from C57BL/6 mice and treated with different doses of sodium nitroferricyanide (III) dihydrate (SNP) which releases nitric oxide into cell culture medium. The pattern of parasite egress was analyzed by flow cytometry. The results showed that exogenous NO released by SNP could trigger egress of *T. gondii* tachyzoites from infected peritoneal macrophages which then underwent necrosis after parasite egress. Our findings provided a novel approach to study the interactions between host immune responses and *T. gondii*.

1. Introduction

Toxoplasma gondii is an intracellular protozoan parasite that can infect virtually all warm-blooded animals (Bradley et al., 2005). Tachyzoites, the asexual stage of *T. gondii*, replicate within host cells with a generation time of 6–8 h (*in vitro*) until they exit the cell to infect neighboring cells, usually after 64–128 parasites have accumulated per host cell (Radke and White, 1998). Invasion and egress are two pivotal events in the life cycle of tachyzoites, but the later has received comparatively less attention (Sibley, 2010). Either Ca^{2+} ionophore A23187 or microinjection of intracellular Ca^{2+} has been used to induce parasite egress (Endo et al., 1982; Schwab et al., 1994). Recently, a variety of chemicals have been reported to regulate egress of *Toxoplasma* from cells. DTT initiate

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egress of *Toxoplasma* from infected host cells through activating powerful *T. gondii* NTPases (Silverman et al., 1998) and increasing concentration of Ca^{2+} within parasitophorous vacuole (PV) lumen (Stommel et al., 1997). Besides Ca^{2+}, the reduction of K^+ concentration in host cells appears to activate a phospholipase C activity in *Toxoplasma* that then causes an increase in cytoplasmic Ca^{2+} within the parasite, leading to parasite egress (Fruth and Arrizabalaga, 2007; Moudy et al., 2001). Interestingly, the phytohormone abscisic acid (ABA) produced by *T. gondii* could stimulate Ca^{2+}-dependent protein secretion, and induce parasite egress from the infected host cell as well (Nagamune et al., 2008). In addition, when host enterocytes are infected by *T. gondii*, the cells can secrete cytotoxic molecules, such as nitric oxide (NO), along with chemokines and cytokines that attract polymorphonuclear leukocytes (PMNs), macrophages and dendritic cells (DCs) (Buzoni-Gatel and Werts, 2006). However, little information on the relationship between NO and parasite egress existed until recently, although studies showed that NO was one of the factors associated with tachyzoite and bradyzoite interconversion (Lyons et al., 2002).

In our study, we attempted to determine whether the cytotoxic molecule NO could induce egress of *Toxoplasma* from infected cells. Our results showed that in *ex vivo* experiments, exogenous NO could trigger the egress of *Toxoplasma* from infected macrophages, followed by necrosis of the host cells. Our study provided a novel approach to study the interactions between host immune responses and *T. gondii*.

2. Materials and methods

2.1. Parasites and mice

In this study, *T. gondii* tachyzoites constitutively expressing yellow fluorescent protein (YFP), i.e. RH–YFP, a transgenic line of RH strain were harvested from monolayers of human foreskin fibroblasts. 6- to 8-week old C57BL/6 mice were infected intraperitoneally with 2×10^6 of these *T. gondii* tachyzoites. Two to three days after infection, when the peritoneal macrophage infection rate achieved about 50% by FACS evaluation, intraperitoneal macrophages were collected by washing peritoneal cavity with about 5 ml cold PBS (Zhao et al., 2009) and centrifuged for the further experiments. Studies were performed in accordance with guidelines of the China Agricultural University Institutional Animal Care and Use Committee.

2.2. SNP treatment and egress assay

The isolated macrophages were treated with different concentrations (10, 20 and 40 mM) of sodium nitroferricyanide (III) dihydrate (SNP) (Sigma–Aldrich, MO, USA) for different time periods (10, 20 or 30 min) at 37 °C followed by flow cytometry analysis (C6, Accuri Cytometers, Inc.) of 100,000 cells. The data were analyzed with FlowJo software (Tree Star). The proportion of parasite egress was calculated by the ratio of free parasites to intracellular parasites. The concentration of NO was detected by the measurement of NO_2/NO_3 by the Griess reaction using NO assay kit (Applygen Tech, Beijing, China) according to the manufacturer's instructions.

2.3. Ex vivo infection and egress assay

For the preparation of peritoneal macrophages, mice were intraperitoneally injected on day 0 with 1 ml 3% thioglycolate. Macrophages were harvested on day 3 by washing the peritoneum with 5 ml invasion medium (RPMI 1640 with 1% FBS). *Ex vivo* infection assay was performed as previously described (Ling et al., 2006; Zhao et al., 2007). Briefly, peritoneal macrophages freshly collected from thioglycolat-treated mice were infected *in vitro* at a multiplicity of infection (MOI) of 0.5 to 1 in 37 °C incubator for 30 min in invasion medium. After removing the extracellular parasites by washing, macrophages were treated with SNP and analyzed as described above.

2.4. Apoptosis assay

10^6 peritoneal macrophages freshly isolated from infected mice were stained with Annexin V-APC (eBioscience), followed by staining with propidium iodide (PI) (eBioscience) both before and after egress induction according to the manufacturer's instructions. Stained cell samples were analyzed on a flow cytometer (C6, Accuri Cytometers, Inc.) and analyzed using FlowJo software (Tree Star).

2.5. Statistical analysis

Statistical analyses were performed using SPSS software (SPSS 15.0 for Windows, Chicago, IL), and all data expressed as means ± SD values.

3. Results

3.1. Effects of NO released by SNP on the egress of T. gondii from infected macrophages

Both invasion and egress are essential for *T. gondii* to complete its life cycle in host cells. Here, we used sodium nitroferricyanide (III) dihydrate (SNP) to determine whether the NO released could induce egress of *T. gondii* tachyzoites from infected macrophages. As shown in Fig. 1A, when infected macrophages were treated with 40 mM SNP for 30 min, the percentage of free parasites in total events of FACS was 28.3%, which was much higher than that of the control group (9.3%). Moreover, when it comes to the ratio of free parasites to intracellular parasites, the ratio of SNP-treated groups (1.33 ± 0.075, in 40 mM SNP, 30 min, Fig. 1C) was significantly higher than in the control group as well (0.27 ± 0.024, in 30 min, Fig. 1C). The parasites underwent egression in a time-dependent manner (Fig. 1A and C). We also evaluated the concentration of NO released by SNP. Consistent with previous studies (Ioannidis et al., 1996), the concentration of NO released from SNP were stable in the early 30 min (Fig. 1B).

3.2. The linkage between egress of T. gondii and parasite developmental stages in macrophages

To investigate whether NO-induced parasite egress could occur shortly after parasite invasion, we performed *ex vivo* infection. However, there was no significant difference in parasite egress pattern between groups treated with or without SNP (Fig. 2), indicating that NO-induced parasite egress may rely on the developmental stage of *T. gondii* in macrophages.

3.3. The fate of macrophage after NO-induced egress of T. gondii tachyzoites

To detect the fate of macrophages from which *T. gondii* egressed, we determined the kinetics of cell death before and after SNP treatment of macrophages using a combination of Annexin-V and PI staining. Interestingly, it was found that the proportion of typical apoptotic cells (Annexin-V^+PI^-) in infected macrophages was statistically higher than that in uninfected cells (38.98 ± 1.60 *vs* 6.68 ± 1.57, Fig. 3A). after treatment with 40 mM SNP, the percentage of Annexin-V^+PI^- cells in infected macrophages decreased apparently (Fig. 3B), indicating that NO mainly induced the para-