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Research Brief

Toxoplasma gondii binds sheep prolactin

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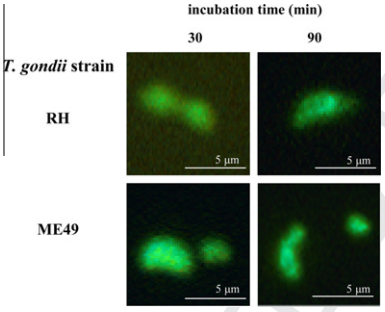
HIGHLIGHTS

- Live tachyzoites of *Toxoplasma gondii* RH and ME49 strains bind sheep prolactin (shPRL).
- Binding of shPRL by *T. gondii* is concentration dependent.
- Binding of shPRL is specific for both RH and ME49 *T. gondii* strains.

GRAPHICAL ABSTRACT

Live RH and ME49 Toxoplasma gondii tachyzoites , bind FITC-labeled sheep prolactin (shPRL).



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ABSTRACT

Taking into account the literature reports on the involvement of prolactin (PRL) in the regulation of immunity against *Toxoplasma gondii*, we decided to check whether this parasite has the ability to bind the lactotrophic hormone. We examined *T. gondii* binding of sheep fluoresceine- and biotine-labeled prolactin isolated from pituitary (shPRL). In this work we announced for the first time that shPRL was bound to live tachyzoites of RH (type I) and ME49 (type II) strains. Furthermore, by use of competitive inhibition analysis, we confirmed that this binding was specific for both tested *T. gondii* strains.

1. Introduction

Toxoplasma gondii (phylum Apicomplexa) is a ubiquitous intracellular obligatory parasite of humans and endothermic animals. In immunocompetent individuals the infection is usually self-limiting and asymptomatic. During the acute infection phase, rapidly multiplying tachyzoites invade a wide variety of nucleated cells and disseminate in the host organism. Under the pressure mediated by acquired cellular immunity, tachyzoites are transformed to dormant bradyzoites, which become enclosed in tissue cysts and a life-lasting latent chronic infection is established. The cellular immune response involves the action of macrophages, dendritic cells, neutrophils, and also CD4+, CD8+ and γδ lymphocytes and proinflammatory mediators (such as IL-12, IFN-γ and TNF-α), controlling tightly the infection course, although the parasite is not eradicated from the host organism (Montoya and Liesenfeld 2004; Denkers et al., 2004). Toxoplasmosis is an opportunistic disease and the parasite is highly pathogenic for immunocompromised individuals (e.g. AIDS patients). Moreover, a primary infection in pregnant women can lead to serious congenital defects of the fetus or spontaneous abortion. *T. gondii* infections in farm animals are also a great veterinary medical problem, particularly toxoplasmosis in sheep deserves special attention because of its economic impact resulting from a high abortion rate.

As the immune system cooperates closely with the nervous and endocrine systems using the same mediators and their receptors, an invasion of a host organism by the parasite may impair the complex neuroimmunoendocrine network, in a different manner. Not

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only may the parasite produce hormones and immunomodulating agents itself, but it can also stimulate immune cells of a host organism to produce hormones and cytokines, and use them as growth factors to establish the infection (Romano et al., 2003; Escobedo et al., 2005; Ouaissi, 2007). On the other hand, hormones produced by the endocrine system may activate different types of immune system cells, thus inhibiting the development of infection and accelerating a recovery process (Zellweger et al. 1996). One of the hormones that exhibit very wide immunomodulatory acitivity is prolactin (PRL), a polypeptide pituitary hormone, structurally related to members of the cytokine hematopoietic family including growth factors (such as GM-CSF) and interleukins (such as IL-2 and IL-7). As a result, PRL interacts with the immune system and may function as a cytokine (Clodi et al., 1992).

In the recent years a few observations concerning the influence of hormones on the course of *T. gondii* infection have been reported (Khalifa et al., 2000; Liesenfeld et al., 2001). A protective role of PRL was described by Benedetto et al. (1995) on a murine experimental toxoplasmosis model. Further studies have confirmed *in vitro* the anti-parasitic activity of PRL, capable of inhibiting multiplication of *Toxoplasma* in murine microglial cell cultures (Benedetto et al., 2001), mouse and human fibroblast lines (L929 and Hs27), and peripheral mononuclear blood cells (PBMC) isolated from women with high levels of serum PRL (Dzitko et al., 2010, 2012), respectively. Moreover, it was well documented that women with hyperprolactinemia showed lower *T. gondii* seroprevalence than those with the normal PRL level (Dzitko et al., 2008). To date, the mechanism of the anti-toxoplasmic action of the hormone has not been explained in detail.

Taking into account all the findings on the protective role of PRL in *T. gondii* infection as well as particular importance of the infection especially in pregnant women, immunosuppressed humans and livestock, we decided to examine whether free *T. gondii* extracellular tachyzoites are able to bind PRL.

2. Material and methods

The tachyzoites of the following *T. gondii* strains: RH – intraspecies type I (ATCC® Number 50174™) and ME49 – intraspecies type II (ATCC® Number 50611™) were grown *in vitro* on an L929 host cell line (ATCC® Number CCL-1™) in the Iscove's Modified Dulbecco's Medium (IMDM, Cytogen GmbH), with the addition of penicillin (100 kU/L; Sigma–Aldrich), streptomycin (100 mg/L; Sigma–Aldrich), β-mercaptoethanol (50 μM/L, Sigma–Aldrich) and 5% fetal calf serum (FCS, Cytogen GmbH).

Experiments intended to assess binding of PRL by live tachyzoites were carried out using prolactin from sheep pituitary (shPRL, Sigma–Aldrich). The hormone was labeled with biotin using the EZ-Link® Sulfo-NHS-Biotinylation Kit (Thermo SCIENTIFIC), according to the protocol provided by the manufacturer. The biotinylated protein was marked as shPRL-BT. Extracellular *T. gondii* of RH and ME49-strain, freshly egressed from host cells, were filtered (5 μm, Sartorius), centrifuged (15 min, 880g, 24 °C), and the samples of 1×10^7 parasites were suspended in 90 μl of the IMDM medium (Cytogen GmbH) supplemented with 5 mM of $CaCl_2$ (Sigma–Aldrich) and 0.1% bovine serum albumin (BSA; Sigma–Aldrich). Finally, 10 μl shPRL-BT was added to the tachyzoites to a final concentration of: 1, 5, 10, 25, 50, and 100 μg/ml (test samples). Tachyzoites suspended in 100 μl of the IMDM medium without shPRL were used as negative control (control sample). Both test and control samples were incubated in standard culture conditions (10% CO_2, 37 °C) for 90 min, and agitated every 15 min. Then, the *Toxoplasma* cells were centrifuged (15 min, 880g, 24 °C), washed three times in 100 μl of the IMDM medium, and finally suspended in 500 μl of 0.5% formaldehyde (Sigma–Aldrich) in buffered saline solution (PBS; Cytogen GmbH) with the addition of 5 mM $CaCl_2$. The tachyzoites were applied (2×10^6 cells/100 μl/well) to the wells of a microtitration plate (Nunc^{TC}) and dried overnight at the temperature of 37 °C in order to fix them. The binding of shPRL-BT by *T. gondii* was evaluated by the cellular enzyme-linked immunosorbent assay (cELISA). Fixed tachyzoites, were washed 4 times in PBS with the addition of 0.05% Tween 20 (wash buffer; Sigma–Aldrich) and blocked with 3% BSA in PBS. After the incubation (24 h, 4 °C), both test and control samples of *T. gondii* were washed (6×5 min), and then treated with streptavidin (1:1000) labeled with HRP (Sigma–Aldrich) in the wash buffer. Thus prepared samples were incubated (24 °C, 2 h), and then washed in the same conditions as above. The color reaction was developed using ABTS solution in a phosphate-citrate buffer (pH = 4.5) as a chromogen with the addition of H_2O_2. The OD values for the samples were read at $\lambda = 405$ nm after 20 min of incubation using a Multiskan EX reader (Thermo SCIENTIFIC).

For competitive inhibition analysis, live tachyzoites of *T. gondii* RH and ME49-strain were filtered (5 μm, Sartorius), centrifuged (15 min, 880g, 24 °C), and 1×10^7 *Toxoplasma* cells were suspended in 100 μl of IMDM medium supplemented with 5 mM $CaCl_2$, 0.1% BSA and 0, 10, 25, 50, 100, 200, 400, 800, 1000, 2000 and 4000-fold excess of unlabeled shPRL. After 90 min incubation (10% CO_2, 37 °C) 25 μg/ml of shPRL-BT was added to each sample. The *Toxoplasma* cells suspensions were then centrifuged (15 min, 880g, 24 °C), washed three times in 100 μl of the IMDM/$CaCl_2$ medium, and finally suspended in 500 μl of 0.5 % formaldehyde in PBS containing 5 mM $CaCl_2$. The subsequent stages of cELISA test were carried out in the same way as described in the protocol provided for the experiments concerning the binding of shPRL-BT.

The experimental results were analysed statistically with the SigmaPlot 12.0 software (Systat Software Inc., San Jose, CA, USA). Normality of the distribution was determined using the Kolomogorov–Smirnov test. A *t*-test was used for normally distributed data to assess differences between groups.

3. Results

The used method (cELISA), involving biotinylated sheep prolactin (shPRL-BT) clearly demonstrated that live *T. gondii* tachyzoites of both RH strain (belonging to type I, extremely pathogenic to mice, $LD_{100} < 10$ tachyzoites, non cyst-forming, widely used in the research and routine diagnostics Sabin–Feldman dye test to recognize toxoplasmosis) and ME49 strain (belonging to type II, intermediate virulent in mice, $LD_{100} > 1000$ tachyzoites, having the opportunity easily establish latent infections characterized by the presence of tissue cysts containing dormant bradyzoites) bind the lactotrophic hormone (Fig. 1). Binding intensity increased proportionally to the hormone concentration up to 25 μg/ml, and above this level reached a plateau. Analysis of the results revealed a statistically significant ($p < 0.001$) increase in the optical density of the samples containing *T. gondii* cells incubated at the presence of 5, 10, 25, 50 and 100 μg/ml of shPTL-BT, as compared to the results in control samples (tachyzoites incubated without hormone). Furthermore, the binding of shPRL by live tachyzoites of both tested parasite strains was confirmed by a strong signal with 25 μg/ml of fluorescein isothiocyanate (FITC)-labeled shPRL after 30 min and 90 min of incubation (Fig. 2). To characterize the binding specificity, a competitive inhibition assay was carried out using shPRL-BT (Fig. 3). It showed that in the presence of an excessive amount of unlabeled shPRL, inhibition of the biotin-labeled homological protein (shPRL-BT) binding by the live *T. gondii* cells occurred. This result confirmed that shPRL binding by both RH and ME49 strain tachyzoites is specific. Ten-fold excess of the unlabeled shPRL caused a decrease of 2.6% (RH strain) and 4.0%

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