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Toxoplasma gondii: Molecular cloning and characterization of a nitric oxide synthase-like protein

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ABSTRACT

Toxoplasma gondii has a nitrite production and a putative nitric oxide synthase (NOS) motif genomic sequence. In order to demonstrate that this sequence is functional and could be involved in the metabolism of L-arginine derivatives, we constructed a baculovirus carrying the previously identified *Toxoplasma* NOS-like DNA sequence. The recombinant protein was expressed into insect Sf9 cells and his activity was tested in serial microplate colorimetric assays. The protein produced 21 nmol/min/ml nitrites per microgram of protein and followed Michaelis–Menten kinetics, with a K_m for L-arginine of 2.3 mM. Furthermore, the optimal pH, temperature and incubation time for the recombinant *Toxoplasma* NOS-like protein were established. *Toxoplasma* NOS runs as a band of 11.6 kDa on tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Our results indicate that the recombinant protein derived from the putative genomic sequence, at the chromosome 1b of *T. gondii,* is able to produce nitrites from L-arginine as substrate.

1. Introduction

Nitric oxide (NO) is a second messenger molecule that participates in a plethora of cellular and systemic physiological conditions, e.g., regulation of vascular tone, neuronal transmission, or in antitumoral or antimicrobial activities (Moncada et al., 1991). NO in mammals is produced from L-arginine by three isoforms of nitric oxide synthases (NOS) that can have natural or inducible activity. It has been also demonstrated that invertebrates, such as certain insect species (Weiske and Wiesner, 1999), molluscs (Huang et al., 1997), nematodes such as *Ascaris suum* (Bascal et al., 1995), *Dirofilaria immitis* (Kaiser et al., 1998) or *Brugia* species (Pfarr and Fuhrman, 2000) and bacteria (Adak et al., 2002) are able to produce NO and have their own NOS-like genes. In this context little is known about protozoan NOS. Different studies show that *Toxoplasma* (Gomez Marin, 2000) in common with other protozoa such as *Tetrahymena* (Christensen, 1996), *Trypanosoma* (Paveto et al., 1995), *Entamoeba* (Hernandez-Campos et al., 2003) and *Plasmodium* (Ghigo et al., 1995) has its own nitrite production that would reflect natural NOS activity (producing 2–6 μM of nitrites). Genestra et al. (2003) have also shown that *Leishmania* produce levels of nitrite similar to those found in *Toxoplasma*. Gutierrez-Escobar and Gómez-Marin (2005) identified the first protozoal genomic sequence that included one putative NOS motif signature and also demonstrated that it was transcriptionally active. This was a strong argument to believe that nitrites in *Toxoplasma* would be derived from a NOS enzyme that could be part of a L-arginine pathway. As a consequence of this previous work, we cloned the *Toxoplasma* sequence containing the universal signature motif NOS (oxygenase domain) into a recombinant baculovirus system in order to demonstrate that *Toxoplasma* has a functional NOS-like protein.

2. Materials and methods

2.1. Parasites

Female Swiss ICR mice (Universidad Nacional, Bogota, Colombia) were inoculated with *Toxoplasma gondii* RH strain. Tachyzoites were recovered from the peritoneal cavity 3–4 days later by instilling 5 ml sterile 0.9% NaCl solution with antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml). Tachyzoites were isolated by centrifugation twice at 200*g* for 10 min. The pellets were resuspended in RPMI medium and filtered through 3 μm pore size polycarbonate membrane (Nucleopore, Cambridge). Cell and parasite viability was tested with the Trypan blue exclusion test (0.4% solution) and only samples with 95% or more viable parasites were used.

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2.2. Cell and baculovirus

Autographa californica nuclear polyhedrosis virus (AcNPV) and its recombinant virus were grown in *Spodoptera frugiperda* (Sf9) cells in a TNM-FH insect medium (Clontech, CA, USA) supplemented with antibiotics (penicillin 100 U/ml and clamoxicillin 100 μg/ml).

2.3. Cloning of the Toxoplasma NOS gene

The template RNA for RT-PCR was extracted from tachyzoites of the *T. gondii* RH strain as described previously (Gutierrez-Escobar and Gómez-Marin, 2005). Two oligonucleotide primers: Tgnos1: AGGCCT-CTGCTTGCCGTTTGTTTCG and Tgnos2: CGCCGGCG-GCACACGCTCAACTAATTAC including the restriction sites Stu1 and Not1 (underlined in the primer sequences) were used to amplify the *Toxoplasma* NOS gene by RT-PCR assay as described previously (Gutierrez-Escobar and Gómez-Marin, 2005) and then the product was recovered from agarose. The transfer vector pAcHLT-A was digested with Stu1 and Not1 restriction enzymes. The purified *Toxoplasma* NOS PCR product amplification was ligated into the Stu1 and Not1 sites of the baculovirus transfer vector pAcHLT-A (Becton–Dickinson, Franklin Lakes, NJ, USA). The resulting plasmid was designated pAcHLT-A-NOS.

2.4. Construction of recombinant baculovirus and DNA sequencing

Sf9 cells were cotransfected with the recombinant transfer vector pAcHLT-A-NOS and linear AcNPV viral DNA (Becton–Dickinson, Franklin Lakes, NJ, USA) following the manufacturer instructions for the Baculovirus Expression and Purification Kit (Clontech, California, USA). After 4 days incubation at 27 °C, the cells and supernatant containing the recombinant virus were harvested and a recombinant baculovirus was obtained. A recombinant transfer vector pAcHLT-XyIE was used as a control virus. Sequencing of recombinant plasmids was performed from *Toxoplasma* NOS PCR products. Specific bands were cut from low-melt agarose gels, followed by recovery using the Wizard PCR Prep kit (Promega, WI). Sequencing was done by using the ABIPRISM model 3100 version 3.7 at the Wadsworth Genetics Institute facility on purified DNA by using 2 pmol of the Tgnos1 and Tgnos primers.

2.5. Recombinant Toxoplasma NOS protein purification and electrophoretic analysis

The sf9 cells were treated with lysis buffer and the supernatant was cleared by centrifugation. Later, they were added to a vial with prewashed Ni–NTA (Ni_2 + nitrilotriacetate) beads (Clontech, CA, USA). The mixture was incubated at 4 °C for 1 h. The beads were collected, washed with lysis buffer and then, the *Toxoplasma* NOS protein that was bound to the beads, was eluted with lysis buffer containing 250 mM imidazole according to the manufacturer instructions. Afterwards, the protein eluted was quantified by the Bradford method. Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (trycine SDS–PAGE) was performed as described by Schägger (2006).

2.6. Animal antisera and fluorescence staining

One New Zealand White rabbit (2 kg) was housed, fed and handled in accordance with the recommendations made by the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA). One hundred fifty micrograms of purified NOS protein emulsified in hydroxide aluminum adjuvant was inoculated intramuscularly at four sites. The same amount of immunogen mixed with aluminum hydroxide was inoculated as a booster on days 8 and 15 and serum was obtained at day 45. ELISA was performed to determine the titers of polyclonal antibody sera and fluorescent staining was performed with 1/10 and 1/50 dilutions of antisera. Controls was undertaken using preimmune antisera and secondary antibody without polyclonal antibodies and testing with two recombinant proteins (*Toxoplasma* metalloprotease and *Entamoeba histolytica* cysteine protease) obtained by the His tagged procedure.

2.7. NO synthase assay

NO synthesis by *Toxoplasma* NOS recombinant protein of *T. gondii* was measured in a microplate assay for nitrite based on the Griess reaction (Stuehr and Marletta, 1985) and using the Nitric Oxide Synthase Assay Kit (Bioxytech, Foster City, USA) as recommended by the manufacturer. The reaction was run at room temperature for 40 min. Residual NADPH, which could interfere with the colorimetric assay, was removed at the end of the incubation by adding 40 U/ml of lactate dehydrogenase. The stability of the protein was determined by incubating the protein for 10 min at a range of temperatures between 0 and 100 °C and then the NOS assay was performed. The pH range for the recombinant NOS was evaluated using sodium acetate buffer at pH 4.5, potassium phosphate buffer at pH 7.0 and 7.5, and Tris–HCl buffer at pH 9.5 in the colorimetric assay.

2.8. Statistics

All data are expressed as means ± SEM from three experiments for NOS activity and in duplicate for kinetic, temperature and pH experiments.

3. Results

3.1. Cloning and bioinformatics analysis of Toxoplasma NOS-like gene

The product of reverse transcription was amplified revealing a band with the expected size (237 pb) for a *Toxoplasma* NOS-like gene (Fig. 1). No amplification product was observed in the negative control, indicating that there was no contamination with genomic DNA. The similar sizes of bands obtained both in PCR and in RT-PCR reactions indicated that *Toxoplasma* NOS-like gene consisted of a single exon and this was further confirmed sequencing the cloning amplification products. During cloning, we used primers with restriction sites obtaining similar bands (Fig. 1).

The *Toxoplasma* NOS product was purified from RT-PCR (Fig. 1, Lane 3) and then ligated into the baculovirus transfer vector pAcHLT-A and cloned into *Escherichia coli* DH5α strain. The recombinant vector was used in a *Toxoplasma* NOS gene PCR assay (Gutierrez-Escobar and Gómez-Marin, 2005) and sequenced (Fig. 2). The analysis of the sequence by using basic local alignment tool (BLAST) in ToxoDB (http://toxoDB.org) showed 100% of identity with our previously reported *Toxoplasma* NOS gene listed in the ToxoDB database (Fig. 2B). Furthermore, the *Toxoplasma* NOS gene was not present in the genome of the Sf9 culture cells after

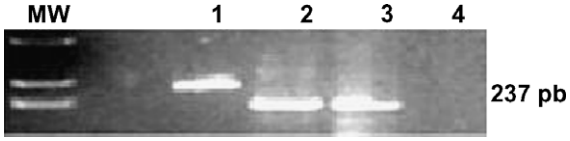

Fig. 1. *Toxoplasma* NOS gene RT-PCR amplification in *Toxoplasma gondii*. Lane 1, positive kit control; lane 2, RT-PCR of DNase-treated total *T. gondii* RNA using the primers without restriction sites; lane 3, the RT-PCR of DNase-treated total *T. gondii* RNA using the primers with restriction sites; lane 4, negative control.