Involvement of unfolded protein response, p53 and Akt in modulation of porcine reproductive and respiratory syndrome virus-mediated JNK activation

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Abstract

Our previous study has shown that activation of JNK plays a critical role in Porcine reproductive and respiratory syndrome virus (PRRSV)-mediated apoptosis. In this follow-up study, we further investigated the mechanisms involved in modulation of PRRSV-mediated JNK activation and apoptosis. We found that unfolded protein response (UPR) was induced in response to PRRSV infection which in turn triggered JNK activation and apoptosis. We also found that p53 and Akt were activated at the early stage of infection and functioned as negative regulator of JNK activation to counteract the PRRSV-mediated apoptosis. Furthermore, induction of UPR, p53 and Akt was not only involved in modulation of PRRSV-mediated apoptosis, but also contributed to the virus replication. Our findings indicated that multiple signaling pathways were involved in modulation of PRRSV-mediated apoptosis of the host cells via regulating JNK signaling pathway and provided novel insights into understanding the mechanisms of pathogenesis of PRRSV infection.

Introduction

The endoplasmic reticulum (ER) is a multifunctional signaling organelle that controls a variety of biological processes. One major function of ER is to serve as the protein-folding factory. The status of protein-folding in ER is tightly monitored and regulated by a primitive, evolutionary conserved signaling pathways, collectively termed the unfolded protein response (UPR) or ER stress response (Walter and Ron, 2011). Three ER resident transmembrane proteins ATF6 (activating transcription factor 6), IRE1 (inositol requiring enzyme 1) and PERK (double-stranded RNA-activated protein kinase (PKR)-like ER kinase) are the stress sensors of ER. Activation of these sensors results in increase of ER protein-folding capacity and decrease of ER protein load. The final outcome of UPR is mitigation of ER stress. However, prolonged activity of the UPR could lead to cell death (Tabas and Ron, 2011). Growing evidence shows that UPR signaling pathways play a critical role in modulation of apoptosis of host cells in response to virus infection (Mahoney et al., 2011; Rodrigues et al., 2012; Wang et al., 2012). The mechanisms involved in UPR modulation of cell death include activating c-Jun N-terminal kinases (JNK)-mediated apoptotic signaling pathways (Ye et al., 2008), activation of ER-resident caspase-12 (Roberson et al., 2012), and modulating functions of Bcl-2 family proteins such as Noxa and Mcl-1 (Rosebeck et al., 2011; Huang et al., 2011).

Porcine reproductive and respiratory syndrome virus (PRRSV) is the etiologic agent of porcine reproductive and respiratory syndrome (PRRS) that causes great economic losses worldwide each year (Lunney et al., 2010). Apoptosis induction of the host cells plays a critical role in pathogenesis of PRRSV infection (Suarez, 2000; Karnyuchuk et al., 2011). It has been demonstrated in our previous study that activation of JNK signaling pathway is a key event in apoptosis induction of the host cells in response to PRRSV infection (Yin et al., 2012). The purpose of the present study was to further decipher the mechanisms involved in modulation of PRRSV-induced JNK activation and apoptosis induction. We found that UPR was induced in response to PRRSV infection, which in turn triggered JNK activation and apoptosis. In addition, p53 and Akt were activated at the early stage of infection and functioned as negative regulator of JNK activation to counteract the PRRSV-mediated apoptosis. Our findings indicated that multiple signaling pathways were involved in modulation of PRRSV-mediated apoptosis of the host cells via regulating JNK signaling pathway.
Results

Activation of UPR signaling pathways contributed to JNK-mediated apoptosis in response to PRRSV infection

Marc-145 cells were infected with PRRSV at MOI = 1. The whole cell lysates from both adherent and floating cells were then prepared at 12 or 24 h after infection. Western blotting was used to analyze changes of ER stress markers in response to PRRSV infection. As shown in Fig. 1A (left), PRRSV infection significantly increased phosphorylation of the ER resident kinase PERK and IRE1, but did not affect expression of ER chaperone BIP. In line with PERK and IRE1 induction, phosphorylation of elf2α and expressions of CHOP and XBP1s were significantly induced in response to the virus infection. The data suggest that PERK and IRE1, the two arms of the UPR are activated in response to PRRSV infection in Marc-145 cells. To investigate if UPR was also activated by PRRSV in porcine alveolar macrophages (PAM), the in vivo target cells of the virus, we analyzed the changes of ER stress-related proteins using western blotting. As shown in Fig. 1A (right), similar changes of the ER stress-related proteins were also observed in PAM, suggesting the general applicability of UPR activation by PRRSV in the host cells.

Fig. 1. Activation of UPR signaling pathways contributed to JNK-mediated apoptosis in response to PRRSV infection. (A) PRRSV induced activation of UPR in Marc-145 cells (left) and porcine alveolar macrophages (PAM) (right). The cells were infected with PRRSV at MOI = 1. At 12 and 24 h post-infection, the cells were harvested for western blot analysis of ER stress markers. (B) Effect of IRE1 inhibition by its inhibitor 3-Ethoxy-5,6-dibromosalicylaldehyde (DBS) on PRRSV-induced JNK activation. The cells were infected with PRRSV in the presence or absence of DBS for 24 h and phosphorylation of JNK and its a substrate c-jun was examined by western blotting; and (C) Effect of IRE1 inhibition on PRRSV-induced apoptosis. The cells were infected with PRRSV in the presence or absence of DBS for 48 h and cell death was measured by Cell Death ELISA Kit (n = 3, p < 0.05).
It has been shown that IRE1 can induce JNK activation through ASK1 (Nishitoh et al., 2002). We then asked if IRE1 induction played a role in PRRSV-mediated JNK activation. We tested effects of IRE1 inhibition by its inhibitor 3-ethoxy-5,6-dibromosalicylaldehyde (Volkmann et al., 2011) on PRRSV-mediated JNK activation measured by Western blotting. As shown in Fig. 1B, inhibition of IRE1 led to a dramatically attenuation of PRRSV-mediated phosphorylation of JNK and its substrate c-jun. We next examined apoptosis induction by PRRSV infection under the condition that IRE1 activation was inhibited by its inhibitor. As shown in Fig. 1C, PRRSV-induced apoptosis was significantly decreased in the presence of IRE1 inhibitor ($p < 0.05$), which was consistent with suppression of JNK activation by the inhibitor. Together, these results indicated that IRE1 activation was a possible mediator to trigger JNK activation in response to PRRSV infection.

**PRRSV replication was inhibited by suppression of IRE1 activation**

It has been shown that UPR signaling pathways including IRE1 play a critical role in a number of viruses’ infection (Hassan et al., 2012; Huang et al., 2005). To determine the role of IRE1 induction in PRRSV replication, we examined effect of IRE1 inactivation on virus yield. The culture supernatant was collected at 48 h post-infection and virus titers were measured as described in materials and methods (A, $p < 0.01$; the infected cells were fixed and the nucleocapsid protein of PRRSV SDOW17 was examined by immunofluorescence assay (B).

**p53 was a negative regulator of PRRSV-mediated-JNK activation and apoptosis**

Our previous study has shown that p53 can negatively regulate ochratoxin A-induced JNK activation (Li et al., 2011). We next asked if p53 was also involved in modulation of PRRSV-mediated
JNK activation. Marc-145 cells were infected with PRRSV at MOI = 1 for 12, 24, 48 and 72 h. Western blotting was used to analyze changes of p53 phosphorylation and its transcriptional target p21; (B) effects of p53 activator nutlin-3 on PRRSV-induced JNK phosphorylation. The cells were infected with PRRSV in the presence or absence of p53 activator Nutlin-3 for 48 h and western blotting was used for detection of JNK phosphorylation; and (C) effect of nutlin-3 on PRRSV-induced apoptosis. The cells were infected with PRRSV in the presence or absence of nutlin-3 for 48 h and cell death was measured by Cell Death ELISA Kit (n = 3, p < 0.01).

Activation of p53 promoted PRRSV replication

To determine the role of p53 in modulation of PRRSV replication, we first measured effects of p53 inactivation by its inhibitor PFT on virus yield. The culture supernatant was collected at 24 h post-infection and virus titers were measured as described in Materials and Methods. As shown in Fig. 4A (left), treatment with p53 inhibitor significantly decreased virus titers compared to mock-treated virus control (p < 0.05), suggesting activation of p53 facilitated PRRSV replication. The enhancing effect of p53 in virus replication was further confirmed by immunofluorescence staining of PRRSV SDOW17 protein. As shown in Fig. 4A (right), the expression of SDOW17 protein was dramatically suppressed when p53 was inhibited by its inhibitor. We next investigated influence of p53 induction by its activator nutlin-3 on virus yield. As we expected, the virus titers were significantly increased in the presence of p53 activator (Fig. 4B, left), which was well correlated with an enhanced PRRSV SDOW17 protein expression (Fig. 4B, right). These results together indicated that p53 was a positive regulator of PRRSV replication.

Activation of Akt suppressed PRRSV-mediated apoptosis via inhibition of JNK activation

It has been shown that the PI3K/Akt pathway can inhibit influenza A virus-induced apoptosis via negatively regulating the JNK pathway (Lu et al., 2010). We then asked if Akt could be also involved in modulation of PRRSV-mediated JNK activation. The cells were infected with PRRSV and then the status of Akt phosphorylation at different time points after infection was assessed by western blotting. As shown in Fig. 5A, PRRSV infection led to a rapid increase in phospho-Akt levels. However, the levels of Akt phosphorylation started to significantly decreased 48 h post-infection. To determine the role of Akt in modulation of PRRSV-mediated JNK activation, we tested effects of Akt inactivation by a PI3K/Akt inhibitor LY294002 on JNK phosphorylation induced by PRRSV infection. As shown in Fig. 5B, phosphorylation of JNK and its substrate c-jun induced by PRRSV infection was further enhanced under the condition that Akt was inhibited by LY294002, suggesting a suppressive role of Akt in PRRSV-mediated JNK activation. Consistent with the inhibitory effect of Akt on JNK activation, apoptosis induction by PRRSV infection was further increased when Akt was blocked by its inhibitor (Fig. 5C). These results suggested that Akt activation inhibited host cell apoptosis early in infection through suppression of JNK activation.

Inhibition of Akt decreased PRRSV replication

Akt has been also found to regulate a number of viruses’ infection (Wei et al., 2012; Urata et al., 2012; Patel and Hardy, 2012). To investigate the role of Akt in modulation of PRRSV replication, we first measured effects of Akt inactivation by its inhibitor LY294002 on virus yield. The culture supernatant was collected at 24 h post-infection and virus titers were measured as described in materials and methods. As shown in Fig. 6A, treatment with PI3K/Akt inhibitor significantly decreased virus titers compared to mock-treated virus control (p < 0.05), suggesting activation of Akt at early stage of infection facilitated PRRSV replication. To further confirm the role of Akt in PRRSV replication, we next measured expression of the virus protein SDOW17 using immunofluorescence staining. As shown in Fig. 6B, the expression of SDOW17 was significantly inhibited under the condition in which Akt activation was blocked by its inhibitor. Together, these results suggested that activation of Akt in response to PRRSV infection at early stage was in favor of the virus replication.

Discussion

Our previous study has shown that activation of JNK plays a critical role in PRRSV-mediated apoptosis. In this follow-up study,
we further investigated the mechanisms involved in modulation of PRRSV-mediated JNK activation and apoptosis. Our findings revealed that induction of ER residue kinase IRE1 contributed to PRRSV-mediated JNK activation, whereas activation of p53 and Akt at the early stage of infection negatively regulated PRRSV-mediated JNK activation. These multiple signaling pathways were coordinated to modulate apoptosis induction in response to PRRSV infection. Our findings provided novel mechanistic explanations for PRRSV-mediated JNK activation and apoptosis induction.

Previous studies have shown that ER stress plays an important role in viral pathogenesis via either modulation of virus replication (Hassan et al., 2012; Huang et al., 2005) or apoptosis induction (Mahoney et al., 2011; Rodrigues et al., 2012; Wang et al., 2012). In the present study, we found that IRE1 and PERK, the two key sensors of ER, were activated in response to PRRSV infection (Fig. 1A). Inhibition of IRE1 resulted in a significant attenuation of PRRSV-induced JNK and its downstream target c-jun phosphorylation, followed by apoptosis reduction (Fig. 1B,C). Moreover, IRE1 activation also facilitated the virus replication (Fig. 2A,B). Our findings provided the first evidence that ER stress was involved in the pathogenesis of PRRSV infection through not only modulation of JNK-mediated apoptosis, but also affected the virus replication. Targeting ER might be a useful approach to fight against PRRSV infection.

p53 is the first identified and the best known tumor suppressor that controls cell cycle checkpoints and apoptosis and DNA repair (Vogelstein et al., 2000). The pro-apoptotic effect of p53 has been well documented (Haupt et al., 2003). However, a growing body of evidence suggests that this protein is also involved in cellular pro-survival response (Li et al., 2011; Yamagishi et al., 1997; Mercer and Bennett, 2006; Garner and Raj, 2007; Amin et al., 2010). It has been shown that some viruses including PRRSV have evolved a variety of strategies to inhibit host cell apoptosis early in infection to ensure their replication (Costers et al., 2008; Nguyen et al., 2006; Sharma-Walia et al., 2010; Liu et al., 2012; Arslan et al., 2012). However, the mechanisms involved in PRRSV-mediated
anti-apoptotic effect at the early stage of infection have not been well elucidated. In the present study, we found that p53 was activated at the early stage of PRRSV infection (Fig. 3A). Activation of p53 suppressed PRRSV-mediated JNK activation and apoptosis induction (Fig. 3B,C), suggesting activation of p53 is a pro-survival signaling, which was in favor of virus replication early infection. This notion was supported by the evidence that the virus titers were increased when p53 was activated by its activator nutlin-3 and IRE1 inhibitor 3-ethoxy-5,6-dibromosalicylaldehyde (DBS) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, MO). A monoclonal anti-PRRSV nucleocapsid (N) antibody SDOW17 was kindly provided by Professor Wenhai Feng (State Key Laboratory for Agrobioengineering, Department of Microbiology and Immunology, College of Biological Science, China Agricultural University).

**Conclusions**

In summary, PRRSV infection caused ER stress response which in turn led to activation of JNK-mediated apoptotic signaling pathways. Activation of p53 and Akt at the early stage of infection negatively regulated ER-dependent JNK activation to control apoptosis process, which was in favor of the virus replication. Our findings provided novel insights into understanding the mechanisms of pathogenesis of PRRSV infection.

**Materials and methods**

**Cell and virus**

Marc-145 (CRL-12231) cells, a subclone of the African green monkey kidney epithelial cell line that is highly permissive for PRRSV replication (Kim et al., 1993), were grown in DMEM (Dulbecco’s Modification of Eagle’s Medium) supplemented with 10% fetal bovine serum. CH-1a, the first PRRSV strain isolated in China, was employed for the study.

**Chemicals and reagents**

Antibodies specific for phospho-JNK, phospho-c-Jun, phospho-p53, phosphor-Akt, Bax, p21 and β-actin were purchased from Cell Signaling Technology (Beverly, MA). The JNK inhibitor SP600125 and PI3K/Akt inhibitor LY294002 were purchased from CalBiochem (La Jolla, CA, USA). The p53 inhibitor pifithrin-α (PFT) was purchased from Alexis Biochemicals (Carlsbad, CA). The p53 activator nutlin-3 and IRE1 inhibitor 3-ethoxy-5,6-dibromosalicylaldehyde (DBS) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, MO). A monoclonal anti-PRRSV nucleocapsid (N) antibody SDOW17 was kindly provided by Professor Wenhai Feng (State Key Laboratory for Agrobioengineering, Department of Microbiology and Immunology, College of Biological Science, China Agricultural University).

**Apoptosis evaluation**

Apoptosis was assessed by a cell death detection ELISA kit to detect cytoplasmic histone-associated-DNA-fragments purchased from Roche Diagnostics. Briefly, cells were cultured in 6-well plates for the desired duration. The spent medium containing floating cells was saved and kept on ice. The adherent cells were collected by gentle trypsinization and were combined with the floaters for pelleting by centrifugation. After gentle lysis of the cells with the buffer provided with detection kit, the cell lysate was used for the ELISA test.

**Western blotting**

The cell lysate was prepared in ice-cold radioimmunoprecipitation assay buffer. Cell lysates were resolved by electrophoresis and transferred to a PVDF (polyvinylidene fluoride) membrane. The blot was then probed with primary antibody followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies. The signal was visualized by enhanced chemiluminescence (Pierce) and recorded on an X-ray film (Kodak).

**Virus yield assay**

Marc-145 cells were infected with PRRSV in the presence or absence of the inhibitors. The culture supernatant was collected at 48 h post-infection. Titrations were performed on Marc-145 cells
in 96-well microtitre plates. The plates were incubated for 3 days and infectivity was analyzed by virus-induced cytopathogenic effect. The titer was calculated according to the method of Reed and Muench (Reed and Muench, 1938).

**Immunofluorescence assay for PRRSV protein detection**

Marc-145 cells were infected with PRRSV at MOI=1. The infected cells were grown in the presence or absence of the inhibitors for 48 h. The cells were then fixed with 4% paraformaldehyde for 15 min at room temperature (RT) and permeabilized with 0.25% Triton X-100 in PBS at RT for 10 min, washed with PBS, and blocked with 1% bovine serum albumin (BSA) for 30 min RT. After blocking, samples were incubated with the MAb SDOW17 at RT for 2 h. Cells were then washed and incubated with secondary fluorescein isothiocyanate (FITC) labeled goat anti-mouse antibody (MBL, Japan) for 1 h at RT. After three washes with PBS, samples were counter-stained with DAPI and examined by fluorescence microscopy.

**Statistical analysis**

Data are presented as mean ± SD. A one-way ANOVA was used for comparison of multiple groups. Dunnett's t-test was used for...
comparision of two groups. Statistical difference was set at $P$ values of $< 0.05$.

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