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BmVDAC upregulation in the midgut of *Rhipicephalus microplus*, during infection with *Babesia bigemina*

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ABSTRACT

The molecular mechanisms involved during the infection of *Rhipicephalus microplus* midgut cells by *Babesia bigemina* are of great relevance and currently unknown. In a previous study, we found a voltage-dependent anion channel (VDAC)-like protein (BmVDAC) that may participate during parasite invasion of midgut cells. In this work, we investigated BmVDAC expression at both mRNA and protein levels and examined BmVDAC localization in midgut cells of ticks infected with *B. bigemina* at different times post-repletion. Based on the RT-PCR results, *Bmvdac* expression levels were significantly higher in infected ticks compared to uninfected ones, reaching their highest values at 24 h post-repletion ($p < 0.0001$). Similar results were obtained at the protein level ($p < 0.0001$). Interestingly, BmVDAC immunolocalization showed that there was an important differential expression and redistribution of BmVDAC protein between the midgut cells of infected and uninfected ticks, which was more evident 24 h post-repletion of infected ticks. This is the first report of BmVDAC upregulation and immunolocalization in *R. microplus* midgut cells during *B. bigemina* infection. Further studies regarding the function of BmVDAC during the infection may provide new insights into the molecular mechanisms between *B. bigemina* and its tick vector and could result in its use as an anti-tick and transmission-blocking vaccine candidate.

1. Introduction

Rhipicephalus microplus is a vector for the protozoan agents of cattle fever, *Babesia bovis* and *Babesia bigemina* (Peter et al., 2005; Estrada-Peña et al., 2006). Because cattle fever is endemic in Mexico and other countries of the temperate world, this threat has triggered the search for new, environmentally safe and effective strategies for the control of *R. microplus*. Anti-tick vaccines are a potential alternative to chemical control methods, but currently only a small number of tick-protective antigens have been identified (Willadsen et al., 1989; de la Fuente and Kocan, 2006). *Babesia* protozoan sexually reproduce in their host vector, giving rise to ray bodies and to zygotes, which penetrate the midgut barrier and are thus adapted to transmit themselves successfully (Bock et al., 2004). Studies on the molecular events that exist in the interphase vector-pathogen are important to implement strategies to block the transmission of the disease (de la Fuente and Kocan, 2003). Nevertheless, protein interactions between the midgut cells of *Rhipicephalus* ticks and sexual stages of *Babesia* have not been widely explored. Our group, by means of a proteomics strategy, previously identified a mitochondrial protein in the midgut of the *R. microplus* tick that interacts with *B. bigemina* sexual stages and which has a high similarity to the mitochondrial voltage-dependent anion-selective channel (BmVDAC) (Rodríguez-Hernández et al., 2012).

The mitochondrion has an important role in the control of apoptosis, through the permeability of its membrane, which allows the release of molecules that activate apoptosis, such as the

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cytochrome c. Pathogens can induce or inhibit the permeability of the mitochondrial membrane, which is understood as a potential regulator of the apoptotic process (Kroemer et al., 2007). The voltage-dependent anion channel (VDAC) is a protein that is found in the external mitochondrial membrane and allows the flux of small molecules into the mitochondrial intermembrane space; it also participates in the complex interactions regulating the metabolism and the apoptosis of cells (Roman et al., 2006; Young et al., 2007). The VDAC porins were initially identified in the external membrane of mitochondria; they are channels with a molecular weight of 30–35 kDa (Sorgato and Moran, 1993), and have been found in plants, fungi, bacteria, protozoa and human, in which they are involved in apoptotic processes. The VDAC channel is an integral protein of the membrane, and it consists of a polypeptide having unfolded alternating hydrophobic and hydrophilic amino acids, forming from 13 to 19 transmembrane β chains composed of a single α-helix at the amino terminus. This conformation provides a means to separate an apolar environment from a polar one, forming a barrel that is inserted into the membrane forming the pore (Bayrhuber et al., 2008). Humans, as well as rats and mice, have three different types of *vdac* genes that codify different isoforms expressed in different tissues (Sampson et al., 1997). The role of the VDAC-1 isoform has been studied and its participation in the permeability transition pore (PTP) has been demonstrated (Shimizu et al., 1999; Bernardi et al., 2001). The activity of VDAC is directly modulated by proteins of the Bcl-2 family (Shimizu et al., 1999; Priault et al., 1999). The Bax/Bak proteins (members of the family Bcl-2) have been discovered to induce the release of cytochrome c in native yeast cells, but not in the mitochondria of yeast cells lacking VDAC-1 (Shimizu et al., 1999; Priault et al., 1999; Shimizu et al., 2000). The presence of porins on extra mitochondrial membranes was initially reported by Thinnes et al. (1989) and, since then, the research and study of the possible existence of porins in domains, organelles and membranes of different cellular types has intensified in order to explain the function of this protein in those compartments.

Currently, it is known that certain intracellular pathogens can activate or inhibit the apoptosis of the host cell at the mitochondrial level through the interaction of any of its proteins with the proteins of the external mitochondrial membrane in which the VDAC, which regulates the permeability of the mitochondrial membrane, is found (Boya et al., 2001).

This study is focused on determining the expression and localization of the BmVDAC protein in the midgut cells of the tick *R. microplus* during the process of invasion by *B. bigemina*.

2. Materials and methods

2.1. Ticks

A *Babesia*-free colony of *R. microplus* (Media Joya strain) was maintained under laboratory conditions at the Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria (CENID-PAVET) in Morelos, México. *R. microplus* larvae hatched from 0.5 g of eggs were placed on a calf and 21 days later replete female ticks were collected. In order to obtain infected ticks, concurrently, *R. microplus* larvae from 0.5 g of eggs were placed on a splenectomized calf. Fourteen days later, the calf was inoculated intravenously with 5 ml of blood infected with *B. bigemina* (Chiapas strain) previously maintained in liquid nitrogen and 21 days later replete female ticks were collected. Replete female ticks fed on infected or uninfected blood were collected and dissected to obtain their midguts at 0, 12, 24, and 72 h post-repletion. To confirm infection, hemolymph smears were examined from 30 females 72 h post-repletion (Burgdorfer, 1970). For confocal microscopy assays, tick midguts were dissected to establish a primary cell culture as described previously (Mosqueda et al., 2008). The cultures were examined after three days using an inverted microscope (IROSCOPE SI-PH, MX).

The protocol for handling and bleeding the cattle and tick collection was approved by the "Comité de Bioética" de la Facultad de Ciencias Naturales, México.

2.2. Transcription analysis by RT-PCR

Total RNA was isolated from uninfected and infected midgut ticks at 0, 12, 24, and 72 h post-repletion using TRIzol (Invitrogen, USA). Then, 2 μg of total RNA were reverse transcribed and used for amplification with SuperScript One Step with Platinum Taq (Invitrogen, USA) and gene specific primers. RT-PCR conditions were 55 °C for 30 min, 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 7 min. The primer sequences were: for the *Bmvdac* gene, primer forward 5′-CGC GGA TCC CCG TGC TAC GCA GAC TTG-3′ and primer reverse 5′-CCC CCA AGC TTG CTA CGC AAC CCA GGC CGA ATC-3′; for the esterase gene used as control: ESTtr forward 5′- CCA TCT ACC ACG ACG CAT TC -3′, ESTtr reverse 5′- GGG CAG GAG ATC TGG CTT C -3′. The "housekeeping" esterase gene was selected from a reference study because it did not vary in the presence of any of the tested conditions (Cossío-Bayúgar et al., 2009).

2.3. Tissue dissection and protein extraction

To obtain midguts from infected or uninfected ticks, replete females were held in place for dissection with wax in Petri dishes and dissected under Hank's Balanced Salt Solution (HBSS) (Invitrogen, USA). The ventral cuticle was excised with a scalpel, the midguts were removed, rinsed in sterile HBSS and groups of ten ticks per tube were either placed in TRIzol reagent for RNA isolation or held on ice to obtain cells. Proteins were isolated using TNTE (50 mM Tris pH 7.4, 150 Mm NaCl, 0.5% Triton and 1 Mm EDTA). Protein isolation was performed in duplicates using midgut tissue from a total of 60 control ticks and 60 infected ticks from all post-repletion times. Then, 0.2 ml of pooled midgut tissue was mixed with 0.5 ml of TNTE containing 0.04 ml (25×) protease inhibitor cocktail complete (Roche, DE). Midgut tissue was homogenized on ice, incubated for 15 min at room temperature and centrifuged at 5000 × *g* for 5 min at 4 °C. Proteins obtained from the interphase were re-suspended in 5× sample buffer.

2.4. Expression and purification of recombinant BmVDAC

A 675 base pair internal region of the *Bmvdac* gene (GenBank no. **GU994210**) was PCR- amplified from *R. microplus* genomic DNA using Forward (5′-CGC**GGATCC**CCGTGCTACGCAGACTTG-3′) and reverse (5′-CCCCC**AAGCTT**GCTACGCAACCCAGGCCGAATC-3′) primers. *Bam*HI and *Hin*dIII restriction sites are in bold. Amplification conditions were: 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 7 min. The amplified PCR fragments were cloned into pCold I expression vector (Takara, Japan) using *Bam*HI and *Hind*III sites. The constructions were verified by sequencing using a 3130 Genetic Analyzer sequencer (Applied Biosystem). Recombinant BmVDAC protein was expressed as His6x-fusion polypeptide in *Escherichia coli* strain BL21 using 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 24 h at 15 °C. Cells were disrupted by sonication and insoluble BmVDAC protein was purified under denaturalized conditions with HisTrap HP affinity columns (GE Healthcare**,** SE) in an AKTA prime Plus FPLC System (GE Healthcare, SE). Recombinant protein concentration was determined by Bradford protein assay (Bradford, 1976) and sample purity was assayed by 12% SDS-PAGE.