Expression of perilipin in human promyelocytic cells in response to *Anaplasma phagocytophilum* infection results in modified lipid metabolism

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The obligate intracellular pathogen *Anaplasma phagocytophilum* is transmitted by ticks and causes human granulocytic anaplasmosis, tick-borne fever of ruminants, and equine and canine granulocytic anaplasmosis. In a previous study, the perilipin (PLIN) gene was identified as one of the genes differentially expressed in human promyelocytic HL-60 cells in response to infection with *A. phagocytophilum*. PLIN is a major adipocyte lipid droplet-associated phosphoprotein that plays a central role in lipolysis and cholesterol synthesis. Host cholesterol and other lipids are required by *A. phagocytophilum* for infection and multiplication in human cells. In this study, it was hypothesized that PLIN may be involved in infection of human HL-60 cells by *A. phagocytophilum*. To test this hypothesis, a combination of real-time RT-PCR, immunofluorescence and RNA interference was used to study the expression of PLIN. The results of these studies demonstrated that *A. phagocytophilum* modulates lipid metabolism by increasing PLIN mRNA levels and facilitates infection of HL-60 cells. The results of these studies expand our knowledge of the role of lipid metabolism in *A. phagocytophilum* infection and multiplication in HL-60 cells and suggest a mechanism by which *A. phagocytophilum* modulates lipid metabolism.

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INTRODUCTION

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is an obligate intracellular tick-borne pathogen that causes human granulocytic anaplasmosis (HGA), tick-borne fever of ruminants, and equine and canine granulocytic anaplasmosis (Dumler *et al.*, 2001). HGA, first described in 1994 in the United States, has become a predominant form of anaplasmosis and is among the most common tick-borne diseases in the United States and Europe (Dumler *et al.*, 2005).

A. phagocytophilum initiates infection by adhering to polymorphonuclear leukocytes, a process which involves adhesins such as the human P-selectin glycoprotein ligand-1 (PSGL-1) that bind cooperatively to neutrophil ligand molecules (Dumler *et al.*, 2005). Host cholesterol is incorporated into the *A. phagocytophilum* membrane and is required for pathogen infection and multiplication, involving cholesterol-rich lipid drafts or caveolae and

glycosylphosphatidylinositol-anchored proteins (Lin & Rikihisa, 2003a, b). After infection, *A. phagocytophilum* undergoes a developmental cycle within a parasitophorous vacuole in which reticulated forms divide by binary fission and then transform into the infective dense forms. This infection cycle modulates host cell growth and differentiation (Carlyon & Fikrig, 2003).

While the main vectors for *A. phagocytophilum* are tick species belonging to the *Ixodes ricinus* complex, the pathogen multiplies in a broad range of terrestrial vertebrates (Dumler *et al.*, 2005; de la Fuente *et al.*, 2005a). In the laboratory, *A. phagocytophilum* can be propagated in the undifferentiated human promyelocytic cell line HL-60. Infection of HL-60 cells with *A. phagocytophilum* results in modulation of host cell gene expression (see, for example, de la Fuente *et al.*, 2005b; Pedra *et al.*, 2005; Sukumaran *et al.*, 2005).

In a previous study, we identified the perilipin (PLIN) gene as one of the genes differentially expressed in human HL-60 cells in response to infection with *A. phagocytophilum* (de la Fuente *et al.*, 2005b). PLIN is a major adipocyte lipid

Abbreviations: p.i., post-infection; PLIN, perilipin; PSGL-1, P-selectin glycoprotein ligand-1; RNAi, RNA interference.

droplet-associated phosphoprotein that plays a central role during lipolysis and cholesterol synthesis (Yeaman, 2004; Moore *et al.*, 2005; Miyoshi *et al.*, 2006; Granneman *et al.*, 2007). Because of the key role of cholesterol and other lipids in *A. phagocytophilum* infection and multiplication of human cells, we hypothesized that PLIN may be involved in the infection of human cells with *A. phagocytophilum*. We tested this hypothesis in the HL-60 human cell line infected with *A. phagocytophilum* using a combination of real-time RT-PCR, immunofluorescence and RNA interference (RNAi).

METHODS

Determination of PLIN mRNA levels in uninfected and infected HL-60 cells. The human HL-60 cell line was cultured and infected with *A. phagocytophilum* as previously described (de la Fuente *et al.*, 2005b). Three independent uninfected and infected cultures were sampled at 0, 12, 24, 48, 72 and 96 h post-infection (p.i.) and human PLIN and *A. phagocytophilum* major surface protein 4 (*msp4*) mRNA levels were determined by real-time RT-PCR using human PLIN (GenBank accession no. NM_002666) and *msp4* (de la Fuente *et al.*, 2005a) sequence-specific primers (PLIN, PLINRT5: 5'-CTCTCGA-TACACCGTGCAGA and PLINRT3: 5'-TGGTCCTCATGATCCT-CCTC; *msp4*, APMSP4RT5: 5'-TGACAGGGGAGGATCTTACG and APMSP4RT3: 5'-TCTAGTCCGCCAATAGCAT) and the QuantiTect SYBR Green RT-PCR kit (Qiagen) in a Bio-Rad iQ5 thermal cycler following the manufacturer's recommendations. mRNA levels were normalized against human β -actin (forward: 5'-TGATATCGCCGCGCTCGTCGTC; reverse: 5'-GCCGATCCACACGGAGTACT; de la Fuente *et al.*, 2005b) and displayed in mRNA arbitrary units. PLIN mRNA levels were compared in infected and uninfected cells using the ANOVA test ($P=0.05$).

PLIN immunofluorescent labelling in uninfected and infected HL-60 cells. Smears of uninfected and *A. phagocytophilum*-infected HL-60 cells collected at 124 h p.i. were prepared using a cytocentrifuge. The cells were dried and fixed immediately with 4% paraformaldehyde in PBS for 20 min at room temperature (RT). The cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min and blocked with 2.5% BSA in PBS for 1 h. The cells were

then incubated with rabbit polyclonal anti-perilipin antibodies (1:10) (Novus Biologicals), rabbit polyclonal antibodies to *A. phagocytophilum* (1:200) (de la Fuente *et al.*, 2006) or rabbit preimmune control serum (1:200) for 1 h at RT. The cells were then incubated with Cy3 or Cy5 goat anti-rabbit IgG (1:100) secondary antibodies (Jackson Immuno Research) for 1 h in the dark at RT. Between each step, the slides were rinsed three times with PBS for 3 min each and kept in PBS before observing them on a confocal microscope (Leica). During all incubations, the cells were rocked on a LabQuake shaker (Barnstead/Thermolyne).

Determination of total cholesterol content in uninfected and infected HL-60 cells. Uninfected and *A. phagocytophilum*-infected HL-60 cells were collected at 72, 120, 144 and 160 h p.i. and the total cholesterol levels were determined using the Amplex Red cholesterol assay kit according to the manufacturer's instructions (Molecular Probes). Total cholesterol content was determined with a Polarstar Optima spectrophotometer (BMG Lab Technologies) and normalized against the total protein concentration as determined by the DC Protein Assay (Bio-Rad). Three replicates were conducted for each time point and the cholesterol content was compared in infected and uninfected cells using the Student's *t*-test ($P=0.05$).

RNA interference in HL-60 cells. Gene expression was silenced in HL-60 cells by RNAi using a combination of two different pre-designed siRNAs to PLIN (NM_002666; siRNA IDs 120985 and 120986) and to actin-related protein 3 (ARP3) (NM_020445; siRNA IDs 127242 and 127243) and PSGL-1 (NM_003006; siRNAs IDs 12441 and 142575) to serve as negative and positive controls, respectively (Ambion). In a 96-well plate, 4×10^5 cells per well were nucleofected with 1 μ g siRNA using the Nucleofector 96-well shuttle system (Amaxa Biosystems) with kit SF and program 96-EN-138 following the manufacturer's instructions (efficiency of transfection $71 \pm 19\%$ after 24 h). After nucleofection, cells were divided into two 96-well plates. Twenty-four hours after nucleofection, cells were collected from one plate for assessment of viability and morphology in Giemsa-stained cytospin smears, RNA extraction (RNeasy 96 kit; Qiagen) and analysis of gene expression by real-time RT-PCR as described above. The second plate was incubated for 24 h with cell-free *A. phagocytophilum* (m.o.i.=10) prepared as described by Thomas & Fikrig (2007), and the level of infection corresponded to approximately 72 h p.i. as depicted in Fig. 1. The cells were then washed three times with PBS and total DNA was extracted (Wizard

Fig. 1. Expression kinetics of PLIN in human HL-60 cells infected with *A. phagocytophilum*. Human PLIN (solid and dotted lines) and *A. phagocytophilum msp4* (dashed line) mRNA levels were determined by real-time RT-PCR in uninfected and infected HL-60 cells. Amplification efficiencies were normalized against human β -actin and displayed in mRNA arbitrary units. PLIN mRNA levels were compared in infected (solid line) and uninfected (dotted line) cells using an ANOVA test (* $P < 0.05$; $n=3$).

SV 96 genomic DNA purification system; Promega). The *A. phagocytophilum* infection levels in HL-60 cells were evaluated after RNAi by real-time PCR of *msh4* and normalizing against human *Alu* sequences (Nicklas & Buel, 2003) using the QuantiTect SYBR Green PCR kit (Qiagen) in an iQ5 thermal cycler (Bio-Rad) as described previously. Known amounts of the full-length *A. phagocytophilum msh4* PCR were used to construct a standard curve for the real-time PCR. PLIN and PSGL-1 mRNA levels were determined after RNAi by real-time RT-PCR, normalized against human β -actin using the comparative Ct (delta delta Ct) method and compared in PLIN or PSGL-1 siRNA- and ARP3 siRNA-treated control cells using the Student's *t*-test ($P=0.05$; $n=4-8$). *A. phagocytophilum msh4* DNA levels were compared in cells nucleofected with PLIN or PSGL-1 siRNAs and control cells treated with ARP3 siRNA using the Student's *t*-test ($P=0.05$; $n=4-8$).

RESULTS AND DISCUSSION

In a previous study in which differential gene expression of HL-60 cells in response to *A. phagocytophilum* infection was characterized by microarray analysis, we demonstrated that expression of PLIN was induced at 72 h p.i. (de la Fuente *et al.*, 2005b). Because of the important role of PLIN during lipolysis and cholesterol synthesis (Yeaman, 2004; Moore *et al.*, 2005; Miyoshi *et al.*, 2006; Granneman *et al.*, 2007), which are essential for *A. phagocytophilum* infection and multiplication (Lin & Rikihisa, 2003a, b; Xiong *et al.*, 2007), we analysed the expression of PLIN at

the mRNA and protein levels in uninfected and *A. phagocytophilum*-infected HL-60 cells.

The PLIN mRNA levels increased in HL-60 cells after 48 h p.i. with *A. phagocytophilum* and reached 37-fold induction at 96 h p.i. (Fig. 1). The increase in PLIN mRNA levels coincided with pathogen multiplication and increasing infection levels (Fig. 1). PLIN protein levels were higher in *A. phagocytophilum*-infected HL-60 cells than in uninfected control cells (Fig. 2a–h). Similar to results in adipocytes (Moore *et al.*, 2005; Miyoshi *et al.*, 2006), PLIN was localized in the cytoplasm and in the periphery of infected HL-60 cells (Fig. 2h), where *A. phagocytophilum* labelling was also found in some cells (see insert on Fig. 2g). In the adipocytes, PLIN preferentially targets a special class of peripheral lipid storage droplets, but not the major or central lipid storage droplets (Moore *et al.*, 2005).

The increase of PLIN mRNA and protein levels in *A. phagocytophilum*-infected HL-60 cells may reflect a protective cellular response to limit rickettsial infection or may be the result of the manipulation by *A. phagocytophilum* of host gene expression to promote pathogen multiplication. To test these hypotheses, the effect of PLIN knockdown by RNAi was evaluated in HL-60 cells infected with *A. phagocytophilum*. After RNAi, expression of PLIN and PSGL-1 was shown to be reduced by $51 \pm 14\%$ ($n=4$) and

Fig. 2. Immunofluorescent labelling for PLIN and *A. phagocytophilum* in uninfected and infected human HL-60 cells. Uninfected (a–d) and infected (e–h) HL-60 cells were incubated with rabbit preimmune control serum (b, f) or anti-*A. phagocytophilum* (c, g) or anti-PLIN (d, h) antibodies. Scale bars, 29.88 μ m (a, b), 50 μ m (c, e, f), 61.89 μ m (d), 88.92 μ m (g; insert, 21.61 μ m) and 40.94 μ m (h).

61 ± 18 % ($n=8$), respectively, as compared with the ARP3 siRNA-nucleofected controls ($P < 0.05$). RNAi also resulted in a reduction in *A. phagocytophilum* DNA in cells nucleofected with PLIN siRNA, similar to results obtained in positive control cells transfected with PSGL-1 siRNA (Fig. 3). If PLIN has a role in the control of *A. phagocytophilum* infection in humans, we would have expected higher infection levels in HL-60 cells with knockdown PLIN. However, the lower infection levels that resulted after RNAi suggested that PLIN was required for *A. phagocytophilum* infection and/or multiplication in HL-60 cells.

A. phagocytophilum infection and multiplication involve cholesterol-rich lipid drafts or caveolae and glycosylphosphatidylinositol-anchored proteins (Lin & Rikihisa, 2003a). Because *A. phagocytophilum* lacks genes for lipid A and peptidoglycan biosynthesis, the pathogen requires the incorporation of host cholesterol into the membrane for survival (Lin & Rikihisa, 2003b). Furthermore, a high-cholesterol diet facilitates *A. phagocytophilum* infection of apolipoprotein E-deficient mice (Xiong *et al.*, 2007). These results are in agreement with the findings reported herein and support a role for PLIN during infection and multiplication of *A. phagocytophilum* in human host cells.

The overexpression of host PLIN in *A. phagocytophilum*-infected HL-60 cells suggested a mechanism by which lipolysis and cholesterol synthesis are affected during pathogen infection and multiplication in human cells. The hormone-sensitive lipase (HSL) catalyses the forma-

Fig. 3. Effect of PLIN on *A. phagocytophilum* infection of human HL-60 cells. *A. phagocytophilum* msp4 DNA levels were determined by real-time PCR in infected HL-60 cells with knockdown PLIN, PSGL-1 (positive control) and ARP3 (negative control). *A. phagocytophilum* msp4 DNA levels were normalized against human *Alu* sequences and infection levels were compared in cells nucleofected with PLIN or PSGL-1 siRNAs and control cells treated with ARP3 siRNA by Student's *t*-test (* $P < 0.05$; $n=4-8$).

tion of cholesterol in macrophages and other cells (Yeaman, 2004). Although contradictory results have been reported on the role of PLIN in promoting or preventing lipolysis (Yeaman, 2004), PLIN was recently shown to promote HSL-mediated adipocyte lipolysis (Moore *et al.*, 2005; Miyoshi *et al.*, 2006; Granneman *et al.*, 2007). Congruent with these findings, the total cholesterol content was higher in *A. phagocytophilum*-infected cells collected at 72 and 120 h p.i. than in uninfected HL-60 cells (Fig. 4). At 144 and 160 h p.i., cholesterol levels were similar in infected and uninfected cells, presumably due to incorporation into the bacterial cells (Lin & Rikihisa, 2003b).

Conclusions

In summary, we have shown that *A. phagocytophilum* infection of human promyelocytic HL-60 cells results in increased PLIN mRNA and protein levels. These results have expanded our knowledge of the important role that lipid metabolism plays in *A. phagocytophilum* infection and multiplication and suggest a new mechanism by which *A. phagocytophilum* modulates lipid metabolism by promoting HSL-mediated lipolysis in infected cells. Furthermore, silencing of PLIN expression reduced pathogen infection/multiplication, thus suggesting that PLIN is not induced in host cells as a defence mechanism to limit pathogen multiplication but rather *A. phagocytophilum* modulates the expression of PLIN to facilitate host cell infection. Further studies are needed to define the mechanism by which *A. phagocytophilum* modulates the expression of PLIN in human HL-60 cells. Nonetheless, the results of these studies suggest that therapeutics and other interventions that target host lipid metabolism will likely aid in the control of *A. phagocytophilum* infection in humans and animals (Xiong *et al.*, 2007).

Fig. 4. Total cholesterol content [μg (mg protein)⁻¹] in *A. phagocytophilum*-infected and uninfected HL-60 cells. Total cholesterol content was determined in uninfected [$39.4 \pm 4.5 \mu\text{g}$ (mg protein)⁻¹] and infected cells at 72, 120, 144 and 160 h p.i. (shown in the graph), normalized against total protein concentration and compared by Student's *t*-test (* $P \leq 0.01$; $n=3$ for each time point).

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