Brucella suis vaccine strain S2-infected immortalized caprine endometrial epithelial cell lines induce non-apoptotic ER-stress

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Abstract *Brucella*, which is regarded as an intracellular pathogen responsible for a zoonotic disease called brucellosis, survives and proliferates within several types of phagocytic and non-phagocytic cells. *Brucella* infects not only their preferred hosts but also other domestic and wild animal species, inducing abortion and infertility. Therefore, the interaction between uterine cells and *Brucella* is important for understanding the pathogenesis of this disease. In this study, we describe the *Brucella suis* vaccine strain S2 (*B.suis.S2*) infection and replication in the immortalized caprine endometrial epithelial cell line hTERT-EECs and the induced cellular and molecular response modulation in vitro. We found that *B.suis.S2* was able to infect and replicate to high titers and inhibit the proliferation of EECs and induce non-apoptotic pathways, as determined by *B.suis.S2* detection using MTT and acridine orange/ethidium bromide (AO/EB) staining and flow cytometry. We explored the evidence of non-apoptotic pathways using real-time quantitative RT-PCR and by western blot analysis. Finally, we discovered the over-expression of GRP78, ATF4, ATF6, PERK, eIF2 α , CHOP, and cytochrome c (Cyt-c) but not IRE1, xbp-1, and caspase-3 in *B.suis.S2* (HK)-attacked and *B.suis.S2*-infected cells, suggesting that the molecular mechanism of ER stress sensor activation by

B.suis.S2 is basically concomitant with that by *B.suis.S2* (HK) and that ER stress, especially the PERK pathway, plays an important role in the process of *B.suis.S2* infecting EEC, which may, in part, explain the role of the uterus in the pathogenesis of *B.suis.S2*.

Keywords *B.suis.S2* · Caprine endometrial epithelial cell · Endoplasmic reticulum stress · Apoptosis · Infection

Abbreviations

T4SS	Type IV secretion system
ER	Endoplasmic reticulum
EEC	Caprine endometrial epithelial cell
<i>B.suis.S2</i>	<i>Brucella suis</i> vaccine strain S2
PERK	Protein kinase RNA-like kinase
IRE1 α	Inositol-requiring enzyme 1 α
ATF6	Activating transcription factor 6
GRP78	78-kDa glucose-regulated protein
CHOP	C/EBP homologous protein
CFU	Colony-forming units
Tm	Tunicamycin
xbp-1	X-box binding protein 1
MTT-3-[4	5-Dimethylthiazol-2-yl]-2
5	Diphenyltetrazolium bromide assay
eIF2 α	α Subunit of eukaryotic initiation factor 2

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Introduction

Bacteria of the genus *Brucella* cause brucellosis, which is recognized as a zoonotic disease of global importance (Scholz et al. 2010). Brucellosis is transmitted via direct contact with animals and/or their secretions or by consumption of milk and dairy products, which has a major economic

affect because the infection causes abortions and stillbirths and reduces fertility in herds (Díaz Aparicio 2013). *Brucella melitensis*, *Brucella abortus* and *Brucella suis*, which are transmitted between animals both vertically and horizontally, cause abortion and infertility in their primary natural hosts, including goats and sheep (*B. melitensis*), cows (*B. abortus*), and sows (*B. suis*). *Brucella* spp. infects not only their preferred hosts but also other domestic and wild animal species, which, in turn, can act as reservoirs of the disease for other animal species and for humans. In animals, *Brucella* vaccination is widely used for the prevention and control of brucellosis. The live attenuated strain *B. suis*.S2, which is the most widely used animal vaccine against brucellosis in China, was isolated from a swine fetus by the China Institute of Veterinary Drug Control (IVDC) in 1952. Thus far, no specific molecular markers have yet been identified for this vaccine strain (Shang et al. 2002).

The ability of *Brucella* to intracellularly replicate is central to its pathogenicity. Within host cells, the *Brucella*-containing vacuole (BCV) transiently interacts with early endosomes, late endosomes, and lysosomes. The acidification of the BCVs is essential because this acidification enables the intracellular expression of the genes encoding the VirB type IV secretion system (T4SS). Then, *Brucella* controls fusion with endoplasmic reticulum (ER) membranes in a VirB T4SS-dependent manner. *Brucella* replicates within ER-derived compartments in both phagocytes and nonprofessional phagocytes (Celli et al. 2003).

The ER stores calcium and is a site of protein synthesis and modification. A change in ER homeostasis leads to stress responses, with the activation of the unfolded protein response (UPR) (Santi-Rocca et al. 2012), which represents a conserved cellular homeostatic mechanism with important roles in normal development and in the pathogenesis of disease (Schröder and Kaufman 2005). The UPR is composed of three different pathways that fall under the control of three respective ER transmembrane proteins: protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 α (IRE1 α), and activating transcription factor 6 (ATF6) (Schröder and Kaufman 2005). Under normal cellular conditions, PERK, IRE1 α , and ATF6 form stable complexes with the ER stress sensor binding immunoglobulin protein (BiP), which is also known as 78-kDa glucose-regulated protein (GRP78), and remain in their inactive state. BiP binds to the ER luminal domains, preventing the homodimerization and activity of PERK and of IRE1 α . The binding of GRP78 to ATF6 blocks its Golgi-localization signals, retaining ATF6 to the ER membrane, which prevents the further processing of ATF6 to its active form. In the presence of cellular stress, the accumulation of misfolded proteins within the ER competitively binds BiP, causing the dissociation of GRP78 from PERK, IRE1 α , and ATF6, thus removing its inhibitory effects. Under moderate ER stress, the UPR can function as a

pro-survival mechanism and return the cell to its state of homeostasis. However, when cellular damage exceeds the capacity of this adaptive response, ER stress is prolonged, and the continued activation of the UPR signals the cell for apoptosis (Oyadomari and Mori 2004). CHOP is a major pro-apoptotic transcription factor that mediates ER-stress induced apoptosis and is a target for upregulation by all three arms of the UPR pathway.

Recent studies have demonstrated that *Brucella* spp. replicate within an intracellular compartment that contains ER resident proteins, the molecular mechanisms by which the pathogen secures this replicative niche remain obscure (Qin et al. 2008). *Brucella* replicate in a vacuole derived from the ER in epithelial cells, macrophages, and dendritic cells (Salcedo et al. 2013). Thus far, factors that mediate *Brucella* infection of host cells remain obscure. Related studies demonstrated that *B. melitensis* infection upregulated the expression of the UPR target genes GRP78, CHOP, and ERdj4 and induced XBP1 mRNA splicing in murine macrophages (Smith et al. 2013). Some studies demonstrated that host cytokine responses to *Brucella abortus* infection were predominantly elicited by the deployment of a type IV secretion system (T4SS), which interacted with the endoplasmic reticulum (ER) chaperone GRP78 and localized to the ER of HeLa cells (de Jong et al. 2013).

To better understand the role of the pathogenesis of *B.suis*.S2 in the uterus, we studied the replication and apoptosis by *B.suis*.S2 infection in the caprine endometrial epithelial cell (EEC) lines. We evaluated whether the ER stress mechanism was involved in the process of *B.suis*.S2 infection into caprine EECs. Our results suggest that ER stress induced by *B.suis*.S2 is required to mediate its intracellular replication within the caprine EECs.

Materials and methods

Bacterial strain preparation and cultivation

Brucella suis vaccine strain S2 (*B.suis*.S2) bacteria were kindly provided by the Shaanxi Provincial Institute for Veterinary Drug Control (Xi'an, Shaanxi, China). *B.suis*.S2 bacteria were grown in 10 ml of tryptic soy broth (TSB) medium with constant agitation at 37 °C. *B.suis*.S2 bacteria were harvested by centrifugation for 15 min at 6,000 $\times$ g at 4 °C and washed three times in 10 ml of phosphate-buffered saline (PBS). The numbers of *B.suis*.S2 were estimated by plating cells onto tryptic soy agar (TSA). To obtain *B.suis*.S2 (HK), *B.suis*.S2 were washed three times for 10 min each in sterile PBS, heat-killed at 70 °C for 20 min, aliquoted, and stored at -80 °C until use. The total absence of *B.suis*.S2 viability after heat killing was verified by the absence of bacterial growth on TSA. To obtain FITC-labeled *B.suis*.S2, *B.suis*.S2 were harvested by

centrifugation for 15 min at $6,000\times g$ at 4°C and washed three times in 10 ml of PBS. Cultures were suspended in PBS, which was supplemented with $200\text{ }\mu\text{g/ml}$ fluorescein isothiocyanate (FITC), in the dark for 1 h at 37°C . Then, *B.suis.S2* were washed three times with PBS to eliminate those FITC molecules that not combine with *B.suis.S2*. Then, the samples were suspended, and the concentration was adjusted with PBS. All procedures involving live bacteria were performed in a biosafety level 3 facility.

B.suis.S2 infection assays

Caprine endometrial epithelial cells (EECs) were immortalized by transfection with the human telomerase reverse transcriptase (hTERT) (Zhang et al. 2010). These cells were preserved by the Key Laboratory of Animal Biotechnology of the Ministry of Agriculture (Northwest A&F University, Yangling, Shaanxi, China). EECs were seeded in six-well plates (5×10^5 cells per well) and inoculated with the *B.suis.S2* or *B.suis.S2* (HK) at a MOI of 100. Multiwell plates were placed in a 5 % CO_2 atmosphere at 37°C . After 4-h incubation, cells were washed three times with phosphate-buffered saline (PBS) (pH 7.4), and the number of total cell-associated *B.suis.S2* bacteria was determined. Cells were treated for 10 min at 37°C with 0.1 ml of 0.1 % Triton X-100 in deionized sterile water, and lysates were serially diluted in PBS and plated on TSA to determine colony-forming units (CFU). To determine the number of intracellular viable *B.suis.S2* bacteria, a standard gentamicin protection assay was performed. Briefly, cells were infected as mentioned above, and after 4-h incubation, cells were washed three times with PBS and further incubated with cell culture medium containing 50 mg/ml gentamicin and 50 mg/ml streptomycin to eliminate remaining extracellular *B.suis.S2* bacteria. After 1 h, cells were washed three times with PBS, the process of FITC-labeled *B.suis.S2* infecting EECs was observed under a Nikon A1R si confocal microscope system, and the number of invasive *B.suis.S2* bacteria was determined. Cells were treated for 10 min with 0.1 ml of 0.1 % Triton X-100, as described above. Lysates were serially diluted in PBS and plated on TSA to determine intracellular CFU. The number of adherent *B.suis.S2* bacteria was calculated as the difference between total cell-associated *B.suis.S2* bacteria and intracellular *B.suis.S2* bacteria (Posadas et al. 2012).

hTERT-EEC cultivation and *B.suis.S2* infection

EECs were cultured in DMEM/F12 (Invitrogen) at 5×10^5 cells well^{-1} , supplemented with 10 % FCS, 5×10^4 IU penicillin and 50 mg streptomycin at 37°C in 5 % CO_2 . Then, the cells were infected with *B.suis.S2* (HK) or *B.suis.S2* at a MOI of 100, as mentioned above, and cells and supernatants were harvested at 8, 12, 24, 48 h post infection (p.i.). Cells were

harvested and stored at -20°C until use. Supernatants were centrifuged at $400\times g$ for 5 min, aliquoted and stored at -80°C until use.

Measurement of EEC viability

To test the effects of *B.suis.S2* (HK) or *B.suis.S2* on changes in cell viability, EECs were seeded into a 96-well plate at 1×10^4 cells/well. And then EECs treated as shown in *B.suis.S2* infection assays. EECs were treated with 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay (MTT) after exposure to *B.suis.S2* (HK) or *B.suis.S2* at 8, 12, 24, 48 h post infection and was then incubated for an additional 4 h. At the end of the experiments, the cell growth medium was replaced by DMSO and colorimetric measurements were performed with an ELISA plate reader at 570 nm using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA).

AO/EB staining and flow cytometry

The cells were seeded into 24-well plates (1×10^5 cells per well) and infected with *B.suis.S2* (HK) and attacked by *B.suis.S2* at a MOI of 100, as mentioned above, respectively. After 24 h, the cells were washed in PBS and stained with 100 mg/ml acridine orange (AO) and with 100 mg/ml ethidium bromide (EB) for 5 min in the dark at room temperature. The stained cells were observed under a Nikon A1R si confocal microscope system.

To determine the percentage of apoptotic cells, the above cells were quantified using an Annexin V-FITC Apoptosis Detection Kit (KGA107, Nanjing Keygen Biotech Co., Ltd.). The EECs were washed twice with cold PBS by centrifugation, and their density was adjusted to 1×10^6 cells/ml. Then, EECs were resuspended in 500 μl binding buffer, which was followed by the addition of 5 μl annexin V-FITC and 10 μl PI, and incubated for 15 min at 4°C in the dark. Detection by flow cytometry (EPICS Altra, Beckman Coulter Cytomics Altra) was conducted within 1 h.

Measurement of caspase-3 activity

Caspase 3 activity was measured using a Caspase 3 Activity Colorimetric Assay Kit (BA3020, Nanjing Biobox Biotech Co., Ltd.) according to the manufacturer's recommendations. Briefly, cell lysates were prepared, and protein concentrations were measured using the BCA Protein Assay Reagent (KGABCA, Nanjing Keygen Biotech Co., Ltd.). Then, 200 mg of protein in each sample was incubated with the caspase 3 substrate (200 mM final concentration) at 37°C in a microplate for 4 h. Samples were read at 405 nm in a microplate reader (Bio-Rad, Inc.)

RNA Extraction and cDNA Synthesis

Total RNA was extracted from EECs using TRIzol (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's instructions and treated with DNase (TaKaRa Bio, Inc.) before reverse transcription to remove genomic DNA contamination. Extracted RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water, and the RNA concentration and purity were estimated by reading the absorbance at 260 and 280 nm, respectively, on a spectrophotometer (Eppendorf, Inc.). The ratios of absorption (260/280 nm) of all preparations were between 1.8 and 2.0. Aliquots of RNA samples were subjected to electrophoresis in a 1.2 % agarose gel with ethidium-bromide staining to verify their integrity. The cDNAs were synthesized using a PrimeScriptTM RT Reagent Kit (TaKaRa Bio, Inc.) according to the manufacturer's instructions. The final reaction volume was 20 µl, including 1,000 ng of total RNA. The reverse transcription product was stored at -20 °C.

Real-time quantitative PCR

The GenBank accession number of the mRNA, the primer sequences, and annealing temperatures are listed in Table 1. Real-time PCR was performed with a LightCycler system (iQ5, Bio-Rad Laboratories, Inc.) using a SYBR[®] Premix Ex TaqTM II Kit (TaKaRa Bio, Inc.) according to the manufacturer's protocol. Each PCR reaction (20 µl total volume)

consisted of 2 µl of the reverse transcription product, 0.8 ml of each 10 µM forward and reverse primer, 10 µl SYBR[®] Premix Ex TaqTM II, and 6.4 µl RNase-free water. Cycling conditions were a denaturation step at 95 °C for 30 s, followed by 40 PCR cycles of 95 °C for 5 s and 60 °C for 20 s. The specificity of the real-time PCR products was confirmed using a melting curve analysis. These reactions were repeated in triplicate for every sample as technical replicates. Gene mRNA quantifications were performed using the $2^{-\Delta\Delta C_t}$ method, and the amount of transcripts in each sample was normalized using GAPDH as the internal control gene to correct for differences in the cDNA used (Qiu et al. 2013).

Western blot analysis

Whole-cell extracts from EECs were prepared using RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.). Protein determination was performed using the BCA assay (Nanjing Keygen Biotech Co., Ltd.). Thirty micrograms of total protein per sample were separated by 12 % sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore; Bedford, MA). After incubation in blocking buffer (5 % nonfat dried milk in Tris-buffered saline (TBS) containing 0.1 % Tween 20) for 1 h at room temperature, the membrane was incubated overnight at 4 °C with antibodies against CHOP (1:1,000; Santa Cruz) and β -actin (1:2,000; Tianjin Sungene Biotech Co., Ltd.). After

Table 1 The primer sequences used for real-time quantitative PCR

TargetGene	GenBank accession no.	Primer sequence	Product size (bp)	Annealing temperature (°C)
GAPDH	XM_005680968.1	AF:5 -GATGGTGAAGGTCGGAGTGAAC-3 AR: 5 -GTCATTGATGGCGACGATGT-3	100	60
GRP78	DQ029323.1	AF:5 -TGAAACTGTGGGAGGTGTCA-3 AR:5 -TCGAAAGTTCCCAGAAGGTG-3	171	60
CHOP	AY943948.1	AF:5 -AGGACCACCAGAGGTCACAC-3 AR:5 -TGCCACTTTCCTTTCGTTTT-3	193	60
ATF4	NM_0 01142518.1	AF:5 -AGATGACCTGGAAACCATGC-3 AR:5 -AGGGGGAAGAGGTTGAAAGA-3	190	60
ATF6	AY942654	AF:5 -AACCAGTCCTTGCTGTTGCT-3 AR:5 -CTTCTTCTTGCGGGACTGAC-3	224	60
Cyt-C	NC_005044.2	AF:5 -AGCCTTACGAGCAGTAGCACA-3 AR:5 -TTCTCCTTCGGTCAGGTCAAA-3	217	60
PERK	XM_005686691.1	AF:5 -CCCCATCCGCTACTGAACG-3 AR:5 -GGGCTGCTGGAGTGTCTTG-3	151	60
eIF2 α	XM_005685995.1	AF:5 -TCGAGCAGATATTGAAGTGGC-3 AR:5 -TCATTACATACCGAGGAGGAGC-3	140	60
IRE1	XM_005694366.1	AF:5 -ACTCCCTCAACATCGTTCACAG-3 AR:5 -CTCCTTGCACTCTTCGCTCA-3	208	60
XBPI	XR_310643.1	AF:5 -CCTGCCTGCTGGATGCTTA-3 AR:5 -GGGAAAGAGTTCACTGGCAAA-3	124	60

washing, the membranes were incubated with biotinylated anti-rabbit (CHOP) (KIT9706, Fuzhou Maixin Biotech Co., Ltd.) and anti-mouse (β -actin) (KIT9701, Fuzhou Maixin Biotech Co., Ltd.) IgG secondary antibodies (Beijing 4A Biotech Co., Ltd.) for 1 h at 37 °C. Sections were washed three times with PBS and then incubated with HRP-labeled streptavidin (SA-HRP) for 30 min at 37 °C. Thereafter, immunoreactive bands were visualized using a diaminobenzidine (DAB)-peroxidase substrate, and the chemiluminescence intensity of each protein band was quantified using Image J software.

Statistical analysis

All experiments were replicated at least three times for each group, and the data were presented as the mean $\pm$ SD. The data were analyzed using an ANOVA, which was followed by Fisher's least significant different test (Fisher LSD) with SPSS software (Version 16.0; SPSS, Inc., Chicago, IL). Differences were considered significant when $P < 0.05$.

Results

B. suis.S2 infect and replicate in caprine EECs

The results from the gentamicin protection assay revealed that *B. suis*.S2 bacteria were able to enter caprine EECs cultured in vitro. *B. suis*.S2 labeled by FITC mainly concentrated within the cell, but the *B. suis*.S2 that are around the cells and in the culture medium were killed by gentamicin and streptomycin. (Fig. 1a). Meanwhile, the intracellular multiplication of *B. suis*.S2 was proliferated in a time-dependent manner, and the bacterial numbers rapidly increased after infecting for 12 h (Fig. 1b). However, the heat-killed *B. suis*.S2 (HK) failed to adhere, infect, and replicate in caprine EECs according to TSA assay (supplementary date 1).

Effect of *B. suis*.S2 on the proliferation and apoptosis of caprine EECs

To clarify the proliferation and apoptosis of caprine EECs infected by *B. suis*.S2, cells were analyzed by MTT and AO/

Fig. 1 Infection and proliferation of *B. suis* S2 in caprine EECs. **a** FITC-labeled *B. suis*.S2 infect the EECs at 4 h post-infection (10 μ m). The white arrows indicate the *B. suis*.S2 intruding into the EECs. **b**

Intracellular multiplication of the *B. suis*.S2 CFU numbers was determined after the lysis of infected cells at the indicated post-infection times

EB staining and by flow cytometry. According to the AO/EB staining results, the nuclei of EECs attacked by not only *B.suis.S2* (HK) but also *B.suis.S2* had uniform green

fluorescence. There were few apoptotic bodies, dense hyperchromatic nuclei, and no orange necrotic cells (Fig. 2a). Although the apoptotic rates of *B.suis.S2* (HK) and

Fig. 2 Morphological changes and cell viability and apoptosis of *B.suis.S2* (HK)-attacked and *B.suis.S2*-infected EECs. **a** Confocal microscope images of *B.suis.S2* (HK)-attacked and *B.suis.S2*-infected EECs at 24 and 48 h p.i.: on the left are representative normal cultured cells (*Control*); in the center are heat-killed *B.suis.S2* (HK)-attacked EECs; on the right are *B.suis.S2*-infected EECs (100 μ m). The white arrows indicate the apoptotic EECs. **b** Statistics of apoptotic EECs as determined by flow cytometry after *B.suis.S2* (HK) attack and *B.suis.S2* infection at 24 and 48 h. **(c)** Cell viability of *B.suis.S2* (HK)-attacked and *B.suis.S2*-infected EECs were measured by MTT Representative data from three independent experiments are shown using the mean $\pm$ SD of two independent experiments performed in duplicate. Bars with different letters are significantly different ($P < 0.05$)

B.suis.S2 groups significantly increased compared with that of the control group, the total numbers of apoptosis cells were lower than 5 % ($P<0.05$, Fig. 2b), suggesting that the infection of *B.suis.S2* in caprine EECs did not induce apoptosis in a time-dependent manner. According to the MTT results, both *B.suis.S2* and *B.suis.S2* (HK) treatment inhibit the proliferation of EECs, and *B.suis.S2* has more intense inhibitory effect than *B.suis.S2* (HK) ($P<0.05$, Fig. 2c).

Expression of cytochrome c and caspase-3 in caprine EECs infected by *B.Suis.S2*

Cyt-c mRNA levels in the *B.suis.S2* (HK) group and in the *B.suis.S2* group were not significantly different from those levels of the control group after 24 h of culture. In contrast, Cyt-c mRNA levels significantly increased in the *B.suis.S2* (HK) group and in the *B.suis.S2* group compared with the control group at 48 h; however, there was no significant difference between the *B.suis.S2* (HK) group and the *B.suis.S2* group ($P<0.05$, Fig. 3a). Meanwhile, we observed that the caspase-3 activities of the three groups were not significantly different during the cultured time ($P<0.05$, Fig. 3b).

Detection of ER stress-related genes in caprine EECs infected by *B.suis.S2*

To verify whether ER stress was involved in the process of caprine EECs infected by *B.suis.S2*, we determined the expression of GRP78, ATF4, ATF6, IRE1, xbp-1, PERK, eIF2 α , and CHOP mRNA using the real-time PCR. As shown in Fig. 4a, GRP78 mRNA levels in the *B.suis.S2* (HK)-attacked and *B.suis.S2*-infected groups cultured for 12 h were higher than those levels in the control group ($P<0.05$), which

followed by a return to basal levels by 24 h and then a significant increase at 48 h ($P<0.05$). The CHOP mRNA levels did not differ among the groups before being cultured for 24 h and significantly increased in the *B.suis.S2* (HK)-attacked and *B.suis.S2*-infected groups at 48 h ($P<0.05$; Fig. 4d). Furthermore, the results of western blotting showed that the expression of the CHOP protein significantly increased with the culture time. The expression of the CHOP protein induced by *B.suis.S2* was higher than that of *B.suis.S2* (HK)-treated group and much lower than that of Tm-treated group ($P<0.05$; Fig. 5a, b). The expression levels of PERK, eIF2 α , ATF4, and ATF6 mRNA were similar to that of GRP78 mRNA during the culture time ($P<0.05$; Fig. 4b, c, e, f). All of the detected gene expression levels in the *B.suis.S2*-infected group were significantly higher than those levels in the *B.suis.S2* (HK)-attacked group by 12 h. However, the expression levels of GRP78, PERK, and eIF2 α in *B.suis.S2* (HK)-attacked group were significantly higher than those levels in the *B.suis.S2*-attacked group. At the same time, the expression levels of IRE1 and xbp-1 were no significant change among the three groups at any time ($P<0.05$; Fig. 4g, h).

Discussion

Brucella bacteria are facultative intracellular pathogens that can survive and replicate within mononuclear phagocytes and within epithelial cells, causing abortion and sterility in domestic animals (Roop et al. 2009). Clinical signs of uterine infection are affected by many factors, such as immunity, the number of pathogenic microorganisms, and the uterine environment (Silva et al. 2012). According to our results, *B. suis.S2* was able to rapidly infect and replicate in caprine

Fig. 3 *B.suis.S2* (HK) and *B.suis.S2* modulate members of the genes involved in the apoptosis pathway. **a** mRNA expression levels of Cyt-c genes are shown for the control at 24 and 48 h. **b** Caspase-3 activity detection is shown for the control at 24 and at 48 h. The results of real-

time PCR (the data are corrected for the expression of the housekeeping gene GAPDH) and the Caspase-3 Activity Colorimetric Assay Kit from three separate experiments are shown (mean $\pm$ SD, $p<0.05$)

EECs, and significantly reduced the proliferation activity of EECs accompanying with the infection and proliferation of

B.suis.S2. These features suggest that the uterus may play an important role in the amplification of the *B.suis.S2* load, which

Fig. 4 *B.suis.S2* (HK) attacked and *B.suis.S2* infections induce the mRNA expression of ER chaperones. mRNA expression levels of GRP78, ATF4, ATF6, PERK, eIF2 α , IRE1, xbp-1, and CHOP are shown for the control, *B.suis.S2* (HK)-incubated and *B.suis.S2*-incubated cells at

8, 12, 24 and 48 h. The results of real-time PCR from three separate experiments are shown (the data are corrected for the expression of the housekeeping gene GAPDH, mean $\pm$ SD)

Fig. 5 *B. suis*.S2 (HK) and *B. suis*.S2 infections induce the protein expression of ER chaperones. Cell lysates were harvested at the indicated time-points after *B. suis*.S2 (HK) attack and *B. suis*.S2 infections. Immunoblotting was performed as described in the “Materials and methods” section. The expression levels of CHOP were quantified using

densitometry and normalized to the housekeeping protein β-actin. Representative data from three independent experiments are shown using the mean ± SD of two independent experiments performed in duplicate. Bars with different letters are significantly different ($P < 0.05$)

is a key element in the pathogenesis of the disease and is considered a predictor of abortion and of infertility (Baud and Greub 2011).

Previous studies have reported that the smooth virulent *Brucella* strains could inhibit macrophage apoptosis; however, many rough *Brucella* strains are cytotoxic to macrophages and induce macrophage cell death (Gross et al. 2000; Pei et al. 2006). Rough *B. abortus* strains RA1 and RB51 induced the

Fig. 6 Updated model of the EEC response to *B. suis*.S2 infection. *B. suis*.S2 took advantage of its virulent factors to manipulate the ER stress of the EECs to inhibit the proliferation and apoptosis of EECs and to promote its proliferation in EECs

apoptosis and necrosis of macrophages by a caspase-2-mediated and caspase-1-independent pathway (Ogata et al. 2006). The apoptotic mechanisms induced by *Brucella* may be complex in different cell lines, and the virulence factors of *Brucella* strains may play an important role. In this study, the percentages of apoptotic EECs attacked by *B. suis*.S2 (HK) and infected with *B. suis*.S2 were both lower than 5 % by 48 h culture, and there were no obvious apoptotic and necrotic morphological changes, as determined by AO/EB staining, suggesting that *B. suis*.S2 could inhibit caprine EEC apoptosis. Meanwhile, caspase-3 activity was not significantly different in *B. suis*.S2 (HK) attack and *B. suis*.S2 infections during the cultured time, although Cyt-c mRNA levels significantly increased at 48 h. Under ER stress conditions, the transcriptional activation of CHOP by p38 leads to the induction of apoptosis by the release of Cyt-c from the mitochondria because of the activation of Bak or of BAX (Li et al. 2006). However, some studies reported that LPS-induced CHOP expression does not induce apoptosis but induces the IL-1β activation pathway (Yoichiro et al. 2010). The ER stress-CHOP pathway is also involved in the pathogenesis of inflammation (Endo et al. 2005, 2006). This study demonstrated that *B. suis*.S2 (HK)-attacked and *B. suis*.S2-infected EECs can inhibit the EECs viability and cause ER stress and lead to expression changes in

Cyt-c but did not induce apoptosis. The most likely reasons to those amazing results were that the ER function-protective system is enhanced before CHOP expression and that the induction level of CHOP is not enough to overcome this already-induced ER function-protective system, only promoting the development of inflammation.

Related studies indicated that under ER stress conditions, GRP78 dissociates from the ER stress sensors and the ER stress sensors are activated. Although the IRE1-XBP1 pathway was promptly activated, the PERK pathway was not activated in LPS-treated RAW 264.7 cells (Oyadomari and Mori 2004). According to our results, PERK pathway but not IRE1-XBP-1 was activated in *B.suis.S2*-treated EECs. In contrast, both sensor systems were promptly activated in TG-treated RAW 264.7 cells (Gotoh et al. 2002). Our results also confirmed that ER stress induced by *B.suis.S2* was significantly lower than that induced by Tm. The induction of CHOP depends strongly on the PERK system and weakly on the other two sensor systems (Harding et al. 2000). Some studies performed an RNAi screen of 240 genes, including 110 ER-associated genes, for molecules that mediate bacterial interactions with the ER (Qin et al. 2008). We assessed the up-regulation of the GRP78 gene, which is a hallmark of ER stress, and the differential expression of the gene coding for ATF4 and for ATF6, which is one of the key hallmarks of ER stress present in the ER stress pathway (Sano and Reed 2013). The expression levels of the GRP78, PERK, eIF2 α , ATF4, and ATF6 in *B.suis.S2*-infected EECs were basically concomitant with *B.suis.S2* (HK)-infected EECs in the same manner as the mRNA levels. The expression of related genes induced by *B.suis.S2* was obviously stronger than that induced by *B.suis.S2* (HK) at 12 h, while this situation reversed at 48 h. Meanwhile, we also detected that the protein expression levels of CHOP significantly increased in *B.suis.S2* (HK)-attacked and *B.suis.S2*-infected EECs throughout the entire process of infection, but the expression levels of CHOP is very weak comparing with that in Tm-treated group. That is to say, ER stress caused by *B.suis.S2* was significantly lower than that induced by traditional revulsant of ER stress, which is unconventional ER stress.

This result suggests that *B.suis.S2* can manipulate the ER stress reaction to inhibit the viability and apoptosis of host cells and to promote its proliferation (Fig. 6). *Brucella* infections, which are associated with the synthesis of many *Brucella* proteins in a short amount of time, perturb ER homeostasis (Schroder and Kaufman 2005). Thus, we suggest that the demand of the synthesis and folding of high amounts of *Brucella* proteins in such a short period leads to ER stress in *B.suis.S2*-infected EECs. Our results also confirmed that PERK-eIF2 α -ATF4-CHOP pathway was more activated in the process of *B.suis.S2* infection.

We also found that the capacity of *B.suis.S2* to elicit non-apoptotic ER stress in EECs was independent of the ability of

the *B.suis.S2* to replicate, given that HK-treated *B.suis.S2* similarly cause non-apoptotic ER stress. This result indicated that *Brucella* replication-associated products might be non-essential for triggering non-apoptotic ER stress but that virulence factors play a main role in the process. Previous studies have shown that LPS, PrpA, and Btp1/TcpB are three significant immunomodulatory molecules with capacities to interfere with the host immune response (Silva et al. 2012). Thus, we speculate that virulence factors play a decisive role in the process of the endoplasmic reticulum stress response and later in the apoptosis of *B.suis.S2*-infected EECs. Although *Brucella* cannot directly cause uterine lesions, but the uterus usually suffer from all sorts of microbial invasion in domestic.

In summary, our main findings were that *B.suis.S2* induced non-apoptotic ER stress in EECs, which are key target cells. We believe that our findings could provide new approaches for better understanding the molecular and cellular mechanisms underlying the pathogenesis of *B.suis.S2* infection. Additionally, due to the limitation of the experimental objective conditions, we only made a preliminary exploration in this paper, remaining the continuing working in the future.

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