Pseudorabies Virus Infection Induces Endoplasmic Reticulum Stress Through PERK and IRE1 Pathways of Unfolded Protein Response

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Abstract

Pseudorabies virus (PRV) is a pathogen of swine resulting in devastating disease. Some viral infections can cause endoplasmic reticulum (ER) stress and unfolded protein response (UPR) to restore ER homeostasis. However, the mechanism of how PRV induces ER stress and UPR activation remains unclear. Here, levels of proteins or transcriptional factors of three UPR pathways were examined in suspension-cultured BHK-21 cells to investigate PRV-induced ER stress. Results showed that PRV triggered ER stress and UPR of the host cells with the upregulated expression of glucose-related protein 78 kD and 94 kD (GRP78 and GRP94). The protein kinase RNA-like ER kinase (PERK) pathway was activated to upregulate ATF4, CHOP, and GADD34 expression. Additionally, the inositol requiring kinase 1 (IRE1) pathway was triggered by splicing of X box-binding protein 1 (XBP1) mRNA and the enhanced expression of p58^{IPK} and EDEM1. Furthermore, our data demonstrated that PRV took advantage of ER stress to accelerate its replication with the activation of the PERK and IRE1 pathways in suspension-cultured BHK-21 cells, and the glycoprotein B played a crucial role in ER stress.

Introduction

Pseudorabies virus (PRV), a member of the *Alphaherpesviridae* subfamily in the family *Herpesviridae*, consists of a linear dsDNA molecule of 143 kilobases [1, 2], which causes Aujeszky's disease in swine and other animals [3]. Swine is the natural host and reservoir of PRV [4]. Before 2011, immunization with Bartha-K61 vaccine had been proved a safe and effective method to prevent the disease until an unprecedented epidemic of pseudorabies affected Chinese pig herds, causing severe impact on domestic swine production [5].

The endoplasmic reticulum (ER) is a vital organelle involved in cell metabolism, calcium homeostasis, protein synthesis, protein folding, and protein trafficking [6]. Additionally, most secreted transmembrane proteins enter the lumen of the ER for maturation before being transported [7]. Various external stimuli, such as pathogen invasion, glucose deprivation, and chemical treatment, may disrupt the ER homeostasis and lead to the accumulation of unfolded or misfolded proteins, which causes ER stress and a cytoprotective signaling cascade termed as unfolded protein response (UPR) [8–10]. The UPR is an immediate cellular response to mediate physiological fluctuations in the folding load of the ER with three main branches triggered by three transmembrane ER stress sensors: the protein kinase RNA-like ER kinase (PERK), activating transcriptional factor-6 (ATF6), and inositol requiring protein-1 (IRE1) [11–13]. Under unstressed conditions, the ER chaperone 78-kDa glucose-regulated protein (GRP78/BIP) binds to the luminal domains of the sensors to keep them inactive [14]. Although the three branches of UPR are independent, they are tightly bound and intercrossed in a signaling network [15, 16]. Dissociation of GRP78 from these sensors caused by the ER stress results in the activation of these sensors, which triggers the stimulation of a complex signal transduction cascade and downstream activation of UPR related genes [17, 18].

The process of PERK activation by ER stress involves oligomerization, autophosphorylation, and direct phosphorylation on serine residue 51 of the α subunit of eukaryotic initiation factor 2 (eIF2 α). Phosphorylated eIF2 α (P-eIF2 α) prevents the formation of ribosomal initiation complexes, leading to global mRNA translational attenuation. However, P-eIF2 α paradoxically upregulates the translation of several other mRNAs, such as encoding activating transcription factor 4 (ATF4) mRNA. This factor can induce the expression of the transcription factor C/EBP homologous protein (CHOP), which further activates transcription of growth arrest and DNA-damage-inducible protein 34 (GADD34) to direct P-eIF2 α dephosphorylation and to restart global mRNA translation [19, 20]. Both IRE1 and ATF6 play essential roles in activating transcriptional pathways that increase the capacity of protein folding, transportation, and degradation in cells [21, 22]. Activated IRE1 cleaves a 26-nucleotide intron from X-box binding protein 1 (XBP1) mRNA under stress conditions. Spliced XBP1 (sXBP1) mediates regulated IRE1-dependent decay (RIDD) and encodes a basic leucine zipper (b-ZIP) transcription factor that upregulates UPR target genes in the ER-associated protein degradation (ERAD) pathway, such as ER-degradation-enhancing- α -mannosidase-like protein1 (EDEMI) and p58IPK [23]. When unfolded or misfolded proteins accumulate in the ER, ATF6 will be translocated to the Golgi, where it is cleaved by Golgi-resident site 1 and site 2 proteases (S1P and S2P) to generate an activated ATF6(N) b-ZIP (basic leucine zipper) transcription factor [24]. Activated ATF6 translocates to the nucleus to induce genes that encode protein chaperones to restore ER homeostasis, such as GRP78, GRP94, protein disulfide isomerase (PDI), and ER protein 57 (ERp57) [25].

Several studies have reported that virus infection induces ER stress, and some viruses utilize host cell membrane to establish their own membrane components for progeny virions in the process of ER stress [26, 27]. To restore ER homeostasis, some viruses activate pathways of the UPR for their replication in infected cells. On the one hand, UPR upregulates the expression of the ER chaperones to promote virus infection. On the other hand, translational attenuation is also mediated by UPR, such as ERAD, RIDD, and apoptosis, which limits viral replication [28]. The ER stress induced by classical swine fever virus (CSFV) promotes CSFV production, in which the IRE1 pathway plays an important role [29]. Transmissible gastroenteritis virus (TGEV) triggers the PERK-eIF2 α pathway of UPR and negatively regulates TGEV replication, representing a vital aspect of host innate responses to invading pathogens [30]. PRV infection induces ER stress and activates the IRE1-XBP1 pathway in PK-15 cells [31]. However, it remains unclear how the ER stress and UPR are induced by PRV infection in suspension-cultured BHK-21, which served as a vaccine manufacturing cell line.

In this study, we systematically examined the induction of the three branches of the UPR and measured the expression levels of UPR sensor genes to explore UPR activation during PRV infection. This work will provide new insights into understanding the mechanisms of replication and pathogenesis of PRV.

Materials And Methods

Cells, viruses and viral propagation

Adherent BHK-21 cells were cultivated in Dulbecco's minimal essential medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Gibco-BRL, Carlsbad, CA, USA) at 37°C and 5% CO₂. Primary suspension-cultured BHK-21 cells were adapted and cultured in a 250 mL shaking flask with 75 mL of MD910 (Merck KGaA, Darmstadt, Germany) containing 1% FBS on Kühner shaker at 180 rpm, 37°C and 5% CO₂. The PRV-NJ strain was isolated and preserved by our laboratory. The Reed–Muench method was used to determine viral titers, using 50% tissue culture infections dose (TCID₅₀) in adherent BHK-21 cells.

Suspension-cultured BHK-21 cells and viruses were cultured in a set of 4 parallel 1-L bioreactors (Applikon, Netherland). The cultivation conditions were 160 rpm, 37°C, pH-controlled at 7.2, and dissolved oxygen (DO) controlled at 50%. When viable cell density reached 4×10^6 cells/mL, the same volume of media was added, followed by the inoculation of the PRV-NJ strain at a 0.01 multiplicity of infection (MOI). The mock-infection and PRV-infection cells were collected at different hours post infection (hpi) until 48 hpi.

Antibodies and reagents

Antibodies against eIF2 α (9722S), P-eIF2 α (3597S), anti-CHOP (5554T), calreticulin (12238T), ATF6 (65880T), ATF4 (11815s), PDI (3501T), α -Tubulin (2144S), β -Actin (4970T), GAPDH (5014S), anti-FLAG (14793S) were purchased from Cell Signaling Technology. Antibodies against GRP78 (ab21685) and GRP94 (ab3674) were obtained from Abcam. Antibody against GAPDH (10494-1-AP) was purchased from Proteintech. HRP-labelled goat anti-rabbit IgG antibody (62-9520) was purchased from Invitrogen. The ER stress inducer thapsigargin (Tg), the ER stress inhibitor tauroursodeoxycholic acid (TUDCA), and 4-phenyl butyric acid (4-PBA) were obtained from Sigma.

RNA extraction, cDNA synthesis, quantitative real-time PCR (qRT-PCR), and XBP1 splicing assays

PRV-infection samples with the same cell counts at different hours post infection were harvested by centrifugation for 10 min at the speed of 1000 rpm. The supernatant was removed, and total RNA was extracted using RNeasy Mini Kit (Qiagen, Cat.74104) and treated with DNase I before reverse transcription to remove genome DNA. QuantiNova Reverse Transcription Kit (Qiagen, Cat.205413) was used for cDNA synthesis, and QuantiFast SYBR Green PCR Kit (Qiagen, Cat.204054) was used for qRT-PCR in LightCycler 480 II (Roche, Germany). As a loading control β -actin gene of BHK-21 cells was used for the relative quantification. The fold changing values of target genes were calculated by a $2^{-\Delta\Delta C_t}$ method using β -actin as the reference gene. The primers used for this study are listed in Table 1.

For XBP1 splicing level analysis, PCR was performed for 35 cycles (94°C for 30 s, 58°C for 30 s, and 72°C for 30 s) with primers, as listed in Table 1. PCR products were separated and visualized on a 2.0% agarose gels by digesting with *Pst*I enzyme under UV ImageQuant. The sizes of the PCR products corresponding to the unspliced XBP1 mRNA (uXBP1) were 317bp and 213 bp.

Western blot

Equal amounts of PRV-infection cells were lysed using RIPA lysis buffer, supplemented with phosphatase and protease inhibitor cocktails (PMSF, Beyotime). The proteins were separated on 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated overnight with primary antibodies at 4°C. Followed by washing three times with TBST, the membranes were incubated with IgG HRP-linked secondary antibodies for 1 h at 37°C, exposed to ECL-2 western blot substrate (Thermo Scientific, Pierce), and imaged using a Tanon 5200 system (Biotanon, Shanghai, China). The protein bands were detected and processed by ImageJ2 software.

Chemical treatment

Three chemicals with different concentrations (0.001, 0.005, 0.01 and 0.02 mM Tg; 20, 40, 80 and 160 µM TUDCA; 0.5, 1, 2 and 4 mM 4-PBA) were respectively supplemented into culture media in combination with PRV inoculation at MOI 0.01 for 48 hours. All the chemicals were maintained during the whole course of virus infection and replication until the virus was harvested. The Counting Kit-8 (CCK-8, Dojindo) was used to measure cell viability. The viral titer was detected as mentioned above.

Transient expression of the PRV structural proteins

The coding sequences of PRV glycoproteins B, C, D, H, I, L, and M (gB, gC, gD, gH, gI, gL, and gM) were synthesized with Flag tag at C-terminal (Tsingke Biotechnology, Jiangsu, China) and cloned into the pcDNA3.1 (-) vector respectively. When BHK-21 cells were incubated to 70% confluence in 6-well culture plates, transient expression of different PRV glycoproteins was performed by transient transfection using Attractene transfection Reagent (Qiagen, Cat.301005) according to the manufacturer's instructions. After 48 hours post transfection, the transfected cells with pcDNA3.1-glycoproteins and pcDNA3.1 empty vector (negative control) and PRV-infection cells (positive control) were lysed using RIPA lysis buffer and analyzed by western blot.

Data analysis

The data were presented as the mean $\pm$ standard deviation (SD). Two-way ANOVA test was applied for statistical significance assessment, and the difference was presented as $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

Results

PRV infection induces ER stress in suspension-cultured BHK-21 cells

The suspension-cultured BHK-21 cells were infected at an MOI of 0.01, and viral titers were detected by TCID₅₀ assay. The growth curve of PRV illustrated that the viral titer exponentially increased and reached a maximal value (8.1 lgTCID₅₀/mL) at 48 hpi (Fig. 1B). The relative cell viability decreased with the increase of PRV replication compared to mock cells (Fig. 1A).

We measured the expression and transcription levels of the ER stress markers GRP78 and GRP94 by western blot and qRT-PCR. As shown in Fig. 1C, 1D, and 1E, proteins and mRNA expression levels of GRP78 and GRP94 were significantly upregulated from 36 hpi in PRV-infection BHK-21 cells compared with mock-infection cells, which suggested that ER stress was potentially triggered by PRV infection and replication afterward.

PRV infection triggers the PERK pathway

The results showed that the level of phosphorylated eIF2 α was upregulated at 36 hpi (Fig. 2A, 2B). We also measured CCAAT/enhancer-binding protein homologous protein (CHOP), cyclic AMP-dependent transcription factor 4 (ATF4), growth arrest, and DNA damage-inducible protein 34 (GADD34). Expectedly, PERK-eIF2 α activation following PRV infection resulted in elevated expression of CHOP, ATF4, and GADD34 downstream of eIF2 α (Fig. 2A, 2B and 2C). Overall, results suggested that PRV infection activated the PERK pathway.

PRV infection activates the IRE1 pathway

Later, splicing in the XBP1 gene, a characteristic marker for activation of IRE1 signaling, was detected for the IRE1 branch investigation. Activated IRE1 spliced a 26-nucleotide (nt) segment from the unspliced XBP1 (uXBP1) mRNA with its endoribonuclease activity. The translation of this mRNA produced the active 371-amino acid isoform and spliced XBP1 (sXBP1). Splicing of uXBP1 results in loss of the *Pst*I restriction site located in the intron. The results showed that the spliced form of XBP1 increased from 36 h by PRV infection, and the RT-PCR products of PRV-infection and mock-infection before 24h were digested by *Pst*I to produce both the 317 bp and 213 bp fragments (Fig. 3A). The activation of the IRE1-XBP1 axis by PRV infection was further confirmed by measuring the transcriptional level of the XBP1 downstream target p58^{IPK} and EDME1 genes (Fig. 3B), suggesting that PRV infection activated the IRE1 pathway under ER stress.

PRV infection does not trigger the ATF6 pathway

An active 50 kDa variant of ATF6 comprising the N-terminus was released by splitting full-length ATF6 protein (90 kDa) in response to the ER stress. To determine whether PRV infection induces the ATF6 pathway of the UPR or not, we compared protein and the mRNA levels of endogenous ATF6 and the chaperones calreticulin, calnexin, ERp57, and PDI using qRT-PCR (Fig. 4A, 4B and 4C). No significant differences were observed in the mRNA level of these genes with infection time elapsed, suggesting that the ATF6 pathway was not activated by PRV infection.

PRV-induced ER stress facilitates viral replication

As mentioned above, our results showed that PRV infection induced ER stress in host cells. The effects of ER stress inducer Tg, inhibitor TUDCA, and 4-PBA treatments on PRV replication were further investigated. Different concentrations of Tg, TUDCA, or 4-PBA were supplemented into culture media in combination with PRV inoculation at MOI 0.01. Different concentrations of chemicals were respectively maintained

during the whole course of virus infection and replication until the virus was harvested at 48 hpi. Our data showed that relative cell viability was not affected at lower Tg concentrations (0.001 and 0.005 μM), compared with mock-infection at 48 h. However, under intense ER stress circumstances, when the concentration of Tg was increased to 0.01 μM , cell viability dramatically reduced (18% reduction) (Fig. 5A). The viral titers significantly increased by almost 0.8 $\text{lgTCID}_{50}/\text{mL}$ at Tg concentrations of 0.005 and 0.01 μM compared to untreated cells (Fig. 5B). Additionally, TUDCA and 4-PBA treatment showed no obvious effect on cell viability (Fig. 5A), but viral titers decreased with increasing TUDCA and 4-PBA concentration (Fig. 5B). Overall, these results demonstrated that the ER stress induced by Tg promoted PRV replication.

PRV structural proteins trigger UPR pathways

The structural proteins of PRV were major effectors for virus entry, virion packaging, virion egress, and cell-to-cell viral spread. To further determine whether the glycoproteins, such as gB, gC, gD, gH, gI, gL, and gM, could trigger UPR pathways, the transient overexpression of these glycoproteins was conducted in BHK-21 cells, respectively. The results indicated that these glycoproteins were successfully expressed in BHK-21 cells (Fig. 6A). Importantly, our results in Fig. 6B and D showed that the overexpression of gB, gD, and gI significantly enhanced GRP78 and GRP94 expression in BHK-21 cells according to immunoblotting analysis. Furthermore, gB expression upregulated the level of phosphorylated eIF2 α , and no significant changes were found in the transfected cells with pcDNA3.1-glycoproteins compared with control (pcDNA3.1 transfected cells) in the ATF6 pathway (Fig. 6B and D). The RT-PCR products were mostly detected by the spliced form of XBP1 in the transfected cells with pcDNA3.1-gB, whereas the RT-PCR products were digested by *Pst* I to generate 317bp and 213bp fragments in other the transfected cells with pcDNA3.1-glycoproteins (Fig. 6C). These results showed that gB triggered PERK and IRE1 pathways and played a key role in ER stress.

Discussion

Viruses develop different kinds of complex strategies to facilitate their propagation through regulating host cell responses and escaping from certain defense mechanisms. ER stress and the UPR pathways induced by virus invasion have been found in many host cells [32–34]. Kaposi's sarcoma-associated herpesvirus (KSHV) infection was involved in the activation of three UPR signaling pathways, but some of UPR downstream transcriptional responses were restricted to support lytic replication [35]. Alphaherpesvirus entry into cells required the coordinated action of several structures on the viral surface. The attachment is mediated by multiple viral glycoproteins and a variety of binding receptors. The glycoprotein B is the viral fusion protein, which is responsible for insertion into the host cell membrane and refolding to drive fusion of the viral envelope and cell membrane[36]. However, whether PRV induces ER stress of the host and activates the UPR pathways, and their correlation with viral production and structural proteins are not clear. This study will provide new insights into the mechanisms of replication and pathogenesis of PRV.

Molecular chaperones, such as GRP78 and GRP94, are stress-response marker proteins whose overexpression triggers host cells under stress. Our study showed that the expression level of GRP78 and GRP94 were upregulated in PRV-infection cells at 36 hpi, suggesting that the ER stress and UPR were activated. Compared with the occasion in other studies [31], the upregulation of GRP78 occurred later by PRV infection in this work. It could be attributed to the low MOI and cell host. Here we used MOI 0.01, which had been optimized as an optimum inoculating parameter in our suspension-cultured BHK-21 cells for PRV vaccine production in bioreactors, while others cultured PRV with adherent cells at a higher MOI of 1.0 in T25 flasks. [37, 38].

Meanwhile, we examined the three branches of UPR pathways, termed PERK, IRE1, and ATF6, using western blot and qRT-PCR. Among the three branches, the PERK pathway was considered to be activated at the early stage of ER stress during virus infection [39], and eIF2 α was phosphorylated at Ser51. However, if the ER stress continues, a downstream signaling molecule of the PERK will be activated, such as ATF4, which further accelerates CHOP expression [40]. In this study, enhanced expression of both ATF4 and GADD34 was observed at 36 hpi. CHOP mRNA expression level significantly increased from 24 hpi and up to 40 folds at 48 hpi, which was initiated a little earlier than other downstream proteins of PERK. We speculated that CHOP was sensitized and activated by another signal cascade [41]. These results showed that PRV infection activated the PERK pathway. IRE1 was critical for the UPR, as it stimulated associated proteins in ER stress. Our results revealed that during PRV infection, the IRE1 pathway was induced at 36 hpi, as demonstrated by the presence of sXBP1 and upregulation of p58^{IPK}, which played a necessary role in the regulation of protein biosynthesis and folding. Although ATF6, a type II ER transmembrane protein, has been verified with a stress sensing domain in the ER lumen and basic region/leucine zipper (bZIP) domain in the cytosol [42], cleaved ATF6 was not found in this work, and other changes of downstream proteins involved ATF6 pathway were also not observed during PRV infection, suggesting the ATF6 pathway was not triggered by PRV infection.

Besides the well-established role of ER stress, several researchers have reported that ER stress has more association with cells apoptosis, autophagy, and innate immunity responses for some viruses [43–45]. Li and colleagues reported that Newcastle disease virus (NDV) promoted cells apoptosis and cytokines secretion by the induction of eIF2 α -CHOP-BCL-2/JNK and IRE1 α -XBP1/JNK signaling cascades, contributing to NDV proliferation [46]. Porcine circovirus type 2 (PCV2) infection or Cap expression induced open reading frame 3 (ORF3)-independent apoptosis by increased cellular reactive oxygen species (ROS) levels and cytosolic and mitochondrial Ca²⁺ levels [40, 47]. Avian metapneumovirus subgroup C induced autophagy through the ATF6 UPR pathway [48], and the IRE1 of ER stress subsequently stimulated natural killer cell immunity in part by regulating c-Myc in NK cells [49].

As an ER stress inducer with the typical function of inhibiting Ca²⁺-ATPase [50], Tg with optimum concentration (0.005 and 0.01 μ M) enormously enhanced PRV proliferation in suspension-cultured BHK-21 cells. On the contrary, PRV titer decreased significantly with the increasing addition of TUDCA and 4-PBA, ER stress inhibitors, which can relieve ER stress and stabilize the UPR [51]. Combined with the results achieved above, it could be concluded that PRV infection induced ER stress in suspension-cultured

BHK-21 cells. Meanwhile, PRV took advantage of ER stress to accelerate its replication. Viroporins, such as coronaviruses, hepatitis C virus (HCV), and influenza A virus (IAV), had been demonstrated to localize to the host ER or its associated membrane networks and play crucial roles in the entry, genome replication, assembly, and release during virus infection [52]. Chikungunya (CHIKV) infection can selectively activate ATF6 and IRE1, but suppress the PERK pathway since CHIKV non-structural protein 4 (nsP4) significantly reduced the phosphorylation (Ser 51) of eIF2 α to ensure translation of viral proteins [53]. Classical swine fever virus can efficiently mediate calcium permeability in the ER by utilizing p7 protein [54]. Therefore, the interaction between ER stress-associated proteins and PRV structural or non-structural proteins would be the major concern in future work.

Conclusion

In summary, for the first time, our study reported that PRV infection induced ER stress and UPR activation in suspension-cultured BHK-21 cells (Fig. 7). The results indicated that the activation of the PERK and IRE1 pathways could benefit PRV replication in suspension-cultured BHK-21 cells, and the glycoprotein B played a key role in ER stress. These data offered details about the intracellular signaling events in PRV infected cells and extended our perspective regarding the mechanisms of ER stress and the UPR pathways.

Declarations

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Conflict of Interest

The authors declare no conflict of interest.

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Table

Table 1 Primers used in this study

Gene	Primer sequence (5'-3')	Size/bp	Application
GRP78	F: TCATCGGACGCACTTGGAA	162	qRT-PCR
	R: TAGTGAGAACCATGGCAGAA		
GRP94	F: CCGAGTTTGATGGGAAGAGGTT	206	qRT-PCR
	R: GGCCACAAGAGCACAAGGAGAT		
ATF4	F: AGCAAAACAAGACAGCAGCCACTA	177	qRT-PCR
	R: TTGCCTTACGGACCTCCTCTATCA		
CHOP	F: AAGAGGAAGATCAAGGAAGAACTA	207	qRT-PCR
	R: CCATGCGGTCAATCAGAG		
GADD34	F: AGCAGCTGACCGAGGCAAGAG	144	qRT-PCR
	R: TTAGGGGCGGTCCAAGGTGA		
XBP1	F: GAGAAGGCGCTGCGGAGGAACTG	530	RT-PCR
	R: GAGAAAGGGAGGCTGGTAAGGAAC		
p58 ^{IPK}	F: AGATGGCGACCCTGATAACTA	246	qRT-PCR
	R: GACTGGGCTTCCTTCTCTTC		
EDEM1	F: GGAAGTCTCCTTTCTGCTCATAG	155	qRT-PCR
	R: GGAATCCCTGTCTTGGTGTT		
Calnexin	F: TGCCGAGCCAGGTGTAGTG	230	qRT-PCR
	R: CCTCTTCATCCCCCTTGTTCTT		
Calreticulin	F: GGAGCCTGCCGTCTACTTC	220	qRT-PCR
	R: GGTCTGGCCCTTGTTACTGA		
ERp57	F: CTAGGACTGCCGATGGGATTGT	180	qRT-PCR
	R: AGTTGCTGGCTGCTTTTAGGAA		
PDI	F: CCCCGGAGGAGGAGGACAAC	105	qRT-PCR
	R: CACACCACGGGGCATAGAAC		
β -actin	F: CCAGGTCATCACCATTGGCAACG	153	qRT-PCR
	R: TTGGCATAGAGGTCTTTGCGGATGT		

Figures

Figure 1

PRV infection induced ER stress in suspension-cultured BHK-21 cells. The cells were infected by PRV at an MOI of 0.01. Mock-infection and PRV-infection cell pellets were collected at 12, 24, 36, and 48 h. (A) The profile of viral titer and relative cell viability of mock-infection and PRV-infection cells. (B) The protein levels of GRP78 and GRP94 were confirmed by western blot; GAPDH was used as a loading control. (C) The relative expression of the targeted proteins/GAPDH was analyzed by densitometric scanning. (D) The

transcriptional levels of GRP78 and GRP94 were measured by qRT-PCR at different time points from 12 to 48 h using β -actin as the reference gene. Means and SD of the results from three independent experiments are shown; Two-way ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2

PRV infection activated the PERK pathway in suspension-cultured BHK-21 cells. The cells were infected by PRV at an MOI of 0.01. Mock-infection and PRV-infection cell pellets were collected at 12, 24, 36, and 48 h. (A) The protein levels of total eIF2 α , P-eIF2 α , ATF4, and CHOP were confirmed by western blot; Tubulin was used as a loading control. (B) The relative expression of the targeted proteins/Tubulin was analyzed by densitometric scanning. (C) The transcriptional levels of the PERK downstream pathway ATF4, CHOP, and GADD34 were measured by qRT-PCR at different time points from 12 to 48 h using β -actin as the reference gene. Means and SD of the results from three independent experiments are shown; Two-way ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 3

PRV infection activated the IRE pathway by in suspension-cultured BHK-21 cells. The cells were infected by PRV at an MOI of 0.01. Mock-infection and PRV-infection cell pellets were collected at 12, 24, 36, and 48 h. (A) XBP1 mRNA was amplified by RT-PCR using XBP1-specific primers, after which the XBP1 fragment was digested by Pst I, and the products were separated by 2% agarose gel electrophoresis. (B) The transcriptional levels of the IRE pathway downstream of p58^{IPK} and EDEM1 were measured by qRT-PCR at different time points from 12 to 48 h using β -actin as the reference gene. Means and SD of the results from three independent experiments are shown; Two-way ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4

PRV infection didn't activate the ATF6 pathway in suspension-cultured BHK-21 cells. The cells were infected by PRV at an MOI of 0.01. Mock-infection and PRV-infection cell pellets were collected at 12, 24, 36, and 48 h. (A) The protein levels of ATF6 and PDI were confirmed by western blot; GAPDH was used as a loading control. (B) The relative expression of the targeted proteins/GAPDH was analyzed by densitometric scanning. (C) The transcriptional levels of ATF6 pathway downstream Calnexin,

Calreticulin, ERp57, and PDI were measured by qRT-PCR at different time points from 12 to 48 h using β -actin as the reference gene. Means and SD of the results from three independent experiments are shown; Two-way ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 5

Chemicals of ER stress affected PRV infection. The suspension-cultured BHK-21 cells were treated with Tg concentrations of 0.001, 0.005, 0.01, and 0.02 μ M, TUDCA concentrations of 20, 40, 80, and 160 μ M and 4-PBA concentrations of 0.5, 1, 2, 4 mM and the cells were infected with PRV for 48 h at the same time. (A) Cell viability was measured using CCK-8 at 48h as described in Materials and methods. (B) Viral titers were measured by TCID₅₀, respectively. Means and SD of the results from three independent experiments are shown; Two-way ANOVA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 6

PRV structural proteins triggered UPR pathways. BHK-21 cells were transfected with pcDNA3.1 vector carrying target genes (glycoproteins B, C, D, H, I, L, or M). The transfected with pcDNA3.1 empty vector and PRV-infection BHK-21 cells were used as negative and positive controls. (A) The overexpression of structural proteins in BHK-21 cells was confirmed by Western blot. (B) The protein levels of GRP78, GRP94, total eIF2α, P-eIF2α, and ATF6 were confirmed by Western blot; GAPDH was used as a loading control. (C) XBP1 mRNA of the overexpressed structural proteins was amplified by RT-PCR using XBP1-specific primers, after which the XBP1 fragment was digested by Pst I, and the products were separated by 2% agarose gel electrophoresis. (D) The relative expression (B) of the targeted proteins/GAPDH was analyzed by densitometric scanning.

Figure 7

Schematic representation of PRV activation and influence over the UPR. PRV infection activated PERK and IRE1 pathways of UPR, but not the ATF6 pathway. Phosphorylated eiF2α upregulated the expression of ATF4, which induced the upregulation of CHOP and GADD34. GADD34 recruited PP1 to the ER to dephosphorylate eiF2α and promoted the return to homeostasis. Activated IRE1 facilitated the splicing of XBP1 mRNA, which encoded a transcription factor leading to the expression of the UPR target genes. Arrows represented the activation of components or processes in the ER stress pathways upon viral infection and pathogenesis. ✓:activated; ✗:non-activated.