Integrated Metabolomics, Transcriptomics and Proteomics Identifies Metabolic Pathways Affected by Anaplasma phagocytophilum Infection in Tick Cells

Margarita Villar†‡§§, Nieves Ayllo´n‡, Pilar Alberdi‡, Andrés Moreno§, María Moreno§, Raquel Tobes¶, Lourdes Mateos-Hernández‡, Sabine Weisheit||**, Lesley Bell-Sakyi||**, and José de la Fuente‡‡‡§§

Anaplasma phagocytophilum is an emerging zoonotic pathogen that causes human granulocytic anaplasmosis. These intracellular bacteria establish infection by affecting cell function in both the vertebrate host and the tick vector, Ixodes scapularis. Previous studies have characterized the tick transcriptome and proteome in response to A. phagocytophilum infection. However, in the post-genomic era, the integration of omics datasets through a systems biology approach allows network-based analyses to describe the complexity and functionality of biological systems such as host–pathogen interactions and the discovery of new targets for prevention and control of infectious diseases. This study reports the first systems biology integration of metabolomics, transcriptomics, and proteomics data to characterize essential metabolic pathways involved in the tick response to A. phagocytophilum infection. The ISE6 tick cells used in this study constitute a model for hemocytes involved in pathogen infection and immune response. The results showed that infection affected protein processing in endoplasmic reticulum and glucose metabolic pathways in tick cells. These results supported tick–Anaplasma co-evolution by providing new evidence of how tick cells limit pathogen infection, while the pathogen benefits from the tick cell response to establish infection. Additionally, ticks benefit from A. phagocytophilum infection by increasing survival while pathogens guarantee transmission. The results suggested that A. phagocytophilum induces protein misfolding to limit the tick cell response and facilitate infection but requires protein degradation to prevent ER stress and cell apoptosis to survive in infected cells. Additionally, A. phagocytophilum may benefit from the tick cell’s ability to limit bacterial infection through PEPCK inhibition leading to decreased glucose metabolism, which also results in the inhibition of cell apoptosis that increases infection of tick cells. These results support the use of this experimental approach to systematically identify cell pathways and molecular mechanisms involved in tick–pathogen interactions. Data are available via ProteomeXchange with identifier PXD002181. Molecular & Cellular Proteomics 14: 10.1074/mcp.M115.051938, 3154–3172, 2015.

A. phagocytophilum (Rickettsiales: Anaplasmataceae) is the causative agent of human granulocytic anaplasmosis, equine and canine granulocytic anaplasmosis and tick-borne fever of ruminants (1). A. phagocytophilum has been reported to be the most common tick-borne pathogens in Europe and the United States where it is vectored by I. ricinus, I. scapularis, and I. pacificus (2, 3). The wide host range of A. phagocytophilum and the extensive distribution of tick vector populations will likely result in establishment of reservoir hosts, followed by the continued emergence of enzootic human granulocytic anaplasmosis in several regions of the world. In addition, tick vector populations are expanding due to changes in climate and human interventions that impact reservoir host movement and human contact with infected ticks (4, 5). All these factors increase the risk of acquiring A. phagocytophilum infection, and thus this tick-borne pathogen is likely to be a growing concern for human and animal health.

The I. scapularis genome is the only tick genome sequenced and assembled (GenBank accession ABJB 010000000) and constitutes a valuable resource for the study
of tick biology and tick–pathogen interactions (6, 7). Postgenomic experimental approaches such as transcriptomics and proteomics have increased our understanding of tick–pathogen interactions. Recent research by our group has focused on the characterization of the vector competency of ticks for *A. phagocytophilum* (8). Previous results demonstrated that tick vector competency involves molecular interactions that ensure that *A. phagocytophilum* bacteria infect, develop, and are transmitted by ticks (1, 8–14). Two studies have characterized the tick transcriptome and proteome in response to *A. phagocytophilum* infection (8, 10) and tick proteins have been identified that mediate *A. phagocytophilum* infection, multiplication, and transmission (1, 8, 9, 11–14). Metabolomics is a postgenomic research field concerned with developing methods for analysis of low molecular weight compounds in biological systems such as cells, organs, and organisms. Metabolomics has been used for the study of infectious diseases (15, 16), but data are not available for ticks. Only a few studies have been published on selected metabolic pathways in tick-borne pathogens grown in culture (17). Rather than focusing on single omics studies, the integration of omics datasets through a systems biology approach allows network-based analyses to describe the complexity and functionality of biological systems such as host–pathogen interactions (17, 18) and the discovery of new targets for prevention and control of infectious diseases (19).

Our objective was that the integration of metabolomics, transcriptomics, and proteomics data to expand the understanding of tick–*Anaplasma* interactions with the discovery of tick metabolic pathways playing a critical role at the tick–pathogen interface. To address this objective, a systems biology approach was developed to integrate metabolomics, transcriptomics, and proteomics data collected from uninfected and *A. phagocytophilum*-infected *I. scapularis* ISE6 cells, which constitute a model for hemocytes involved in pathogen infection and immune response (20). The results showed that infection with *A. phagocytophilum* affected protein processing in endoplasmic reticulum (ER) and glucose metabolic pathways in tick cells and suggested new coevolved mechanisms involved in pathogen infection and the tick cell response to infection.

**EXPERIMENTAL PROCEDURES**

*I. scapularis* Tick Cells and Sample Preparation—The *I. scapularis* embryo-derived tick cell line ISE6, provided by Ulrike Munderloh, University of Minnesota, was cultured in L-15B300 medium as described previously (20), except that the osmotic pressure was lowered by the addition of one-fourth sterile water by volume. The ISE6 cells were first inoculated with *A. phagocytophilum* (human NY18 isolate)-infected HL-60 cells (21) and maintained according to Munderloh et al. (22) until infection was established and routinely passaged. Infected ISE6 cells were frozen in liquid nitrogen and served as inoculum for uninfected cells. Uninfected and infected cultures (n = 3 independent cultures with ~10^7 cells each) were sampled at 7 days postinfection (percentage infected cells 71–77% (Ave 1 ± SD, 74 ± 3). The percentage of cells infected with *A. phagocytophilum* was calculated by examining at least 200 cells using a 100x oil immersion objective. The cells were centrifuged at 10,000 g for 3 min, and cell pellets were lyophilized for H NMR spectroscopy used in the present study allows a rapid quantitative analysis of complex matrices (27). Furthermore, the 13C nucleus sweeps a wider range of chemical shifts, therefore improving resolution and facilitating assignments and quantitative analysis. For all NMR spectra, the lyophilized tick cell samples were dissolved in 500 μl deuterium oxide and transferred to 5 mm NMR tubes. All proton and carbon NMR spectra were recorded on a Varian Inova 500 MHz spectrometer in deuterium oxide at 278 °K and at 499.769 MHz and 125.678 MHz for H and 13C NMR, respectively. Chemical shifts were referenced to trimethylsilyl propionate. 1H NMR experiments were recorded with the spectral width of 7,000 Hz, 90° pulse and 256 scans, the relaxation delay was 25 s, and the number of data points was 30,256. The 13C NMR data were acquired in order to identify the main compounds using the following conditions: spectral width 20,000 Hz, 90° pulse, 256 scans, the relaxation delay was 25 s, and the number of data points were 81,726; inverse gated decoupling sequence was applied for proton decoupling during acquisition. Assignment of spectra was carried out using NOESY-1D, g-COSY and Total Correlation Spectroscopy. The spectra were acquired employing the inverse gated decoupling pulse sequence in order to suppress nuclear overhauser effects. The following parameters were used: spectral width, acquisition time 0.64 s, and 160 scans. In all cases, probe temperatures were adjusted at 25 °C. The pulse programs were taken from the standard Varian pulse sequence library. All spectra were Fourier-transformed with MestreNova 8.1 software (MestreLab Research S.L., Santiago de Compostela, Spain) and ACD/Specmannger 7.00 software (Advanced Chemistry, Inc., Toronto, ON, Canada). Apodization of 0.2 Hz and zero filling (32,000) were used prior to Fourier transformation. The spectra were phased.
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Proteomics Data Collection and Analysis—The protein extracts (150 μg) from control and infected tick cells were precipitated following the methanol/chloroform procedure (38), resuspended in 100 μl Laemmli sample buffer, and loaded into 1.2-cm wide wells of a conventional SDS-PAGE gel (0.5 mm-thick, 4% stacking, 10% resolving). The electrophoretic run was stopped as soon as the front entered 3 mm into the resolving gel so that the whole proteome became concentrated in the stacking/resolving gel interface. The unseparated protein band was visualized by staining with GelCode Blue Stain Reagent (Thermo Scientific, San Jose, CA), excised, cut into cubes (2 × 2 mm), and submitted to water and acetoneitrile washes prior to in-gel reduction with 10 mM dithiothreitol for 1 h. The gel pieces were then acetoneitrile washed and treated for 1 h in the dark with 50 mM iodoacetamide. Protein samples were digested overnight at 37 °C with 60 ng/μl sequencing grade trypsin (Promega, Madison, WI) at a 5:1 protein:trypsin (w/w) ratio in 50 mM ammonium bicarbonate, pH 8.8, containing 10% (v/v) acetoneitrile. The resulting trypic peptides from each proteome were extracted by incubation (30 min) in 12 mM ammonium bicarbonate, pH 8.8. Trifluoroacetic acid was added to a final concentration of 1% and the peptides were finally desalted onto OmniPipette tips C18 (Agilent Technologies), dried down, and stored at −20 °C until reverse-phase high-performance liquid chromatography (RP-HPLC)-(Linear Ion Trap) analysis. The desalted protein digests were resuspended in 0.1% formic acid and analyzed by liquid chromatography-tandem MS (LC-MS/MS) using an Easy-nLC II system coupled to an linear ion trap mass spectrometer model LTQ (Thermo Scientific). The peptides were concentrated (on-line) by reverse phase chromatography using a 0.1 × 20 mm C18 RP precolumn (Thermo Scientific) and then separated using a 0.075 × 100 mm C18 RP column (Thermo Scientific) operating at 300 nl/min. Peptides were eluted using a 180-min gradient from 5 to 40% solvent B in solvent A (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile). (Electrospray ionization) ionization was done using a fused-silica PicoTip Emitter ID 10 mm (New Objective, Woburn, MA) interface. Peptides were detected in survey scans from 400 to 1,600 amu (1 μscans), followed by 15 data-dependent MS/MS scans (Top 15), using an isolation width of 2 mass-to-charge ratio units, normalized collision energy of 35%, and dynamic exclusion applied during 30 s periods.

The MS/MS raw files generated with Xcalibur (version 2.1, Thermo Scientific) were searched against a compiled database containing all sequences from Ixodidea (77,195 Uniprot entries in March 2015) and Anaplasmataceae (64,677 Uniprot entries in March 2015) supplemented with the sequence of porcine trypsin. Searches for MS/MS fragment ions, and the searches were performed allowing coverer 1.4, Thermo Scientific). The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 1 Da for precursor ions and 0.8 Da for MS/MS fragment ions, and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation. Searches were also performed against a decoy database in an integrated decoy approach. A false discovery rate (FDR) < 0.05 was considered as condition for successful peptide assignments and at least two peptides per protein were the necessary condition for protein identification. Three biological replicates were used for each of uninfected and infected tick cells, and genes differentially expressed in response to A. phagocytophilum infection were selected with p ≤ 0.05. Gene ontology (GO) analysis for biological process (BP) was done with Blast2GO software (version 3.0; www.blast2go.com) (37). The RNAseq data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE68881 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE68881). Supplemental Table III contains RNAseq statistics with read information for replicates 1 and 2 in analyzed samples. Supplemental Table III contains all transcripts identified in analyzed samples and data quantitation.

and baseline corrected by the Whitaker smoother. 1H NMR spectra were integrated manually, choosing the area of each peak of interest. The integrals were normalized in order to carry out relative quantification of the interesting compounds. 1H spectral resonances peak assignments were based on published data (28–31; Human Metabolome Database version 3.0, http://www.hmdb.ca) and Chenomx Profiler software (Chenomx NMR Suite, Edmonton, Canada). Assignments were confirmed through the addition of pure standard compounds (Sigma). Three biological replicates were used for each of uninfected and infected tick cells, and results were compared between uninfected and infected tick cells by Student’s t test for unequal variance (p = 0.05). Supplemental Table II contains all 1H NMR spectra (parts per million; ppm) for all identified metabolites in analyzed samples and data quantitation. Transcriptomics Data Collection and Analysis—Purified RNAs were used for library preparation using the TruSeq-based NEB-Next Ultra Directional RNA Library Prep kit (New England Biolabs, Ipswick, MA). Briefly, 1 μg total RNA was used as starting material for library preparation. Messenger RNA was captured using oligo-dT magnetic beads (Poly(A) RNA Magnetic Isolation Module; New England Biolabs) and purified poly(A) + RNA was chemically fragmented and reverse-transcribed. A controlled fragmentation period was applied to obtain an insert size of ~320 nucleotides, best suited to generate nonoverlapping reads after pair-end sequencing. The second strand was generated and double-stranded DNA was purified using AMPure SPRI-based magnetic beads (Beckman Coulter, IZASA, Spain). Next, samples were end-repaired followed by adaptor ligation and removal of excess oligonucleotides by double AMPure selection, made according to NEB-Next recommendations as a function of library size. Adapter oligonucleotides contained the signals for subsequent amplification and sequencing as well as sample-specific identifiers, which allowed multiplexing in the sequencing run. An enrichment procedure based on PCR was then performed to ensure that all molecules in the library contained the adapters at both ends. The number of PCR cycles was adjusted to 13 for all four samples. The final amplified library was checked again on a BioAnalyzer 2100 (Agilent, Santa Clara, CA), pooled, quantified by fluorometric methods (PicoGreen®, and titrated by real-time PCR using a well-controlled library as standard. For RNAseq, libraries were denatured and seeded on the surface of a Pair-End Flowcell, where clusters were generated and sequenced using a HiSeq2000 equipment (Illumina, San Diego, CA), under a 2× 100 bp recipe. Libraries were diluted to obtain 50.7 (Uninfected 1) 52.8 (Uninfected 2) 57.2 (Infected 1) and 57.6 (Infected 2) million pair-end reads per sample. The 1×100 bp pair-end reads were mapped to the l. scapularis reference genome sequence (assembly JCVI-ISG_33_1.0; http://www.ncbi.nlm.nih.gov/nucleotide/NZ_ABJR00000000). Reads were mapped to the reference genome using the scaffolds and GFF (General Feature Format) files (https://www.vectorbase.org/) with the tool Bowtie (32) integrated in the TopHat suite (33). The pipeline used for bioinformatics analysis of RNAseq data was similar to that described by Trapnell et al. (34) using a TopHat-Cufflinks-Cuffmerge-Cuffquant-Cuffdiff pipeline (35, 36) (Supplemental Fig. 1). Two biological replicates were used for each of uninfected and infected tick cells, and genes differentially expressed in response to A. phagocytophilum infection were selected with p ≤ 0.05. Gene ontology (GO) analysis for biological process (BP) was done with Blast2GO software (version 3.0; www.blast2go.com) (37). The RNAseq data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE68881 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE68881). Supplemental Table III contains RNAseq statistics with read information for replicates 1 and 2 in analyzed samples. Supplemental Table III contains all transcripts identified in analyzed samples and data quantitation.
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Discard proteins with high variation between replicates. The mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium (http://proteomexchange.org) via the PRoteomXchange Identifications (PRIDE) repository with the dataset identifier PXD002181 and doi: 10.6019/PXD002181. Supp. Table V contains all tick proteins identified with FDR < 0.05 and at least two peptides per protein in at least one of the analyzed samples and data quantitation. Supplementary Table VI contains all tick peptide sequences assigned with FDR < 0.05 and at least two PSMs per peptide in at least one of the analyzed samples. Supp. Table VII contains all *Anaplasma* proteins identified in infected tick cells with FDR < 0.05 and at least two peptides per protein in at least one of the analyzed samples.

**Systems Biology Analysis—**A systems biology pipeline was implemented for this study (Supplemental Fig. 2). The Uniprot (http://www.uniprot.org) and Biosystems (http://www.ncbi.nlm.nih.gov/biosystems/; ref. 39) databases were searched for *I. scapularis* and *A. phagocytophilum* genes/proteins with functional annotations that could be related to the metabolites affected in response to infection of tick cells. Then the genes/proteins that were selected as related to these metabolites in these databases (Uniprot and Biosystems) were searched in the set of genes/proteins differentially expressed/represented in response to *A. phagocytophilum* infection and only those found in this set were used for further characterization. The proteins with hits to ticks other than *I. scapularis* were blasted to select the closest ortholog in *I. scapularis*. Finally, these genes/proteins were searched for GO and pathway annotations in VectorBase (www.vectorbase.org) to identify putative pathways affected by *A. phagocytophilum* infection of tick cells.

**RNA Interference (RNAi) for Gene Knockdown in Tick Cells—**Oligonucleotide primers homologous to *I. scapularis* shSF gene containing T7 promoters (Supplemental Table I) were used for *in vitro* transcription and synthesis of dsRNA as described previously (12), using the Access RT-PCR system (Promega) and the Megascript RNAi kit (Ambion, Austin, TX). The unrelated Rs86 dsRNA was synthesized using the same methods described previously and used as negative control (12). The dsRNA was purified and quantified by spectrophotometry. RNAi experiments were conducted in cell cultures by incubating ISE6 tick cells with 10 μl dsRNA (5 × 10^11–5 × 10^13 molecules/μl) and 90 μl 15%BS medium in 24-well plates using five wells per treatment (12). Control cells were incubated with the unrelated Rs86 dsRNA. After 48 h of dsRNA exposure, tick cells were infected with cell-free *A. phagocytophilum* NY18 obtained from −5 to −10^6 infected HL60 cells (90–100% infected cells) (48) and resuspended in 24 ml culture medium to use 1 ml/well or mock infected by adding the same volume of culture medium alone. Cells were incubated for an additional 72 h, harvested, and used for Annexin V–FITC staining to detect cell apoptosis, DNA, and RNA extraction. RNA was used to analyze gene knockdown by real-time RT-PCR with respect to Rs86 control. Control RNA was used to quantify the *A. phagocytophilum* infection levels by major surface protein 4 gene (msp4) PCR.

**Pharmacological Studies in Cultured Tick Cells—**ISE6 tick cells infected with *A. phagocytophilum* were left untreated or treated for 48 h with 100 μg/ml methyl methanesulfonate (Sigma) to stimulate M KK (41), 10 μM nicotinic acid (Sigma) to stimulate PEPCK (42) or PEPCK inhibitors, 100 μg/ml cycloheximide (Sigma) and 1 μg/ml actinomycin D (Sigma) (42). Cells were harvested and used for Annexin V–FITC staining to detect cell apoptosis and for DNA extraction. DNA was used to quantify the *A. phagocytophilum* infection levels by msp4 PCR. All treatments were done in triplicate.

**Annexin V–FITC Staining to Detect Cell Apoptosis after Experimental Infection with *A. phagocytophilum*—**Approximately 5 × 10^5–1 × 10^6 uninfected and *A. phagocytophilum*-infected ISE6 tick cells were collected after different treatments. Apoptosis was measured by flow cytometry using the Annexin V–fluorescein isothiocyanate (FITC) apoptosis detection kit (Immunostep, Salamanca, Spain) following the manufacturer’s protocols. The technique detects changes in phospholipid symmetry analyzed by measuring Annexin V (labeled with FITC) binding to phosphatidylserine, which is exposed in the external surface of the cell membrane in apoptotic cells. Cells were stained simultaneously with the nonvital dye propidium iodide, allowing the discrimination of intact cells (Annexin V–FITC negative, propidium iodide negative) and early apoptotic cells (Annexin V–FITC positive, propidium iodide negative). All samples were analyzed on a FAC Scallibur flow cytometer equipped with CellQuest Pro software (BD Bio-Sciences, Madrid, Spain). The viable cell population was gated according to forward-scatter and side-scatter parameters. The percentage of apoptotic cells was determined by flow cytometry after Annexin V–FITC and propidium iodide labeling and compared between both treated and untreated infected and uninfected cells by Student’s *t* test with unequal variance (*p* = 0.05). All treatments were conducted in triplicate.

**Determination of *A. phagocytophilum* Infection by Real-Time PCR—** *A. phagocytophilum* DNA levels were characterized by msp4 real-time PCR normalizing against tick 16S rDNA as described previously (12). Normalized Ct values were compared between untreated and treated cells by Student’s *t* test with unequal variance (*p* = 0.05; *n* = 3).

**Determination of TmRNA levels by Real-Time RT-PCR—** The expression of selected genes was characterized using total RNA extracted from infected and uninfected ISE6 tick cells. Real-time RT-PCR was performed on RNA samples using gene-specific oligonucleotide primers (Supplemental Table I) and the iScript One-Step RT-PCR Kit with SYBR Green and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicons denatured consistently in the same temperature range for every sample. The mRNA levels were normalized against tick 16S RNA and cyclophilin using the genNorm method (Delta-Delta-Ct (ddCt) method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0) as described previously (12). Normalized Ct values were compared between test dsRNA-treated tick cells and controls treated with Rs86 dsRNA or between infected and uninfected tick cells by Student’s *t* test with unequal variance (*p* = 0.05; *n* = 3–5).

**Western Blot Analysis—** Antibodies against MKK, Hsp70 and Hsp90 (Sigma) and ISE6 tick cell protein extract were used for Western blot analysis. Total proteins from infected and uninfected ISE6 tick cells (50 μg from each sample) were methanol/chloroform precipitated, resuspended in Laemmli sample buffer, and separated on a 12% SDS-PAGE gel under reducing conditions. After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad), blocked with SuperBlock blocking buffer in TBS and immunoreactive proteins were detected with 3,3′, 5,5′-tetramethylbenzidine stabilized substrate for HRP (Promega) for 10 min, quantified using the ImageQuant TL 7.0 software (GE Healthcare, Madrid, Spain) and normalized against ISE6 tick cell protein extract. Normalized protein levels were compared between samples by *χ*^2^ test (*p* = 0.05; *n* = 2).

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RESULTS

Metabolomics of the Tick Cell Response to A. phagocytophilum Infection—In the 1H NMR analysis, all the metabolite spectra were similar between uninfected and infected tick cells (Fig. 1A) but differed in metabolite concentration ratios (Fig. 1B). Although the 1H NMR spectrum was derived from a complex mixture of different metabolites and most of the signals overlapped, the identification of some metabolites by bidimensional NMR experiments was possible. Chemical shift values were based on those reported in the literature and public databases (see Experimental Procedures). Furthermore, in some cases the addition of reference compounds was performed to ensure peak assignments. As illustrated in the 1H NMR spectrum, free amino acids such as proline, serine, glutamate, and lysine were the most abundant metabolites identified (Fig. 1A). However, high levels of ethanol, lactate betaine, and its derivatives (dimethylamine) were also identified (Fig. 1A).

The 1H NMR spectra were integrated manually choosing the area of each peak of interest. Due to the coprecipitation effect by the addition of some compounds such as trimethylsilyl propionate, no internal standard was added to the mixture but a small amount of trimethylsilyl propionate was added to generate reference spectra. Therefore, all integrals were normalized and relative metabolite concentrations were calculated. In infected tick cells, significant changes in the concentration of different metabolites were observed when compared with uninfected cells ($p < .05$; Table I and Supplemental Table II). The metabolites identified in tick cells belonged to amino acid, glucose, nucleic acid, and methane metabolic pathways.
pathways (Fig. 2). However, 88% of the metabolites identified were in the amino acid and glucose metabolic pathways, suggesting that these pathways are important in the tick cell response to *A. phagocytophilum* infection, and they were therefore selected for further characterization (Fig. 2). Infected tick cells had higher or lower proportion of some amino acids while other did not change in response to infection (Fig. 2 and Table I). The levels of the metabolites in the glucose metabolic pathway were lower in infected than uninfected cells (Fig. 2 and Table I).

**Fig. 2.** Metabolic pathways and metabolites affected in response to *A. phagocytophilum* infection of tick cells. Metabolites and corresponding metabolic pathways identified in tick cells are shown for metabolites increased, decreased or with no difference in infected tick cells when compared with uninfected cells.

### Table I

| Metabolite                                | Uninfected tick cells (ppm; Ave ± SD) | Infected tick cells (ppm; Ave ± SD) | Infected/uninfected ratio |
|-------------------------------------------|---------------------------------------|--------------------------------------|---------------------------|
| Betaine                                   | 1.45 ± 0.19                           | 2.52 ± 0.09                         | 1.74<sup>a</sup>          |
| Lysine                                    | 7.09 ± 0.90                           | 9.45 ± 0.40                         | 1.33<sup>b</sup>          |
| Phenylalanine                             | 0.51 ± 0.10                           | 1.11 ± 0.12                         | 2.20<sup>b</sup>          |
| Proline                                   | 8.03 ± 1.12                           | 12.96 ± 0.98                        | 1.61<sup>a</sup>          |
| Serine                                    | 16.49 ± 0.92                          | 22.45 ± 1.26                        | 1.36<sup>a</sup>          |
| Tyrosine                                  | 1.16 ± 0.08                           | 1.44 ± 0.15                         | 1.24<sup>b</sup>          |
| Uridine                                   | 0.14 ± 0.07                           | 0.30 ± 0.06                         | 2.14<sup>b</sup>          |
| Acetone                                   | 1.94 ± 0.25                           | 1.19 ± 0.19                         | 0.61<sup>b</sup>          |
| Alanine                                   | 7.02 ± 0.53                           | 3.27 ± 0.48                         | 0.47<sup>b</sup>          |
| Adenosine monophosphate (AMP)             | 0.53 ± 0.02                           | 0.45 ± 0.04                         | 0.86<sup>a</sup>          |
| Dimethylamine                             | 1.27 ± 0.16                           | 0.74 ± 0.07                         | 0.58<sup>b</sup>          |
| Ethanol                                   | 10.93 ± 0.39                          | 7.74 ± 0.46                         | 0.71<sup>c</sup>          |
| Glucose                                   | 0.51 ± 0.06                           | 0.31 ± 0.05                         | 0.59<sup>b</sup>          |
| Glutamate                                 | 13.02 ± 0.43                          | 11.37 ± 0.56                        | 0.87<sup>b</sup>          |
| Glutamine                                 | 4.99 ± 0.95                           | 3.92 ± 0.33                         | 0.79<sup>b</sup>          |
| Glycine                                   | 6.41 ± 0.75                           | 5.06 ± 0.28                         | 0.79<sup>b</sup>          |
| Lactate                                   | 6.63 ± 0.50                           | 5.38 ± 0.25                         | 0.81<sup>c</sup>          |
| Methanol                                  | 2.95 ± 0.52                           | 1.68 ± 0.14                         | 0.57<sup>b</sup>          |
| Methionine                                | 1.36 ± 0.52                           | 0.48 ± 0.07                         | 0.35<sup>b</sup>          |
| Succinate                                 | 0.62 ± 0.22                           | 0.43 ± 0.08                         | 0.70<sup>b</sup>          |
| Cholines                                  | 0.69 ± 0.22                           | 0.74 ± 0.06                         | 1.07<sup>b</sup>          |
| Isoleucine                                | 1.70 ± 0.27                           | 1.60 ± 0.19                         | 0.95<sup>b</sup>          |
| Leucine                                   | 1.69 ± 0.12                           | 1.64 ± 0.15                         | 0.97<sup>b</sup>          |
| Valine                                    | 2.88 ± 0.29                           | 2.66 ± 0.17                         | 0.92<sup>b</sup>          |

All experiments were performed in triplicate and results were compared between uninfected and infected tick cells by Student’s t-test for unequal variance (<sup>a</sup>p < .005; <sup>b</sup>p < .05; <sup>c</sup>p < .0005).
were aligned to the *I. scapularis* reference genome and resulted in 37,990 transcripts (Supplemental Table III). Of these, 2,566 were differentially expressed in response to infection (*p* ≤ 0.05) with 460 up-regulated and 2,106 down-regulated genes (Supplemental Table IV). In accordance with metabolomics results, the GO analysis showed that metabolic and cellular processes were the biological processes (BP) most affected by *A. phagocytophilum* infection of tick cells (Figs. 3A and 3B). Possibly due to the higher number of down-regulated genes, more BP were represented in this category (Fig. 3B) when compared with up-regulated genes (Fig. 3A). However, a similar distribution was observed for BP represented in both up- and down-regulated genes (Figs. 3A and 3B).

Proteomics of the Tick Cell Response to *A. phagocytophilum* Infection—After MS analysis, a total of 14,234 tick proteins were identified (Supplemental Table V). Of these, 284 proteins were differentially represented in response to infection (*p* ≤ 0.05) with 129 overrepresented and 155 underrepresented (Supplemental Table V). Not surprisingly, the GO analysis showed a profile of BP very similar to that obtained for the transcriptome and in agreement with metabolomics results (Figs. 3A–3D). Metabolic and cellular processes were the BP most affected in infected cells for both overrepresented and underrepresented proteins (Figs. 3C and 3D). As shown for transcriptomics data, a similar distribution was observed for BP represented in both over- and underrepresented proteins (Figs. 3C and 3D).

The response to stimulus BP was represented at the mRNA and protein levels for both up-regulated/overrepresented and down-regulated/underrepresented genes/proteins (Figs. 3A–3D). Furthermore, the number of overrepresented proteins was higher than the number of underrepresented proteins for this BP (Figs. 3C and 3D), suggesting that these proteins may be relevant for tick–pathogen interactions. Among the proteins annotated in the response to stimulus BP, heat shock proteins Hsp70 (E4W3Z2, B7PAR6, B7PEN4) and Hsp90 (L7MEG0, M9WB33) were overrepresented in *A. phagocytophilum*-infected ISE6 tick cells when compared with uninfected cells (Supplemental Table V).

**A. phagocytophilum Proteome in Infected Tick Cells**—A total of 935 *Anaplasma* proteins were identified in infected tick cells (Supplemental Table VII). Of them, the most represented proteins included uncharacterized proteins, ankyrin-related proteins, major surface proteins, and glutamate dehydrogenase (GDG) (Supplemental Table VII) These proteins are involved in host–pathogen interactions and may be relevant for *A. phagocytophilum* infection of tick cells.
Metabolic Pathways Affected by *Anaplasma* in Tick Cells

Systems Biology of the Tick Cell Response to *A. phagocytophilum* infection—A systems biology approach was used to integrate metabolomics, transcriptomics, and proteomics data (Supplemental Fig. 2). The analysis was based on the metabolites identified in tick cells with emphasis on those significantly affected in response to *A. phagocytophilum* infection when compared with uninfected cells (Table I). The terms related to these metabolites were searched in the annotations of Uniprot tick proteins and tick-specific Byosys-tems entries in order to select a set of genes/proteins that could be related to these metabolites in *I. scapularis*. Then the genes/proteins that were selected as related to these metabolites were compared with the differentially expressed/repre-sented genes/proteins in response to *A. phagocytophilum* infection, and those also found in this set were used for further characterization (Table II). Finally, tick genes/proteins were searched for GO and pathway annotations in VectorBase to identify putative pathways affected by *A. phagocytophilum* infection of tick cells with emphasis on the most affected metabolic pathways.

The metabolomics results showed that the most affected metabolic pathway corresponded to amino acids metabolism (Fig. 2). In agreement with this finding, the results showed that the protein processing in ER pathway (http://www.kegg.jp/ pathway/isc04141+._ISCW006944%09orange) was affected in response to *A. phagocytophilum* infection (Fig. 4A). Accumulation of misfolded proteins in the ER causes ER stress (43), and in tick cells a signaling pathway was activated in response to infection, resulting in the attenuation of protein synthesis and activation of amino acid metabolism to recover from ER stress (Fig. 4A). At the same time, the signaling pathway that results in cell apoptosis if ER normal function cannot be recovered (44, 45) was inhibited in infected tick cells (Fig. 4A). Finally, ER protein degradation that results in proteasome proteolysis (46) was also activated in response to rickettsial infection (Fig. 4A). These signaling pathways involved other genes/proteins that were also differentially expressed/represented in infected tick cells (Table II). The attenuation of protein synthesis in infected cells correlated with the down-regulation of *exostosin*-2 and *chordin* genes. The activation of amino acid metabolism in infected cells correlated with the overrepresentation of arginase, underrepresentation of myosin heavy chain and down-regulation of *netrin*-4, *laminin alpha*-1 and leucine-rich transmembrane protein genes. The activation of protein degradation in infected cells also included the down-regulated *F-spondin* gene coding for a serine-type endopeptidase inhibitor and the overrepresented Hsp, Ubiquitin carboxyl-terminal hydrolase, 26S proteasome regulatory complex and syntaxin-18. The apoptosis inhibition also included the underrepresentation in infected tick cells of the ubiquitin protein ligase E3, a protein that has been shown to be required for *A. phagocytophilum* infection in ticks (47). Finally, some of the genes differentially expressed in response to *A. phagocytophilum* infection of tick cells directly correlated with the metabolomics results for certain amino acids (Fig. 4B).

The second metabolic pathway most affected according to metabolomics results corresponded to glucose metabolism (Fig. 2). The results showed that glucose metabolism was affected in different ways, with lower hexokinase, and phosphoenolpyruvate carboxykinase (PEPCK) protein levels in infected tick cells (Fig. 5). Lower protein levels for these enzymes correlated with lower methanol, acetone, glucose, lactate, glutamate, glutamine, and alanine metabolites (Figs. 2 and 5). Additionally, GDG and pyruvate kinase (PK) were identified in the *A. phagocytophilum* proteome (Supplemental Table VII and Fig. 5). These proteins are involved in glucose metabolism (Fig. 5), and GDG was one of the most represented bacterial proteins in infected tick cells (Supplemental Table VII). These results suggested a possible interplay between tick and *A. phagocytophilum* proteins for glucose metabolism in infected tick cells.

**Dynamics and Tissue Specificity of the Effect of *A. phagocytophilum* Infection on Tick Metabolic Pathways**—In this research, we focused on uninfected and *A. phagocytophilum*-infected ISE6 tick cells to identify metabolic pathways affected by infection through systems biology integration of metabolomics, transcriptomics, and proteomics data. However, it has been demonstrated that the tick response to infection is a complex and dynamic process with tissue-specific differences (8). In an attempt to address this issue, a preliminary and complementary analysis of tissue-specific differences in response to infection of *I. scapularis* nymphs and adult female guts and salivary glands was included in the study.

The tick cell line ISE6 was derived from embryonated eggs and contains cells with morphology and behavior similar to hemocytes involved in bacterial infection and immune response to control infection (20, 48–50). Additionally, infection of *I. scapularis* hemocytes by *A. phagocytophilum* is mediated by the P11 tick protein, which is required for successful migration of the pathogen from the midgut to salivary glands (51), showing that pathogen phagocytosis or engulfment by tick hemocytes does not necessarily cause its elimination. The hemocytes are freely circulating cells in the hemolymph, and tick cell lines constitute a good model for the study of tick–pathogen interactions in this fluid that is otherwise difficult to study in vivo (52). The transcript coding for P11 and the related protein B7P9I7 were identified in ISE6 tick cells but did not show differences in mRNA or protein levels between infected and uninfected cells (Supplemental Tables IV and V). Other hemolymph effector proteins associated with tick hemocytes such as defensins, Complement-like molecules and antimicrobial peptides were also identified and, although not differentially represented between infected and uninfected cells (Supplemental Table V), could serve as biomarkers for hemocyte-like function of ISE6 tick cells (51).
### Metabolic Pathways Affected by *Anaplasma* in Tick Cells

#### Table II

| Gene/protein accession numbers | Description (metabolic pathway) | Gene expression | Protein representation |
|-------------------------------|---------------------------------|-----------------|------------------------|
| ISCW000326 B7P147             | Exostosin-2 (synthesis of methionine and cysteine) | –1.4           | NS                     |
| ISCW017029 B7P0C7             | Chordin (inhibitor of bone morphogenic protein (BMP), a growth factor promoting protein synthesis) | –1.3           | NS                     |
| ISCW006658 B7PRM5             | ABC transporter (transport of amino acids within, out or into the cell) | –1.6           | +∞                     |
| ISCW006727 B7PN44             | Netrin-4 (tyrosine catabolism) | –2.4           | NS                     |
| ISCW006728 B7PN45             | Laminin alpha-1, 2 chain (tyrosine catabolism) | –1.8           | NS                     |
| ISCW019298 B7PW4R             | Leucine-rich transmembrane protein (hydrolase activity, protein catabolism) | –1.6           | NS                     |
| ISCW020983 B7P19             | Bone morphogenic protein receptor, type II (receptor signaling for BMP with serine/threonine kinase activity) | –1.6           | NS                     |
| ISCW012675 B7Q0C6             | F-spondin (serine-type endopeptidase inhibitor) | –1.4           | NS                     |
| ISCW014885 B7QH1             | Myotubulin (peptidyl-tyrosine dephosphorylation) | –1.6           | NS                     |
| ISCW023752 B7QH5O             | Ribokinase (α-ribose/glucose metabolism) | –1.6           | NS                     |
| ISCW014242 B7Q069             | Sprouty protein evh1 domain-containing protein (developmental signal-transduction protein with tyrosine kinase substrate that inhibits growth factor-mediated activation of MAP kinase) | –1.5           | NS                     |
| ISCW014078 B7QJY2             | Serine/threonine protein kinase (protein phosphorylation) | –1.7           | ∞                      |
| ISCW016175 B7P258             | Actin (cytoskeleton, cell division) | NS             | –0.5                   |
| ISCW017456 B7Q0R6             | Heat shock protein (stress response, protein folding and unfolding) | NS             | +0.7                   |
| ISCW000190 B7P3D7             | Heat shock protein (Hsp70) (stress response, protein folding and unfolding) | NS             | +0.8                   |
| ISCW024057 B7P4X5             | Heat shock protein 70 (stress response, protein folding and unfolding) | NS             | +0.8                   |
| ISCW014265 B7Q0I1             | Heat shock protein 90 (Hsp90) (stress response, maintenance of steroid receptors and transcription factors) | NS             | +0.9                   |
| ISCW005108 B7P331             | Myosin heavy chain (peptidyl-tyrosine dephosphorylation, tyrosine catabolism) | NS             | –2.3                   |
| ISCW016227 B7POP3             | Calcium-independent phospholipase A2 (lipid metabolism) | NS             | ∞                      |
| ISCW008211 B7PUK7             | Phosphoenolpyruvate carboxykinase (PEPCK) (glucose synthesis) | NS             | ∞                      |
| ISCW023971 B7QNN3             | Low-density lipoprotein (LDL) receptor (endocytosis of LDL) | NS             | –3.1                   |
| ISCW023507 B7QDM7             | Ubiquitin protein ligase E3 mdm2 (protein lysine modification, negative regulation of apoptotic process) | NS             | –3.1                   |
| ISCW001109 B7PP27             | DNA (Cytosine-5)-methyltransferase (DNA methylation) | NS             | –3.1                   |
| ISCW019676 B7PPW8             | Beta-galactosidase (carbohydrate metabolism) | NS             | ∞                      |
| ISCW0013414 B7QSB2             | DEAD box ATP-dependent RNA helicase (RNA metabolism) | NS             | ∞                      |
| ISCW001739 B7P9S9              | Proline and glutamine-rich splicing factor (SFPQ) (spliceosome formation, nucleic acid metabolism) | NS             | ∞                      |
| ISCW015924 B7Q0P8             | Ubiquitin carboxyl-terminal hydrolase (proteolysis) | NS             | ∞                      |
| ISCW013998 B7QX86             | Nuclear pore complex protein nap98 (transport of glucose within, out or into the cell) | NS             | ∞                      |
| ISCW012387 B7Q4S5              | Hexokinase (HK) (glucose metabolism) | NS             | ∞                      |
| ISCW021966 B7Q5X4             | Mitogen-activated protein kinase (protein phosphorylation) | NS             | ∞                      |
### Table II — continued

| Gene/protein accession numbers<sup>a</sup> | Description (metabolic pathway)<sup>b</sup>                                                                 | Gene expression<sup>c</sup> | Protein representation<sup>c</sup> |
|------------------------------------------|---------------------------------------------------------------------------------------------------------------|----------------------------|-----------------------------------|
| ISCW004748 B7P4A0                       | Protein disulfide isomerase (protein folding, breakage of disulfide bonds between cysteine residues)         | NS                         | +4.9                              |
| ISCW016161 B7P4U1                       | Protein disulfide-isomerase (protein folding, breakage of disulfide bonds between cysteine residues)         | NS                         | +4.9                              |
| ISCW020699 B7PXW4                       | Dihydroorotate dehydrogenase (pyrimidine biosynthesis)                                                        | NS                         | −∞                                |
| ISCW017452 B7PAR2                       | Methionyl-tRNA synthetase (charge tRNA with methionine)                                                         | NS                         | −∞                                |
| ISCW001113 B7P346                       | Dystrophin (cytoskeleton, cell division)                                                                       | −1.4                       | −∞                                |
| ISCW010101 B7Q1Y2                       | 6-phosphogluconate dehydrogenase, decarboxylating (nucleotide synthesis)                                      | NS                         | −∞                                |
| ISCW008555 B7Q054                       | Lysophosphatidic acid acyltransferase endophilin (endocytosis, protein and lipid metabolism)                  | NS                         | −∞                                |
| ISCW013122 B7QEL2                       | Glycolate oxidase (oxidoreductase, glucose metabolism)                                                          | NS                         | −∞                                |
| ISCW016252 B7P0R8                       | Helicase (separation of annealed nucleic acids, translation, transcription)                                     | NS                         | +1.9                              |
| ISCW008410 B7PW7                        | E3 ubiquitin ligase (protein ubiquitination)                                                                  | NS                         | +2.1                              |
| ISCW018771 B7PNU5                       | Glutamine synthetase (nitrogen metabolism, catalyses the formation of glutamine from glutamate)                | NS                         | +2.3                              |
| ISCW015924 B7P3R8                       | Ubiquitin carboxyl-terminal hydrolase (proteolysis)                                                             | NS                         | +3.1                              |
| ISCW006944 B7PV74                       | Ataxin (DUB) (proteolysis)                                                                                     | NS                         | +3.2                              |
| ISCW012689 B7QBR7                       | Arginase (arginine, glutamate, glutamine and proline metabolism)                                              | NS                         | −∞                                |
| ISCW005309 B7PLM8                       | Dehydrogenase (oxidoreductase, glucose metabolism)                                                              | NS                         | −∞                                |
| ISCW001480 B7P159                       | Dolichyl-diphosphooligosaccharide protein glycosyltransferase (protein N-linked glycosylation via asparagine) | NS                         | −∞                                |
| ISCW007233 B7PQ45                       | Dihydroxyacetone kinase (glycerol and alcohol metabolism)                                                       | NS                         | −∞                                |
| ISCW013321 B7QDL0                       | Isoleucyl-tRNA synthetase (charge tRNA with isoleucine)                                                         | NS                         | −∞                                |
| ISCW001922 B7PFA1                       | 26S proteasome regulatory complex, ATPase RPT1 (protein degradation)                                             | NS                         | −∞                                |
| ISCW018346 B7PF9                        | Vacular H⁺ ATPase (proton transport)                                                                           | NS                         | −∞                                |
| ISCW009494 B7PY0                        | Beta-1,3-N-acetylglucosaminyltransferase (transfer of glycosyl groups)                                          | NS                         | −∞                                |
| ISCW019106 B7PQ25                       | Syntaxin-18 (protein degradation)                                                                               | NS                         | −∞                                |
| ISCW020443 B7PVU5                       | Phosphoglycerate mutase (glucose catabolism)                                                                    | NS                         | −∞                                |
| ISCW017818 B7PQ0                        | Calponin homology (CH) domain-containing, chdc/lrch (dephosphorylation, molybdate and amino acid metabolism)  | −1.3                       | −∞                                |
| ISCW012339 B7QCE8                      | Acetylcholinesterase (degrades acetylcholine into choline and acetate)                                          | NS                         | −∞                                |
| ISCW000457 B7P3D0                       | Transcription factor E2F4 (transcription)                                                                      | NS                         | −∞                                |
| ISCW007452 B7PX04                       | Galactosyltransferase (transfer of glycosyl groups)                                                             | NS                         | −∞                                |
| ISCW020745 B7Q0V5                       | Cytoplasmic dynein 1 intermediate chain (peptidyl lysine acetylation)                                          | NS                         | −∞                                |
| ISCW015936 B7P2M8                       | Ceramide glucosyltransferase (Catalysis of the transfer of a glucosyl group from UDP-glucose to an acceptor molecule) | NS                         | −∞                                |
| ISCW004239 B7P6C9                       | Leukocyte receptor cluster (Lrc) member 4 protein (immunoglobulin-like receptor, amino acid metabolism)        | NS                         | −∞                                |
| ISCW009367 B7PYE7                       | B-cell receptor-associated protein (Bap31) (intracellular protein transport)                                    | +0.6                       | NS                                |
| ISCW001762 B7P7F7                       | Heat shock protein (shSF) (stress response, protein folding and unfolding)                                       | −0.8                       | +0.8                              |
Metabolic Pathways Affected by Anaplasma in Tick Cells

| Gene/protein accession numbers | Description (metabolic pathway)                      | Gene expression | Protein representation |
|-------------------------------|------------------------------------------------------|-----------------|------------------------|
| ISCW017360                   | Translation initiation factor 2, alpha subunit (eIF2a) (protein biosynthesis) | NS              | +3.1                   |
| B7P037                       | Apoptosis signal-regulating kinase 1 (ASK1) (apoptosis, protein metabolism) | −1.2            | NS                     |
| ISCW001564                   | Mitogen-activated protein kinase (MKK) (apoptosis, protein metabolism) | NS              | −∞                     |
| B7P594                       | Heat shock protein 70-interacting protein (CHIP) (cytoplasm ubiquitin ligase complex, protein metabolism) | −0.7            | NS                     |
| ISCW006910                   | UDP-glucose dehydrogenase (glucose metabolism) | NS              | −∞                     |
| B7PVJ6                       | ISCW020876                                            |                 |                        |
| B7Q116                       | ISCW016065                                            |                 |                        |
| B7PBA4                       | ISCW017360                                            |                 |                        |

For redundant genes/proteins only one accession number is shown.
The analysis was based on the metabolites identified in tick cells with emphasis on those significantly affected in response to A. phagocytophilum infection when compared to uninfected cells. The terms related to these metabolites were searched in the annotations of Uniprot tick proteins and tick-specific Byosystems entries in order to select a set of genes/proteins that could be related to these metabolites in I. scapularis. Then the genes/proteins that were selected as related to these metabolites were compared to the differentially expressed genes and differentially represented proteins in response to A. phagocytophilum infection and those also found in this set were used for further characterization. Finally, tick genes/proteins were searched for GO and pathway annotations in VectorBase to identify putative pathways affected by A. phagocytophilum infection of tick cells with emphasis on the most affected metabolic pathways.

Gene expression and protein representation were calculated for genes and/or proteins with significant (p ≤ .05) differences between infected and uninfected tick cells as the Infected to Uninfected Log2 ratio.
Abbreviation: NS, differences between infected and uninfected cells not significant.

Recent results showed how the I. scapularis nymphs and adult female guts and salivary glands respond to A. phagocytophilum infection at both RNA and protein levels (8). Using those results and the results reported in the present study, the expression/representation of selected genes/proteins involved in the protein processing in ER and glucose metabolic pathways were compared between in vitro and in vivo studies (Fig. 6). The results showed differences between ISE6 tick cells, guts, and salivary glands and between mRNA and protein levels (Fig. 6). The results in cultured tick cells resembled more closely the in vivo results in tick nymphs in which whole tick tissues were used for analysis when compared with individual tissues from adult females. Furthermore, the results obtained here probably reflected the fact that ISE6 tick cells show some properties similar to hemocytes that are freely circulating in the hemolymph, therefore supporting differences in the response to infection when compared with tick guts and salivary glands. These results further emphasize the presence of tissue-specific differences in the tick response to A. phagocytophilum infection.

**Functional Characterization of Selected Tick Genes/Proteins**—The validation of RNAseq and proteomics data is important in order to provide additional support for the results obtained in these studies. Therefore, nine genes were selected to validate the RNAseq results by real-time RT-PCR (Fig. 7A). The results showed a 78% correlation between RNAseq and RT-PCR results (Fig. 7A). The differences observed between the results of the two analyses could be attributed to intrinsic variations in gene expression (8). For the validation of proteomics data, antibodies against Hsp70, Hsp90, and MKK were used for Western blot analysis and corroborated results at the protein level (Fig. 7B).

The function of selected genes and proteins involved in protein processing in ER and glucose metabolic pathways was characterized with respect to tick cell viability and A. phagocytophilum infection. RNAi was used to characterize the effect of sHSF gene knockdown on tick cell apoptosis and rickettsial infection. This gene was up-regulated in infected tick cells and is part of the protein processing in ER pathway (Fig. 4). The results showed that gene knockdown (52 ± 9% expression silencing with respect to Rs86 control treated cells) resulted in lower A. phagocytophilum DNA levels without affecting tick cell apoptosis (Fig. 8A). Furthermore, using MM to stimulate MKK that was underrepresented in infected tick cells (Fig. 4) also resulted in increased apoptosis (Fig. 8B) and lower infection (Fig. 8C). These results supported the function of these genes/proteins in tick–pathogen interactions and provided support for the role of the protein processing in ER pathway in response to A. phagocytophilum infection of tick cells.

PEPCK is a mitochondria-related enzyme involved in glucose metabolism that was underrepresented in infected tick cells (Fig. 5). After nicotinic acid treatment, glycogen is depleted and PEPCK activity increases, an effect that is prevented by cycloheximide or actinomycin D (42). In infected tick cells, treatment with nicotinic acid resulted in a higher percentage of apoptotic cells without affecting A. phagocytophilum DNA levels, while cycloheximide or actinomycin D treatment reduced apoptosis and increased infection when compared with untreated cells (Figs. 8D and 8E).

These results supported the view that the metabolic pathways in tick cells that are affected in response to A. phagocytophilum infection are crucial for tick–pathogen interactions.
DISCUSSION

The results obtained in this study proved our hypothesis by showing that the systems biology integration of metabolomics, transcriptomics, and proteomics data resulted in the discovery of new metabolic pathways affected by Anaplasma phagocytophilum infection of tick cells. It has been previously shown that the impact of Anaplasma phagocytophilum infection on tick cell function is reflected by changes in the transcriptome (1, 8, 9, 11, 12, 14, 55). However, despite the impact of metabolomics on the study of infectious diseases (15, 16), data were not available for ticks. Furthermore, only the integration of metabolomics, transcriptomics, and proteomics data using a systems biology approach resulted in the identification of protein processing in ER and glucose metabolic pathways playing a critical role at the tick–pathogen interface.

Intracellular bacteria such as Anaplasma phagocytophilum have evolved to manipulate host cell mechanisms to establish infection (1, 8, 9, 11, 12, 14, 55). Recently, Ayllón et al. (8) demonstrated that Anaplasma phagocytophilum inhibits cell apoptosis through the Janus kinase/signal transducers and activators of transcription and intrinsic apoptosis pathways to facilitate and establish infection in tick guts and salivary glands, respectively. The results obtained here suggested that the effect of rickettsial infection on the protein processing in ER pathway may represent an additional mechanism by which Anaplasma phagocytophilum inhibits cell apoptosis to increase infection of tick cells. Additionally, it could also represent a mechanism by
which tick cells suppress apoptosis as they recover from ER stress through activation of amino acid metabolism. *A. phagocytophilum* could promote protein misfolding, a process that activates protein targeting and degradation to prevent ER stress and cell apoptosis, to counteract the tick cell response to infection. These processes may have resulted from tick–pathogen coevolution as *A. phagocytophilum* induces protein misfolding to limit the tick cell response and facilitate infection but requires protein degradation to prevent ER stress and cell apoptosis in order to survive in infected cells.

Glucose metabolism was recently characterized in the *Rhipicephalus microplus* BME26 embryo-derived cell line (56), and glucose was identified as one of the metabolites involved in chemosensation during tick blood feeding (57). These results highlight the importance of glucose metabolism in ticks, but information was not available on the role of this pathway during pathogen infection. Glucose and hexokinase levels do not change in bovine erythrocytes in response to *Anaplasma marginale* infection (58). However, hexokinase activity decreases during *A. phagocytophilum* infection of ISE6 tick cells (12), a finding that correlated with the lower protein levels found here for this protein in infected tick cells. As an obligatory intracellular bacterium, *A. phagocytophilum* has significantly less coding capacity for biosynthesis and central intermediary metabolism than do free-living bacteria (59), thus requiring the acquisition of various compounds from host cells. Huang *et al.* (60) demonstrated that the isolated outer membrane of *A. phagocytophilum* has porin activity mediated by the outer membrane protein P44 (MSP2) for different compounds such as glutamine and glucose that were found at lower levels in infected tick cells. Furthermore, they demonstrated that the tricarboxylic acid cycle of *A. phagocytophilum* is incomplete and requires the exogenous acquisition of L-glutamine or L-glutamate for function. Additionally, *A. phagocytophilum* needs glucose for the glycosylation of the MSP2 paralogs (61), which is probably also acquired at least in part from the host cell. Considering the requirements of *A. phagocytophilum* for host cell metabolites such as glucose, glutamine, and glutamate and the coupling between glucose metabolism and glutamate cycling (62), it is possible that tick cells decreased glucose metabolism to limit rickettsial infection. Pharmacological inhibition of PEPCK enhances apopto-

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**Fig. 5. Glucose metabolism pathway.** Simplified scheme of glucose metabolism pathway showing genes/proteins differentially expressed/represented or with no differences between infected and uninfected cells and biological processes affected in response to *A. phagocytophilum* infection of tick cells. Differences refer to infected versus uninfected tick cells. Discontinuous arrows indicate the presence of other enzymes in the pathway. Abbreviations: IAH, isoamyl acetate-hydrolizing esterase (ISCW002753, B7PB86); HK, hexokinase; GP, glucose-6-phosphatase (ISCW017459, B7PAR9); PK, pyruvate kinase; GS, glucose synthase; GSK, glycogen synthase (ISCW006570, B7PPS1); LDH, lactate dehydrogenase (ISCW013290, B7QA60); GDG, Glutamate dehydrogenase; PEPCK, Phosphoenolpyruvate carboxykinase; PEP, phosphoenolpyruvate; OAA, oxaloacetic acid. Accession numbers are listed for genes/proteins that did not change in expression/representation between infected and uninfected tick cells; the remaining accession numbers are given in Table II.
sis produced by glucose depletion in certain human cancer cells (63). However, in hepatic cells PEPCK inhibition reduces apoptosis and ameliorates mitochondrial dysfunction (64). Our results showed that PEPCK stimulation increases apoptosis in tick cells, thus suggesting again a coevolutionary mechanism by which *A. phagocytophilum* may benefit from...
the tick cell’s ability to limit rickettsial infection by inhibiting PEPCK and thereby decreasing glucose metabolism, which also results in the inhibition of cell apoptosis that increases infection in ticks (8, 12).

Intracellular bacteria such as *A. phagocytophilum* produce proteins that could contribute to changes in the metabolome of infected tick cells (65). Although the subcellular localization of these proteins has not been defined, the identification of *A. phagocytophilum* GDG and PK in infected tick cells may contribute to glucose metabolism to counteract the tick cell response to infection. Some of the metabolites identified in this study such as glutamine, proline, and glucose were part of the L-15B300 medium used for culture of ISE6 tick cells (66). Other metabolites could have been derived from culture medium components. Nevertheless, the medium was used to culture both uninfected and infected cells, and the presence of these metabolites could contribute to the results obtained in this study. For example, lower glucose levels in infected tick cells could be due to the fact that *A. phagocytophilum* infection affected the glucose synthesis pathway and thus tick cells were forced to metabolize more glucose collected from the culture medium.

As in previous reports (10), the transcriptomics and proteomics results showed differences between cultured tick cells and tick developmental stages and adult tissues in response to pathogen infection, highlighting the presence of
tissue-specific differences in the tick cell response to infection (8, 12, 67). Furthermore, the results of this study are probably relevant for the characterization of the response of tick hemocytes to *A. phagocytophilum* infection (50, 51). This possibility could explain the differences observed with the response in tick guts and salivary glands and further support the presence of tissue-specific signatures in the tick response to *A. phagocytophilum* infection. The results also showed differences between mRNA and protein levels suggesting that this discrepancy could be due, at least in part, by delay between mRNA synthesis and protein accumulation that could be minimized by sampling at different time points and/or the role for posttranscriptional and posttranslational modifications in tick cell response to *A. phagocytophilum* infection (8). For example, as supported by the results presented here, Hsp is often regulated at the posttranscriptional level (8, 68).

Recent results have shown how ticks benefit from infection by *A. phagocytophilum* (69, 70). Neelakanta et al. (69) demonstrated that infection with *A. phagocytophilum* induces *I. scapularis* ticks to express an antifreeze glycoprotein that increases tick cold tolerance. The heat shock and other stress responses are a conserved reaction of cells and organisms to stress conditions such as elevated temperatures, toxicity, and pathogen infection (71). Busby et al. (70) demonstrated that Hsp is involved in the control of *I. scapularis* response to the stress produced by heat shock, blood feeding, and *A. phagocytophilum* infection. These studies showed that at high temperatures and during blood feeding, when hsp is overexpressed, *I. scapularis* ticks are protected from stress and *Anaplasma* infection and have a higher questing speed. In the study reported here, Hsp70 and Hsp90 were overrepresented in infected tick cells. The increase in Hsp levels counteracts the negative effect of heat shock and pathogen infection on tick questing behavior and increases tick survival, those playing an important role in pathogen transmission and the adaptation of tick populations to challenging environmental conditions (70, 71). These results also highlight coevolutionary mechanisms by which ticks benefit from *A. phagocytophilum* infection by increasing survival while pathogens guarantee transmission to susceptible hosts.

**CONCLUSIONS**

This study reported for the first time changes in the tick metabolome in response to *A. phagocytophilum* infection and a systems biology integration of metabolomics, transcriptomics, and proteomics data to characterize essential metabolic pathways involved in this process. This experimental approach resulted in the identification of new metabolic pathways affected by *A. phagocytophilum* infection of tick cells, probably relevant for the hemocyte response to infection. The results showed that pathogen infection affected protein processing in ER and glucose metabolic pathways in tick cells. These results supported the tick-*Anaplasma* co-evolution hypothesis by providing new evidences of how tick cells limit pathogen infection while the pathogen benefits from the tick cell response to establish infection (Fig. 9). Additionally, the overrepresentation of tick Hsp in infected cells suggested a coevolutionary mechanism by which ticks benefit from *A. phagocytophilum* infection by increasing survival while pathogens guarantee transmission to susceptible hosts. The results reported here, Hsp70 and Hsp90 were overrepresented in infected tick cells. The increase in Hsp levels counteracts the negative effect of heat shock and pathogen infection on tick questing behavior and increases tick survival, those playing an important role in pathogen transmission and the adaptation of tick populations to challenging environmental conditions (70, 71). These results also highlight coevolutionary mechanisms by which ticks benefit from *A. phagocytophilum* infection by increasing survival while pathogens guarantee transmission to susceptible hosts.

**Fig. 9. Evidence of tick-*Anaplasma* co-evolution.** *A. phagocytophilum* infection affected protein processing in ER and glucose metabolic pathways in tick cells. The results suggested that *A. phagocytophilum* induces protein misfolding to limit the tick cell response and facilitate infection but requires protein degradation to prevent ER stress and cell apoptosis to survive in infected cells. Additionally, *A. phagocytophilum* may benefit from the tick cell’s ability to limit pathogen infection through PEPCK inhibition thereby decreasing glucose metabolism, which also results in the inhibition of cell apoptosis that increases infection of tick cells. *A. phagocytophilum* PK and GDG in infected tick cells may contribute to glucose metabolism to counteract the tick cell response to infection. Question mark indicates that the role of bacterial protein in glucose metabolism remains to be proved. Abbreviations: Ap, *A. phagocytophilum*; PK, pyruvate kinase; GDG, glutamate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase.
suggested that A. phagocytophilum induces protein misfolding to limit the tick cell response and facilitate infection but requires protein degradation to prevent ER stress and cell apoptosis to survive in infected cells. Additionally, A. phagocytophilum may benefit from the tick cell’s ability to limit rickettsial infection through PEPCK inhibition, leading to decreased glucose metabolism, which also results in the inhibition of cell apoptosis that increases infection of tick cells. As demonstrated in previous studies (8, 12), the inhibition of cell apoptosis seems to play a central role in A. phagocytophilum infection of tick cells.

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