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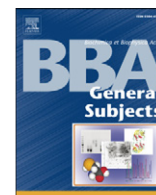

The conserved role of the AKT/GSK3 axis in cell survival and glycogen metabolism in *Rhipicephalus* (*Boophilus*) *microplus* embryo tick cell line BME26

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ABSTRACT

Background: Tick embryogenesis is a metabolically intensive process developed under tightly controlled conditions and whose components are poorly understood.
Methods: In order to characterize the role of AKT (protein kinase B) in glycogen metabolism and cell viability, glycogen determination, identification and cloning of an AKT from *Rhipicephalus microplus* were carried out, in parallel with experiments using RNA interference (RNAi) and chemical inhibition.
Results: A decrease in glycogen content was observed when AKT was chemically inhibited by 10-DEBC treatment, while GSK3 inhibition by alsterpaullone had an opposing effect. RmAKT ORF is 1584-bp long and encodes a polypeptide chain of 60.1 kDa. Phylogenetic and sequence analyses showed significant differences between vertebrate and tick AKTs. Either AKT or GSK3 knocked down cells showed a 70% reduction in target transcript levels, but decrease in AKT also reduced glycogen content, cell viability and altered cell membrane permeability. However, the GSK3 reduction promoted an increase in glycogen content. Additionally, either GSK3 inhibition or gene silencing had a protective effect on BME26 viability after exposure to ultraviolet radiation. *R. microplus* AKT and GSK3 were widely expressed during embryo development. Taken together, our data support an antagonistic role for AKT and GSK3, and strongly suggest that such a signaling axis is conserved in tick embryos, with AKT located upstream of GSK3.
General significance: The AKT/GSK3 axis is conserved in tick in a way that integrates glycogen metabolism and cell survival, and exhibits phylogenic differences that could be important for the development of novel control methods.

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1. Introduction

Recent studies on energy metabolism during tick embryo development demonstrate that it is strongly associated with carbohydrate mobilization. Glucose and glycogen levels have been shown to oscillate during *Rhipicephalus microplus* embryogenesis in a way greatly supported by gluconeogenesis [1]. Additionally, during tick embryo development, the transition from syncytial to cellular blastoderm represents not only a decisive moment in embryo formation, but also a shift in embryo metabolism in order to reduce the consumption of glucose reserves coupled with increased protein degradation to sustain gluconeogenesis [1,2]. Interestingly, cellular processes that take place before and after *Aedes aegypti* mosquito embryo germ band retraction are known to greatly affect glucose metabolism as well [3].

Globally, ectoparasites have medical, veterinary and economic importance, primarily due to their ability to transmit a diverse range of pathogens, and also due to the toxic and immunosuppressive components in their saliva [4]. Thus, elucidating the physiological processes of disease vectors is central to the development of new control methods. The establishment of tick cell lines provided a powerful tool for studying vector-pathogen interactions at cellular level and also to understand the mechanisms behind pesticide resistance [5–8]. A better

Abbreviations: 10-DEBC, 10-(4′-(N,N-Diethylamino) butyl)-2-chlorophenoxazine hydrochloride; AKT, Protein kinase B; CN, Negative control; DMSO, Dimethyl sulfoxide; dsRNA, Double-stranded RNA; dsCN, Unrelated dsRNA; dsAKT, Double-stranded RNA for protein kinase B; dsGSK, Double-stranded RNA for glycogen synthase kinase 3; FBS, Fetal bovine serum; GSK3, Glycogen synthase kinase 3; IC_{50}, Inhibitory concentration to reduce cell viability by 50%; ISP, Insulin/insulin-like growth factor signaling pathway; IGF, Insulin-like growth factor; MTT, 3-(4,5 Dymethil-2-thiazolyn)-2-5-dyphenyl-2H-tetrazolium bromide); ORF, Open reading frame; PI3K, Phosphatidylinositol 3-OH kinase; RTK, Receptor tyrosine kinase; RNAi, Ribonucleotic acid interference; RACE, Rapid amplification of cDNA ends; RmAKT, Rhipicephalus microplus protein kinase B

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understanding of the physiological and metabolic processes used by these cells may contribute to the discovery of novel pathways that can be targeted in the design of anti-tick control measures [9,10].

Cell metabolism, growth, survival and replication are dependent on extracellular signals that require receptor-mediated transduction to intracellular components [11]. Growth factors responsive machinery coordinates such events in a mechanism whose components are highly conserved in metazoan species: the phosphoinositide 3-kinase (PI3K)/AKT (also known as protein kinase B, PKB) pathway [12,13]. In mammals, the activation of PI3K/AKT pathway facilitates glucose uptake in, but not limited to insulin-responsive tissues [14–16]. Normally, PI3K is activated upon stimulation of membrane receptor tyrosine kinase (RTK), G protein-coupled receptors (GPCRs) and cytokines receptors *via* extracellular growth factors [17]. Activated PI3K generates phosphatidylinositol lipids which lead to recruitment and activation of additional kinases, especially AKT. AKT activation is fully accomplished by phosphorylation at two specific residues and represents a node of major regulation of the many cellular effects of insulin/insulin-like growth factors signaling pathway (ISP) [18–21]. A diversity of AKT substrates is associated with the promotion of cell survival, inhibition of apoptosis, stimulation of glycogen, protein and lipid synthesis, inhibition of gluconeogenesis and promotion of glycolytic enzymes [12].

Previous studies reported the immunodetection of insulin-like proteins in tick synganglion [22–24]. Although such endogenous peptides remain to be identified, IGF-binding proteins (IGFBP) have been characterized in ticks before, suggesting modulation of the insulin/IGF pathway to complete bloodmeal acquisition [25]. Moreover, the BME26 cell line was shown to be responsive to the addition of exogenous insulin that is marked by an increase in glycogen content, and this effect was completely abolished in the presence of PI3K inhibitors [26]. Insulin addition alone was also able to alter the relative transcription of PI3K regulatory subunit p85. Recent identification of AKT in parthenogenic *Haemaphysalis longicornis* ticks points to its role in blood feeding and cell and organ growth in adult stages [27].

One of the target proteins negatively regulated by the PI3K/AKT pathway is glycogen synthase kinase 3 (GSK3), a conserved eukaryotic serine/threonine kinase, which is inhibited by phosphorylation at Ser^{21} (GSKα) or Ser^{9} (GSKβ) positions by AKT [28]. GSK3 is reported to affect several cellular functions such as cell cycle, gene transcription, apoptosis and development, besides its role in carbohydrate metabolism, where it inhibits glycogen synthase by phosphorylation. Tick GSK3 exists only in one isoform, and it has been found that its enzymatic activity is inversely related to embryo glycogen content [29]. Similarly, mosquito GSK3 activity also varied oppositely with glycogen content during initial stages of *A. aegypti* embryo development [3]. Importantly, GSK3 activity is also necessary for tick oviposition and egg viability, as evidenced from both chemical inhibition and adult gene knockdown [30].

Although GSK3 role in oviposition and embryo development has been characterized in other models [31], the upstream regulators that control tick metabolism are unknown. Herein, we report the identification and cloning of an AKT from *R. microplus*, and characterized its role in glycogen metabolism and cell viability as an upstream regulator of GSK3 in BME26 cells using both RNA interference (RNAi) and chemical inhibition methods. The antagonistic roles observed for AKT and GSK3 strongly suggest that such a signaling axis is conserved in tick embryos.

2. Experimental procedures

2.1. Chemicals

The AKT inhibitor 10-DEBC {10-[4′-(N,N-Diethylamino)butyl]-2-chlorophenoxazine hydrochloride} and PI3K inhibitor, wortmannin, were purchased from Tocris Bioscience (Ellisville, MO, USA). LY294002, an additional inhibitor for PI3K was purchased from Calbiochem (San Diego, CA, USA). GSK3 inhibitor, alsterpaullone {9-Nitro-7, 12-dihydroindolo [3,2-d][1]benzazepin-6(5H)-one}, Hoechst 33342, Propidium iodide, MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], Leibovitz's 15 culture medium and α-amiloglucosidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Nutricell Nutrientes Celulares (Campinas, SP, Brazil). Other reagents and chemicals used were of analytical grade and purchased locally.

2.2. BME26 cell line

Cells were maintained as previously described [32]. Attached cells from confluent flasks (25 cm^2) were resuspended in fresh complete medium using a 22-gauge needle with bent tip fitted to a 5-mL plastic syringe. Cells were passaged every 3–4 weeks, and the medium replaced weekly. Culture density was determined using a Neubauer hemocytometer and cell viability was determined by trypan blue exclusion (0.4%, Sigma) technique. Two weeks prior to their use in experiments, synchronized cells were prepared by transferring 1×10^7 cells into 5 mL of fresh complete medium (final volume), and incubated at 34 °C to promote doubling (within 2 weeks), and the medium replaced weekly. BME26 cell line is derived from embryos of different ages of *Rhipicephalus microplus* ticks, first isolated in the 1980s and described in 2008 [32].

2.3. Viability assay

BME26 cell suspension was seeded into 24-well plates (5×10^5 cells/well) to a final volume of 500 μL of complete medium and allowed to attach. After 24-h incubation at 34 °C, chemical inhibitors were added at the final concentrations indicated, and whereas 0.05% DMSO was used in negative control wells. After 24 h of treatment, 50 μL MTT prepared in serum-free medium (5 mg/mL) was added to each well. After additional 2-h incubation, media were completely discarded and 1 mL of acid-isopropyl alcohol (0.15% HCl in isopropyl alcohol) was added to dissolve the formazan crystals. The mixture was transferred to 1.5-mL tubes, spun at 6000 ×*g* for 15 min, and the clear supernatant collected in new tubes for absorbance measurement at 570 nm using quartz cuvettes in an UVmini-1240 UV–VIS spectrophotometer (Shimadzu, Japan). Unless otherwise stated, absorbance values of control treatment were used for normalization (100% viability).

2.4. RmAKT cloning

The degenerate primers, forward 5′-CARTGGACNACNGTNATYGA-3′ and reverse 5′-CCRCACATCATYTCRTACAT-3′ [33] were used to amplify a partial AKT sequence from *R. microplus* egg cDNA containing the conserved regions of AKT, the PH (pleckstrin homology) and kinase domains, by touchdown PCR. The cycling program was as follows: 94 °C for 5 min; 95 °C for 30s, 60 °C to 50 °C (decreasing 0.6 °C at each cycle) for 45 s, 72 °C for 1 min, 17 cycles followed by 95 °C for 30 s, 48 °C for 45 s, 72 °C for 1 min, 40 cycles; and a final extension at 72 °C for 5 min, in 200-μL microtubes. A 793-bp PCR product was purified using the Gene Clean III DNA purification kit (QBiogene, Carlsbad, CA, USA), and cloned into pGEM-T vector (Promega, Madison, WI, USA) according to the manufacturer's instructions, followed by propagation in *Escherichia coli* (DH5α strain). Recombinant plasmid DNA was extracted using Qiagen Miniprep kit (Qiagen, Hilden, Germany) and DNA sequencing performed by CEQ 2000 Dye Terminator Cycle with Quick Start kit (Beckman Coulter, Inc., Fullerton, CA, USA.). To obtain the entire cDNA sequence coding for RmAKT, 3′ and 5′-RACE (rapid amplification of cDNA ends) amplifications were performed using the nucleotide sequence of the 793-bp amplified fragment. The first-strand cDNA for 3′-RACE was obtained through the reaction of SuperScript™ II Reverse Transcriptase (Invitrogen) with total RNA and an oligo (dT) adaptor primer. The cDNA was amplified with the

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