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Modulation of host HIF-1α activity and the tryptophan pathway contributes to the anti-*Toxoplasma gondii* potential of nanoparticles

Oluyomi Stephen Adeyemi[a,b], Yuho Murata[a], Tatsuki Sugi[a], Yongmei Han[a], Kentaro Kato[a,*]

[a] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[b] Medicinal Biochemistry and Toxicology Laboratory, Department of Biological Sciences, Landmark University, PMB 1004, Ipetu Road, Omu-Aran 370102, Nigeria

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ABSTRACT

Background: Toxoplasmosis constitutes a large global burden that is further exacerbated by the shortcomings of available therapeutic options, thus underscoring the urgent need for better anti-*Toxoplasma gondii* therapy or strategies. Recently, we showed that the anti-parasitic action of inorganic nanoparticles (NPs) could, in part, be due to changes in redox status as well as in the parasite mitochondrial membrane potential.
Methods: In the present study, we explored the in vitro mode of action of the anti-*T. gondii* effect of NPs by evaluating the contributions of host cellular processes, including the tryptophan pathway and hypoxia-inducing factor activity. NPs, at concentrations ranging from 0.01 to 200 μg/ml were screened for anti-parasitic activity. Sulfadiazine and/or pyrimethamine served as positive controls.
Results: We found that interplay among multiple host cellular processes, including HIF-1α activity, indoleamine 2,3-dioxygenase activity, and to a larger extent the tryptophan pathway, contribute to the anti-parasitic action of NPs.
Conclusion: To our knowledge, this is the first study to demonstrate an effect of NPs on the tryptophan and/or kynurenine pathway.
General significance: Our findings deepen our understanding of the mechanism of action of NPs and suggest that modulation of the host nutrient pool may represent a viable approach to the development of new and effective anti-parasitic agents.

1. Introduction

Toxoplasma gondii is the causative agent of toxoplasmosis, a parasitic disease that constitutes a serious public health challenge worldwide [1,2]. *T. gondii* has low specificity and infects a range of hosts; accordingly, the parasitic disease it causes is common and widespread, affecting more than 60% of the world population [3,4]. The *T. gondii* infection is usually asymptomatic in healthy individuals, but can be fatal in pregnant or immunocompromised individuals [5]. In healthy individuals, the *T. gondii* infection is controlled by the immune system and appropriate medication, but cysts remain in all infected tissues including the brain and these may serve as a source for exacerbations particularly in immunocompromised individuals. Available treatment options for toxoplasmosis patients are limited, but include the use of anti-malarial drugs or antibiotics, which often cause serious side effects including bone marrow suppression and rashes [5]. Consequently, toxoplasmosis remains a large global burden that is further enhanced by the shortcomings of current therapeutic options. These factors drive the search for better anti-*T. gondii* drugs and/or new approaches to the treatment of toxoplasmosis.

Recently, we showed that inorganic nanoparticles (NPs) including Au, Ag, and Pt nanoparticles caused *T. gondii* death partially via changes in redox status and parasite mitochondria membrane potential [6]. However, since nanomedicine is still in its infancy, the modes of action of many NPs that appear to be bioactive remain poorly understood [7]. To further our understanding of the mode of action of NPs as it relates to their anti-*T. gondii* activity [6], we examined the host contribution to the anti-parasitic action of nanoparticles. In our earlier report [6], we determined that oxidative stress plays a part in the anti-parasitic action of NPs, but evidence [6] suggests that modulation of host cellular processes also contributes to the NP-induced anti-parasitic effect. Interestingly, NPs have the potential to affect several cellular signaling processes, including the activity of hypoxia-inducible factor 1 (HIF-1) [8–10]. HIF-1 is a heterodimer consisting of α and β subunits. It plays a remarkable role in *T. gondii* survival in the host by regulating pro-parasite genes including glycolytic metabolic genes, transferrin receptor, and vascular endothelial growth factors [11–13]. Moreover, increased levels of HIF-1 protein and activity are not restricted to

* Corresponding author.
E-mail address: kkato@obihiro.ac.jp (K. Kato).

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hypoxic stress as many pathogens including *T. gondii* activate HIF-1 [14], and loss of the HIF-1α subunit has been shown to cause a significant reduction in parasite growth at physiological oxygen levels [15].

Furthermore, given that *T. gondii* is an obligate intracellular parasite, it must satisfy its nutritional needs by scavenging essential nutrients such as tryptophan from its host [16]. Therefore, this may represent an opportunity for the host to naturally restrict parasite growth by modulating nutrient pools. For example, in human cells, the inducible enzyme indoleamine 2,3-dioxygenase (IDO) reduces local tryptophan levels and is therefore able to mediate broad spectrum effector functions including restricting the growth of various clinically relevant pathogens [17]. IDO belongs to the family of heme enzymes that catalyze the oxidative degradation of tryptophan, which the parasite cannot synthesize *de novo* [18]. Previous studies have shown that the parasite grows unhindered if IDO function is impaired [17] and the suppressive effect of IDO on parasite growth can be reversed by the addition of excess tryptophan to the growth medium [18]. Taken together, these studies suggest that tryptophan starvation may represent a critical anti-parasitic pathway. Moreover, hypoxia with a concomitant increase in HIF-1α level has been linked to reduced IDO expression [17] leading to a sparing effect on the local tryptophan pool that consequently may support parasite growth. Therefore, we asked whether NP treatment affects host cellular processes in a way that helps to restrict parasite growth and sought to determine likely host cellular processes involved in mediating the anti-parasitic action of NPs. The present study provides evidence that modulation of HIF-1α levels, IDO activity, and the tryptophan pathway in host cells partially mediates the anti-parasitic action of NPs.

2. Materials and methods

2.1. Materials

Nanoparticles (NPs), including gold (AuNP, 5 nm), silver (AgNP, 10 nm), and platinum (PtNP, 3 nm), were purchased from Sigma-Aldrich (St. Louis, MO, USA). The NPs were used as supplied after evaluation to confirm the supplier's specifications. The NPs were reconstituted in fresh culture medium prior to each use. L-tryptophan, L-kynurenine, cobalt (II) chloride ($CoCl_2$), 4-(dimethylamino) benzaldehyde, 1-Methyl-D-tryptophan (DMT), and 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) were obtained from Sigma-Aldrich. Dexamethasone sodium phosphate and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were obtained from Wako Pure Chemicals (Osaka, Japan); (±)3,4-dihydro-3-hydroxy-2,2-dimethyl-4-[(phenylmethyl)amino]-2H-naphtho[2,3-b]pyran-5,10-dione (a naphthoquinone derivative – NQ) was obtained from Cayman Chemicals (Ann Arbor, MI, USA). All reagents were of analytical grade and used as supplied unless otherwise stated.

2.2. Parasite strain

A luciferase-expressing parasite strain, *T. gondii* RH-2F [19], was used for this study. The parasite was maintained by repeated passages in monolayers of human foreskin fibroblast cells (HFF; ATCC®, Manassas, VA, USA) cultured in Dulbecco's Modified Eagle Medium (DMEM; Nissui, Tokyo, Japan) and supplemented with GlutaMAX™-I (Gibco, Invitrogen, Waltham, MA, USA), 10% (v/v) fetal calf serum (FCS; Gibco, Invitrogen, Waltham, MA, USA), and penicillin and streptomycin (10,000 U/ml; Leicestershire, UK). The number of *T. gondii* tachyzoites was determined through a luminescence-based assay of β-galactosidase (β-gal) activity expressed by the parasite strain RH-2F. To obtain a purified parasite suspension for the assays, infected cells were passed through a 27-gauge needle to lyse them and the lysates were filtered to remove cell debris. The parasite suspension free of host cell debris was then washed with fresh culture medium. Parasite density was measured with a hemocytometer and adjusted for in vitro experimental infection analysis.

2.2.1. The anti-T. gondii potential of NPs in vitro

NP doses were selected on the basis of our previous findings [6], and in vitro growth inhibition assays were performed as previously described [6]. Briefly, purified parasite suspension plus the NPs (reconstituted in culture medium prior to use) was added to growing HFF monolayers and incubated for 48 h. The untreated but infected cells served as controls, whereas the culture medium only well was used to correct for the background signal. Sulfadiazine (Sigma, St Louis, MO, USA) and/or pyrimethamine (Wako Pure Chemical, Osaka, Japan) were included as positive controls. After the 48-h incubation at 37 °C in a 5% CO_2 atmosphere, the viability of the RH-2F parasite strain was determined by assaying for galactosidase activity by using a Beta-Glo luminescent assay kit (Promega, Madison, WI, USA). The assay was performed in triplicate and repeated three times independently. All experiments were performed in 96-well solid white plates (Nunc; Fisher Scientific, Pittsburgh, PA, USA) unless otherwise stated.

2.3. Determination of indoleamine 2,3-dioxygenase (IDO EC 1.13.11.52) activity and kynurenine levels

Briefly, growing HFF monolayers were treated with NPs in the presence or absence of RH-2F infection. After a 24- or 48-h incubation at 37 °C, cells were scrapped and washed three times with cold PBS at 2500 × *g* for 10 min (Cold centrifuge; Hitachi, Japan). The cells were resuspended in M-PER lysis buffer (Thermo-Fisher, Waltham, MA, USA). The mixture was gently shaken for 10 min and cell debris removed by centrifugation at 14,000 × *g* for 15 min. The supernatant was transferred to a new tube for immediate biochemical analysis. For IDO activity determination, a Sandwich human ELISA assay kit (Cloud-Clone, Houston, TX, USA) was used. The assay was performed according to the manufacturer's instructions.

To determine the concentration of kynurenine in cell supernatant, we used the protocol described by Braun et al. [20] with slight modification. Briefly, 100 µL of 30% trichloroacetic acid (TCA) was added to 100 µL of culture supernatant and incubated for 30 min at 50 °C to hydrolyze N-formylkynurenine to kynurenine. This was then vortexed, and centrifuged at 8500 × *g* for 5 min. An aliquot (100 µL) of the supernatant was then mixed with an equal volume of freshly prepared Ehrlich reagent (2%; 100 mg *P*-dimethylbenzaldehyde in 5 ml of glacial acetic acid) in a micro-titer plate well (96-well format). After a 10-min incubation at room temperature, the optical density was measured at 492 nm by using a microplate reader (MTP 500; Corona Electric, Hitachinaka, Japan). The level of kynurenine in the culture supernatant was extrapolated from a calibration curve of defined kynurenine concentrations (0–250 µM).

2.4. Chemical induction of hypoxia

Chemical hypoxia was induced in HFF cells by following the procedure described by Wu and Yotnda (2011). Briefly, growing HFF cells were treated with $CoCl_2$ (0.1 µM final concentration) and incubated for 24 h at 37 °C. Successful hypoxia induction was confirmed by measuring the HIF-1α level and comparing it with that of the untreated control.

2.4.1. Determination of hypoxia-inducing factor 1-alpha (HIF-1α) levels

HIF-1α was detected by using a cell-based human ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA) developed for rapid detection of HIF-1α in fixed cells. The assay was performed according to the manufacturer's instructions. Briefly, growing HFF monolayers in solid white microplate wells (96-well format) were treated with NPs in the presence or absence of RH-2F infection. After a 24-h incubation at 37 °C, cells were fixed, permeabilized, and then neutralized in the well. HIF-1α was