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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**A B S T R A C T**

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 and, 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 phylogenetic 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
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 (IGFBPs) 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 pathogenetic 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$^{3}$ (GSKox) or Ser$^{9}$ (GSK$^{\alpha}$) 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 glycosylation. Previous studies reported the immunodetection of insulin-like protein BME26 cells using both RNA interference (RNAi) and chemical inhibition methods. The antagonistic roles observed for Akt and GSK3 in ticks before, suggesting modulation of the insulin/IGF pathway [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].

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-dihydropodolo[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 trypsinization. Two weeks prior to their use in experiments, synchronized cells were prepared by transferring 1 x 10$^5$ 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 x 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 x g for 15 min, and the clear supernatant collected in new tubes for absorbance measurement at 570 nm using quartz cuvettes in a 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′-CARTGACACNAGNTATNYA-3′ and reverse 5′-CCRCACATCYCTRCATC-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 sequencing in (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...
gene-specific forward primer and adaptor primer. First-strand cDNA for 5′-RACE was synthesized from total RNA using the oligonucleotide 5′-CTCTGAGGAACTCAAAG-3′ and reverse transcriptase enzyme, as specified by the manufacturer (Invitrogen, Carlsbad, CA, USA). The PCR-amplified cDNA was made using the abridged anchor primer AUAP and a nested gene-specific primer 5′-CTCTGAGGAACTCAAAG-3′. A second nested specific primer 5′-CGACTGCTGCTCC TGGGCC-3′ was used to perform a second PCR. The product was cloned into pGEM-T and sequenced. Subsequently, using primers designed from the 5′-RACE (forward primer ATGATGGAAGCGCC ATTGCC) and 3′-RACE (reverse primer TACAGAGAGATGCTGCTGTC products), the full-coding cDNA was amplified by RT-PCR and sequenced to confirm the coding region of AKT. The amplified fragment was cloned into pGEM-T and sequenced.

2.5. Sequence analyses

Nucleotide analysis and deduced amino acid sequences were carried out using a commercial software package, GENETYX-Win version 4.04 (Software development Co. LTD., Tokyo, Japan) and with BioEdit version 7.0.9.0 [34]. The new sequence was aligned with related sequences retrieved from the Genbank using CLUSTAL W [35], and conserved regions identified by the PROSITE algorithm [36]. A phylogenetic tree was constructed using neighbor-joining method [37] executed in Mega version 5.0. module.

2.6. Double-stranded RNA (dsRNA) synthesis

A 595-bp dsRNA was designed for *R. microplus* AKT coding sequence. Since *R. microplus* genome is not yet available, dsRNA specificity and potential off-targets were estimated from similar genes in other species with dsCheck (*Drosophila melanogaster* and *Rattus novergicus*) [38] and DEQOR (*D. melanogaster* and *Anopheles gambiense*) [39] programs. Oligonucleotide primers containing T7 promoter sequences (lower case nucleotides) were designed by gel electrophoresis. Double-stranded RNA synthesis for *RmGSK3* and *RmAKT* was performed as described previously [30]. The negative control for RNAi was dsCheck- (forward), 5′-CCGTCGAGATGCTGAACTTGT-3′ (reverse) for *RmAKT* (Ta = 56 °C, Amplicon 86 bp), Specific primers for *R. microplus* Expression Factor 1A (EFlA) (accession number EF142066) [29] and for Rm40S ribosomal protein (accession no. EW679928) [41] were the same as described previously. Relative expression was determined using the Cp values from each run on Relative Expression Software Tool—REST [42]. EFlA was used as reference gene for *RmAKT* analysis and Rm40S was the reference gene for *RmGSK3* analysis. cDNA from BME26 not exposed to dsRNA was used as calibrator.

2.9. Glycogen content determination

Cell lysates (in triplicate) were incubated with 1 unit α-amylglucosidase in acetate buffer (200 mM, pH 4.8) for 4 h at 40 °C. The reaction was stopped with phosphate buffer (100 mM, pH 7.4). Liberated glucose was detected with a commercial kit for glucose concentration (Glucost®, Doles) at 510 nm. Control conditions (without α-amylglucosidase) were used to determine baseline glucose level and subtracted from test conditions. Glycogen content was determined using a standard curve submitted to the same conditions [1], and normalized by total protein content. Results are presented as mean and standard deviation from three independent experiments.

2.10. Membrane integrity analysis

BME26 cells were plated (3 × 10⁵ cell/well) over glass coverslips placed at the bottom of 24-well plate. Cells were treated either with dsRNA for tick AKT, or with unrelated dsRNA. After prolonged exposure assay, BME26 cells were directly stained by adding Hoechst 33342 (0.4 μg/mL, final concentration) for 5 min, and then propidium iodide (2 μg/mL, final concentration) for additional 2 min. Incubations at room temperature were performed protected from light. Well content was discarded, coverslips were washed with PBS and mounted over glass slides with 5 μL of glycerol. Coverslips were observed under florescence microscope (model Eclipse 80i, Nikon, Japan), and pictures were obtained at 400× magnification.

2.11. Protein determination

Total protein was determined according to Bradford [43], using bovine serum albumin as standard.

2.12. *RmAKT* expression during embryogenesis

Total RNA was extracted from *R. microplus* eggs on different days after oviposition with TRIZOL reagent (Invitrogen) according to the manufacturer’s protocol. One microgram of total RNA was converted into complementary DNA with the High Capacity cDNA Reverse transcription kit (Applied Biosciences). Quantitative PCR analysis was performed using 200 ng of cDNA in 10-μL reactions in a LightCycler platform, as described above. Relative expression determination was performed as described earlier, using one-day-old eggs as calibrators.
2.13. Ultraviolet light exposure

BME26 cells treated with dsRNA for tick GSK3 were exposed to UV light (254 nm) within a distance of 54 cm, for 8 min in order to induce cell death [44]. After treatment, cells were incubated for 24 h and viability determined by the MTT assay. Values were normalized with cells not treated with dsRNA and comparisons done with treatment with unrelated dsRNA.

In other experiments, BME26 cells were incubated with alsterpaullone or vehicle alone (0.05% DMSO) for 60 min at 34 °C prior to UV light exposure (15 min), and cell viability was determined by MTT after additional 24-h incubation. Viability values were normalized by those obtained for cells in vehicle alone not exposed to UV.

2.14. Statistical analysis

Non-parametric analysis was used in glycogen and viability comparisons between inhibition/knock down of AKT and GSK3. Results for AKT expression were analyzed by one-way ANOVA. The GraphPad Prism 5.03 Software was used in all analyses.

3. Results

3.1. AKT or GSK3 inhibition has different effects on BME26 cell viability

To examine the effects of AKT inhibition on viability, BME26 cells were treated with different concentrations of 10-DEBC (AKT inhibitor) or vehicle alone (0.05% DMSO) for 24 h. Cell viability as determined by MTT, which revealed that AKT inhibition with 10-DEBC decreased viability in a dose-dependent manner within the range of 24 and 96 μM (Fig. 1A). Cell viability was slightly altered up to 24 μM 10-DEBC, but it significantly decreased in the presence of 48–96 μM 10-DEBC. The IC50 value for 10-DEBC inhibitor was determined to be approximately 48 μM (insert). In order to determine the effects of 10-DEBC on glycogen content, we used concentrations lower than the corresponding IC50. Alsterpaullone, a chemical inhibitor of GSK3, had no significant effect on BME26 viability, when used in concentrations ranging from 4 to 400 nM (Fig. 1B). Treatment with PI3K inhibitors LY294002 and wortmannin mildly affected BME26 viability (Supplementary Fig. 2).

3.2. Effects of AKT and GSK3 inhibition on BME26 glycogen content

The participation of AKT and GSK3β in glycogen metabolism in BME26 cells was determined by culturing them in the presence of inhibitors in the growth medium. Glycogen content decreased 30.4% and 36.5% in cells exposed to 6 and 12 μM 10-DEBC, respectively, compared to cells treated with vehicle alone (0.05% DMSO) (Fig. 2A). On the other hand, cells treated with 1 μM alsterpaullone (GSK3 inhibitor) showed a nearly 50% increase in glycogen level (Fig. 2B).

3.3. Cloning and sequence analysis of RmAKT

PCR with egg cDNA yielded a fragment that encodes an AKT-like protein sequence when searched against protein sequences in the NCBI. Full-length tick AKT cDNA (1584 bp) was determined from sequence data obtained by cloning RACE PCR products and from sequences available in nucleotide database. The deduced RmAKT amino acid sequence (527 aa) was aligned with homologous proteins with ClustalW (Supplementary Fig. 1). ScanProsite analysis showed that the Pleckstrin Homology (PH) domain ranges from positions 39 to 141, the Serine/Threonine (Ser/Thr) kinase domain is between positions 180 and 437, and a hydrophobic motif is present from positions 501 to 506. The putative phosphorylation sites are also conserved at Thr336 and Ser505 residues.

RmAKT and amino acid sequences of related proteins were subjected to phylogenetic analysis, and a tree was constructed (Fig. 3). The phylogeny consists of two protein clades: one that includes RmAKT and other AKTs from arthropods, and one bearing mammalian AKTs. In addition, the RmAKT sequence pairs with AKT homologue from H. longicornis, with high bootstrap values.

3.4. Effects of RmAKT and RmGSK3 silencing on BME26 cells

Possible accidental targets of AKT and GSK3 silencing by RNAi were selected based on orthologous sequences of D. melanogaster and C. elegans, since the entire R. microplus genome is unavailable. Both DEQOR and dsCheck analyses showed high efficiency and specificity of the iRNAs, and revealed that the chances of non-target inhibition would be significantly lower in comparison with the desired targets (data not shown).

The RNAi mediated gene silencing for AKT and GSK3β in BME26 cells was greater than 70% (Fig. 4A and B), as determined by relative expression analysis using RT-qPCR within the first 24 h of dsRNA exposure. Additionally, glycogen content in AKT silenced cells decreased by approximately 30% (Fig. 5A). On the other hand, glycogen content was increased in cells silenced for GSK3β by almost 50% (Fig. 5B). Glycogen
determination was performed in samples obtained after prolonged exposure assay.

BME26 cells silenced for AKT exhibited a near 30% reduction in cell viability, as determined by the MTT assay (Fig. 6A). It was also observed that cells treated with dsAKT double-stained with propidium iodide and Hoechst 33342 (pink nuclei in Fig. 6C) at higher intensity, compared to cells treated with dsRNA (Hoechst 33342 blue stained nuclei in Fig. 6B).

BME26 cells were exposed to UV light for 8 min and cell viability determined by the MTT assay after 24 h (Fig. 7A). There was no significant difference between cells treated with test dsRNA (black bar) and those treated with unrelated control dsRNA (white bar). However, cells previously silenced for GSK3β showed a significant increase (17%) in cell viability (dashed bars). Chemical inhibition of GSK3 by alsterpaullone treatment prior to UV light exposure also protected BME26 viability (Fig. 7B) in a dose-dependent manner. Cells treated with 1 μM alsterpaullone were as viable as those not exposed to UV.

Fig. 8 is a schematic representation of the proposed model of the conserved role of the AKT/GSK3 axis in cell viability and glycogen metabolism in R. microplus tick embryo cell line BME26.

3.5. RmAKT is transcribed during tick embryogenesis

In tick embryos of different ages, the AKT gene was found to be expressed at the highest level in day 1 embryos, at significantly lesser amounts in day 3 embryos, and thereafter remained unaltered until day 18 (Fig. 9).

4. Discussion

Here, we describe an important signal transduction axis that integrates our previous knowledge about the PI3K/AKT signaling pathway in BME26 cells and the importance of GSK3 in tick embryogenesis [26,30]. The importance of AKT signaling has been demonstrated in a number of organisms, and supports a conserved role in the regulation of cell metabolism [45]. It is strongly associated with some types of human cancer, and acts mainly by increasing the inhibition of pro-apoptotic components and improving energy metabolism [46]. AKT kinase is an important downstream effector of the PI3K pathway that can be activated by growth factors, cytokines and peptides related to the insulin family [11,12]. Remarkably, dysfunctional AKT activity has emerged as a promising target in the development of drugs against some forms of cancer [47].

In D. melanogaster, AKT activity has been shown to be at the core of nutrient sensing mechanisms that incorporate hormonal and growth factors pathways, and processes associated with reproduction [45,48]. More recently, activation of AKT signaling in Anopheles stephensi mosquitoes was shown to reduce malaria parasite infectivity [49].

Moreover, Bombyx mori AKT has been shown to be phosphorylated following stimulation by bombyxin, an endogenous insulin-like peptide, as well as by dietary intake [50].

AKT regulation during glycogen metabolism has largely been deduced from correlating the phosphorylation status of both AKT (active upon phosphorylation at Ser Thr residues) and GSK3 (is inhibited upon phosphorylation at Ser63/21 by AKT) [19,31,51]. In the present study, we show that glycogen distribution is tilted in opposite directions when either AKT or GSK3β is genetically silenced (Fig. 5). Our data also strongly suggest that GSK3 is a downstream target of AKT regulation during glycogen metabolism in tick cells (Fig. 8). Chemical inhibition of either AKT or GSK3 (Fig. 2) promoted opposing effects in glycogen content, which reinforces our observations. Interestingly, even though cells treated with low concentrations of alsterpaullone exhibited an increase in glycogen content, cell viability was not altered within the range of 4 and 400 nM (Figs. 1B and 2B). On the other hand, a
decrease in glycogen content was observed following AKT inhibition at concentrations below the IC50 determined (Figs. 1A and 2A). These data support a prevalent role of AKT in the regulation of BME26 cell survival.

Besides the suggested roles of AKT/GSK3 pathway in cell metabolism and survival, the data presented here also support its participation in vivo during R. microplus embryogenesis (Fig. 9). In such context, tick embryogenesis emerges as a tempting target in identifying novel pathways whose inhibition would have significant impact on tick physiology [52,53]. Studies using neuronal cell lines depict a differential modulation of AKT/GSK3β pathway in the regulation of apoptotic and cytoprotective signaling responses [54]. Similarly, in the current study, AKT knockdown resulted in reduced viability of BME26 cells (Fig. 6A) and disturbed cell permeability (Fig. 6B and C), which strongly suggests that an apoptotic pathway may also occur in BME26 cells. However, the type of cell death promoted by AKT knockdown in BME26 cells was not the focus of the present work. Previous studies reported programmed cell death in adult Rhipicephalus sanguineus [55,56], R. microplus [57] and Dermacentor variabilis [58] ticks. The observations that both AKT (Fig. 9) and GSK3β [29] are expressed during tick embryogenesis do not rule out the possibility that AKT/GSK3β signaling exerts similar importance in vivo, and at other life stages.

The results presented here further suggest that GSK3β is also involved in cell survival in addition to glycogen metabolism in BME26 cell line. UV light exposure is thought to induce cell death mainly through the deleterious effects on DNA, which then compromise some protein functions [59]. GSK3β knocked-down BME26 cells challenged by UV exposure exhibited extended longevity as determined by MMT assay, in comparison with controls (Fig. 7A). Moreover, GSK3β inhibition prior to UV exposure promoted a protective effect for cell viability (Fig. 7B). These results support the importance and functional conservation of GSK3β activity for BME26 cell line. In vitro studies in mammal cell culture demonstrate that GSK3β inhibition attenuates or fully abrogates apoptosis [60]. Taken together with findings from other studies, these observations place GSK3β as a promoter of apoptosis due to inhibition of prosurvival transcription factors and facilitation of proapoptotic transcription factors [61]. Such protective effect of deleting GSK3β is also focus of developing novel radioprotectors. The use of GSK3β inhibitors in animals undergoing radiotherapy were shown to protect normal tissues from radiation-induced apoptosis and attenuate neurocognitive dysfunction resulting from cranial irradiation [62].

Conversely, overexpression of wild-type GSK3β induces apoptosis in various cell types in culture, and specific inhibition of GSK3β ameliorates apoptosis [63,64]. GSK3 inhibition was shown to inhibit apoptotic cell death in neuroblastoma cell line SH-SY5Y upon treatment with fipronil [65]. More recently, GSK3β inhibitors have also been used in pharmacologic prophylaxis to protect normal tissues during radiotherapy in cancer patients, due to its key role in regulation of radiation-induced apoptosis [62]. Previous observations of GSK3β role during tick and mosquito embryogenesis support a strict control in respect to glycogen metabolism and mobilization [3,29]. Additionally, GSK3β activity was recently shown to greatly influence tick oviposition and normal embryo development [30].

Targeting ISP components has been proposed for Giardia intestinalis by selective inhibition of PI3K [66]. In insects, ISP regulates growth, nutritional status and reproduction. Inhibition of PI3K mimics the effects of starvation and reduction of Insulin Receptor signaling reduces

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**Fig. 4.** Confirmation of RNAi mediated gene silencing by RT-qPCR in BME26 cells. BME26 cells were treated with dsRNA for AKT (A) or GSK3 (B) genes independently, and relative transcription was determined by RT-qPCR. AKT silencing was confirmed 24 h after dsRNA treatment. GSK3 silencing was confirmed at the end of the prolonged exposure assay. The plots represent mean±SE from three independent experiments. *= p<0.05, with respect to cells treated with unrelated dsRNA (dsCN), and using EltRa (A) or 40S (B) as reference genes to determine relative transcription using the Pfaffl (2001) method.

**Fig. 5.** Glycogen content is differently affected by AKT and GSK3 gene silencing. BME26 cells were treated with dsRNA for AKT (A) or GSK3 (B) genes independently, and glycogen content was enzymatically determined (see Methodology) by the end of the prolonged exposure assay. Glycogen content was decreased upon AKT silencing but increased by GSK3 silencing. The plots represent mean±SD from three independent experiments in triplicates. *= p<0.05.
fertility [10]. In vitro treatment of adult Schistosoma japonicum with specific inhibitors for or antiserum against ligand binding domains of Insulin Receptor blocks glucose uptake [67]. These observations suggested that designed inhibitors capable of blocking the binding site of Insulin receptor or inhibiting downstream signals could starve the worms. The growing reports on the insulin signaling pathway present a new opportunity on the emerging need for expertise in arachno-entomology [68].

Characterizing gene functions by RNAi silencing has become an essential procedure for tick biologists [69]. RNAi using dsRNA was successfully performed on BME26 cells with a transcription reduction over 70%, being detected 24 h post-exposure (Fig. 4). Past reports on gene silencing in BME26 cells focused on the mechanisms underlying RNAi pathway in ticks [70] and to determine genes that are differentially expressed in response to infection with Anaplasma marginale [8]. Moreover, the majority of studies that have reported using RNAi in tick cell lines have focused on tick-borne pathogen pathology and the function of genes either in vector-pathogen relationships or as candidate targets for anti-tick control methods [71]. In the present work, we extend the application of RNAi in tick cell lines to examine the AKT/GSK3β axis. Our data suggest that such a pathway is conserved in ticks and tick cell lines in a way that integrates both glycogen metabolism and cell survival (Fig. 8). Both processes are essential for proper embryo development, and cannot be ignored in adult life stages of ticks. Further studies are necessary to better understand the mechanisms involved on the regulation of cell survival in ticks. The correlation between signal transduction and metabolic pathways emerges as an attractive area for tick cell line research, and may be extrapolated to other tick life stages.

Although mammalian and arthropod AKT proteins have similar biochemical properties, sequence and phylogenic analyses coupled
with RmAKT silencing indicate that these enzymes differ significantly (Fig. 3). Therefore, it could be possible to exploit these differences in the design of new inhibitors that selectively impair the arthropod vector while causing minimal effects on the mammalian host, a prerequisite for commercials acaricides. With existing acaricidal products rapidly losing their efficacy, there is urgent need to explore alternative control methods against ticks. The discovery and characterization of physiological pathways could lead to novel drug targets, candidate drugs or antigens while at the same time reveal new processes that are essential to ticks’ complex physiological adaptations.

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Fig. 8. Schematic model showing the conserved role of the AKT-GSK3 axis on cell viability and glycogen metabolism in BME26 cells. BME26 cells were treated as indicated by the left side of the white boxes in order to block the activity of either AKT or GSK3. The effects on cell viability and glycogen content are indicated on the right side of the white boxes. •: decrease, blunt arrow head: direct inhibition, dashed arrow: multi step signal transduction. ★: GSK3 knocked down cells exposed to U.V., PI3K: phosphatidylinositol 3-OH kinase.

Fig. 9. RmAKT expression during embryo development. Relative transcription of RmAKT was determined by RT-qPCR using cDNA obtained from eggs collected on the days indicated (see details on Methodology). Elf1A was used as reference gene. Plot represents mean±SE from three experiments in triplicates. *** = p<0.001, compared to first day after oviposition.

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