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Porcine reproductive and respiratory syndrome virus (PRRSV) could be sensed by professional beta interferon-producing system and had mechanisms to inhibit this action in MARC-145 cells

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1. Introduction

PRRSV, a positive-stranded RNA virus, is a member of family Arteriviridae (Meulenberg, 2000). Since it was firstly identified in the United States in 1987 and in Europe in 1990, PRRS has caused one of the most economically important diseases of swine which is characterized by severe reproductive failure in sows and respiratory distress in piglets and growing pigs (Rossow, 1998). Infection with PRRSV also predisposes pigs to secondary infection by bacterial and viral pathogens, which may be due to the immunosuppression induced by the virus (Feng et al., 2001; Mateu and Diaz, 2008).

IFN-β is the first responder against animal virus infection (Muller et al., 1994; Weber et al., 2004). When virus infects, the virus could be recognized by the pathogen-associated molecular patterns (PAMPs) such as membrane bound Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I). These PAMPs recruit different adaptor proteins, for example, TLRs recruit the adaptor molecule myeloid differentiation primary-response gene 88(MyD88) and Toll/IL-1 receptor domain-containing adaptor inducing IFN(Trif) while RIG-I recruits virus-induced signaling adapter (VISA), to make TANK-binding kinase 1 (TBK1) or IκB kinase-ε (IKK-ε) phosphorylate IRF-3 and finally to induce IFN-β transcription (Bowie and Unterholzner, 2008). Then, IFN-β induces the IFN-regulated genes responsible for the antiviral response (Sadler and Williams, 2008). However, during the co-evolution with the host cells, many viruses have developed defensive mechanisms to inhibit IFN-β production, making it difficult for host cells to defeat viral infection (Bowie and Unterholzner, 2008; Weber et al., 2004). Luo et al. (2008) and Miller et al. (2004) concluded that PRRSV does not induce IFN-β in MARC-145 cells infected with PRRSV, but Luo et al. did not detect the level of IFN-β mRNA by RT-PCR, and in Miller’s paper, the level of IFN-β appears a little higher in MARC-145 cells infected by PRRSV than that in control group, which may lead to a suspicion that whereas PRRSV could induce IFN-β production or not may be valued for verifying. Furthermore, Genini et al. (2008) and Loving et al. (2007) reported that PRRSV could induce the production of IFN-β in primary swine cells, which supply a clue that maybe PRRSV could also induce the IFN-β production in MARC-145 cells. Previous studies have documented that SARS-CoV nsp3 could inhibit the IFN-β production by its papain-like protease domain (Devaraj et al., 2007) and SARS-CoV N was capable of inhibiting IFN-β production (Kopecky-Bromberg et al., 2007). It is a coincidence that PRRSV nsp1 also contained papain-like protease domain (den Boon et al., 1995) and the crystal structure of PRRSV N protein was similar to that of SARS-CoV N protein (Yu et al., 2006). So, the purpose of the present experiments is to analyze the patterns of IFN-β promoter activity in MARC-145 cells.

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cells during infection with PRRSV and to analyze whether PRRSV nsp1 and N protein could inhibit IFN-β production.

2. Materials and methods

2.1. Cell, virus and primary antibodies

MARC-145 cell, a fetal green monkey fibroblast cell line derived from MA-104 (Kim et al., 1993), was maintained in Dulbecco’s modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone). PRRSV strain BJ-4, a kind gift from Dr. Hanchun Yang (China Agricultural University), was propagated in MARC-145 cells, which after 96 h post-infection (p.i.), the cells were frozen and thawed and clarified by low-speed centrifugation, and then the supernatants were stocked at −80°C. By the same methods, the supernatants of cells which were not infected with PRRSV were also prepared as the sham virus infection in the experiment. Primary antibodies used for this study were anti-IRF-3, anti-serine 396-phosphorylated species of IRF-3 (pIRF-3) (Cell Signaling Technology), anti-actin and anti-His tag (Beijing Zhongshan Goldenbridge Biotechnology Company, China).

2.2. Plasmids

The PRRSV nsp1 and N, which contained 6× His-tag in C-terminus in their reverse primers, were cloned from PRRSV RNA, and the PCR products were cloned into pMD19-T vector (Takara) and then ligated into pcDNA3.1 (Invitrogen), TBK1, VISA, and TRIF genes were cloned from MARC-145 cells by RT-PCR and were ligated into pcDNA3.1. RIG-N gene, the constitutive active caspase recruitment domain in RIG-I, was cloned from pEF-BOS-flag RIG-I (Wallac) which was kindly provided by Dr. Takashi Fujita (Institute for Medical Research Kyoto University, Tokyo, Japan) (Yoneyama et al., 2004) and was ligated into pcDNA3.1. pcDNA3.1-IRF-3(5D)-Luciferase plasmid which has been described (Shi et al., 2008). Briefly, the total protein concentration was quantified with the Bradford protein assay (Bio-dene difluoride membranes (Millipore Company), and then probed with appropriate antibodies. Proteins were detected by using an ECL detection system (Cell Signaling Technology).

2.5. RT-PCR

MARC-145 cells were transfected with pcDNA3.1-His (control vector) and pcDNA3.1-nsp1. Thirty hours later, the total cellular RNA was extracted by using TRIzol reagent (Invitrogen), and treated with DNase I (Takara) to remove genomic DNA contamination. Then, the RNA was reverse-transcribed to cDNA by reverse transcriptase M–MLV (Takara). The IRF-3 RT primers used in semi-quantitative analysis of the IRF-3 mRNA were listed in Table 1.

2.6. Statistical analysis

Statistical analyses was performed by Student’s t-test, and the comparisons were considered as statistical significance when \( p < 0.05 \).

3. Results

3.1. PRRSV sensed by professional beta interferon-producing system

It has been extensively documented that cytopathic effect (CPE), induced by PRRSV infection in MARC-145 cells, only appeared after 72 h p.i. (Cafruny et al., 2006; Kim et al., 2002), which was also confirmed in our experiment (data not shown). Cell viability was assessed by trypan blue staining, and approximately 93% of PRRSV-infected cells were viable at 48 h p.i. (data not shown).
To investigate whether PRRSV could induce the IFN-β response in cell culture, MARC-145 cells were infected with PRRSV at an MOI of 0.05. At a certain time, cells were co-transfected with p-284 Luc and phRL-TK, and 18 h later, the cells were harvested and subjected to a dual luciferase reporter assay system. As shown in Fig. 1A, PRRSV could activate IFN-β promoter in MARC-145 Cells at 24 h p.i., but the activity of IFN-β promoter was much lower than that triggered by Poly(I:C). And the activity of IFN-β promoter was rapidly inhibited in the following infection. The expression of IFN-β mRNA was also analyzed by semi-quantitative RT-PCR at the different time points after PRRSV infection and gotten the results similar to that of Fig. 1A (data not shown). To confirm this result, the level of pIRF-3 was also detected because pIRF-3 was a necessary transcription factor to the activation of IFN-β promoter (Peters et al., 2002; Yoneyama et al., 1998). In consistent with the results of Fig. 1A, PRRSV could phosphorylate IRF-3 in early stage of infection (24 h p.i.), but after that, no detectable levels of pIRF-3 showed at certain time interval (Fig. 1B).

3.2. PRRSV nsp1 inhibited the activation of IFN-β promoter induced by Poly(I:C)

Previous studies have documented that SARS-CoV nsp3 could inhibit the IFN-β production by its papain-like protease domain (Devaraj et al., 2007) and SARS-CoV N was capable of inhibiting IFN-β production (Kopecky-Bromberg et al., 2007). Since PRRSV nsp1 also contains papain-like protease domain (den Boon et al., 1995) and the crystal structure of PRRSV N protein was similar to that of SARS-CoV N protein (Yu et al., 2006), PRRSV nsp1 and N proteins were chosen to probe which component of PRRSV could inhibit IFN-β production.

The expressions of pcDNA3.1 nsp1 and pcDNA3.1 N in MARC-145 cells were confirmed by RT-PCR (Fig. 2A) and western blot (Fig. 2B). Since nsp1 should be cleaved into nsp1α and nsp1β sub-units (den Boon et al., 1995) and the 6× His tag was ligated to the C-terminal of nsp1 in this study, here only nsp1β could be detected (Fig. 2B).

MARC-145 cells were co-transfected with pcDNA3.1-nsp1 or pcDNA3.1-N, p-284 Luc and phRL-TK plasmids. As control group, pcDNA3.1-His was in place of pcDNA3.1 nsp1. Twenty four hours later, MARC-145 cells were either mock-treated or Poly(I:C)-treated for 6 h, and then the cells were harvested for luciferase reporter assay. The experimental data showed that only nsp1 suppressed the activation of IFN-β promoter (Fig. 2C), but N protein did not.

Because the pIRF-3 was a necessary component to the activation of IFN-β promoter, after the Poly(I:C) treatment or the mock treatment, pIRF-3-dependent synthetic promoter, p55C1B-Luc (Devaraj et al., 2007; Yoneyama et al., 1998, 2004) was detected with luciferase reporter assays. As shown in Fig. 2D, only nsp1 could inhibit the activation of p55C1B-Luc, while N protein could not. The results in Fig. 2D confirmed the results in Fig. 2C.

3.3. PRRSV nsp1 inhibited the activity of IFN-β promoter induced by RIG-N, VISA, TRIF or TBK1, but did not inhibit the activity of IFN-β promoter induced by IRF-3(5D)

RIG-I, VISA, TRIF and TBK1 were the upstream signaling proteins of IRF-3 in the signal pathway of IFN-β production. The over-
expression of RIG-I, VISA, TRIF or TBK1 could induce the activation of IRF-3 and promote the activation of IFN-β promoter (Devaraj et al., 2007; Yoneyama et al., 2004; Zhong et al., 2008). RIG-N was the constitutive active caspase recruitment domain in RIG-I (Yoneyama et al., 2004). PRRSV nsp1 strongly suppressed the activation of IFN-β promoter induced by over-expression of RIG-N or ectopic expression of VISA, TRIF, TBK1 (Fig. 3A). In contrast, IRF-3(5D), a constitutively active and phospho-mimetic IRF-3 mutant (Lin et al., 1998), activated the IFN-β promoter, and that was no difference between cells expressing nsp1 and cells transfected with a control vector (Fig. 3A). Similar results were obtained when p55C1B Luc was in place of p-284 Luc (Fig. 3B).

3.4. PRRSV nsp1 down-regulated the protein level of IRF-3 and inhibited the phosphorylation of IRF-3 induced by Poly(I:C)

Immunoblot analyzed the activation and latent status of IRF-3. And the results showed that nsp1 not only down-regulated the protein level of IRF-3, but also inhibited the phosphorylation of IRF-3 induced by Poly(I:C) (Fig. 4A).

The expression of IRF-3 mRNA was analyzed by semi-quantitative RT-PCR. The results in Fig. 4B demonstrated that nsp1 have no effect on the transcription of IRF-3.

4. Discussion

Our present work was for the first time to analyze the dynamic activities of IFN-β promoter and IRF-3, a necessary transcription factor to the activity of IFN-β promoter (Yoneyama et al., 1998) during PRRSV infection in MARC-145 cell line which was capable of producing IFN-β (McKimm-Breschkin and Holmes, 1982) and supported the replication of PRRSV (Kim et al., 1993). The results indicated that PRRSV could trigger the activation of IRF-3 as well as induce the activation of IFN-β promoter at 24 h p.i., but the activities were much lower than that triggered by the Poly(I:C). And their activities were rapidly inhibited in following infection (Fig. 1). So it is reasonable to speculate that PRRSV could be sensed by professional IFN-β-producing system and had mechanisms to inhibit this action in MARC-145 cells.

PRRSV nsp1 contained papain-like protease domain (den Boon et al., 1995) and the crystal structure of PRRSV N protein was similar to that of SARS-CoV N (Yu et al., 2006). Previous studies have documented that SARS-CoV nsp3 could inhibit the IFN-β production by its papain-like protease domain (Devaraj et al., 2007) and SARS-CoV N was capable of inhibiting IFN-β production (Kopecky-Bromberg et al., 2007). Our present work indicated that PRRSV nsp1 inhibited the IFN-β production induced by Poly(I:C), while N protein did not (Fig. 2). It’s unexpected that PRRSV N protein could not inhibit the IFN-β production, because a protein’s function generally depended on its three-dimension structure or conformation. Perhaps, SARS-CoV N protein could inhibit IFN-β production by its peptide or other unclear mechanism.

Poly(I:C), a double-stranded RNA, could be recognized by TLR3 (Yamamoto et al., 2003) and RIG-I (Kato et al., 2006). Then through TBK1, TLR3 recruited TRIF and RIG-1 recruited VISA to phosphorylate IRF-3 and finally activate IFN-β promoter (Bowie and Unterholzner, 2008). Over-expression of RIG-1, VISA, TBK1 or TRIF could induce the activation of IRF-3 and promote the activation of IFN-β promoter (Devaraj et al., 2007; Yoneyama et al., 2004; Zhong et al., 2008). Our data showed that nsp1 inhibited the IFN-β production induced by RIG-1, VISA, TBK1 or TRIF, which indicated that nsp1 inhibited the IFN-β production induced by both TLR3 and RIG-1 pathways (Fig. 3). Nevertheless, nsp1 had no effect on the activity of IFN-β promoter induced by IRF-3(5D), which suggested...
that nsp1 inhibited IFN-β production by suppressing the activation of IRF-3. This speculation has been confirmed. PRRSV nsp1 down-regulated the protein level of IRF-3 and inhibited Poly(I:C)-induced phosphorylation of IRF-3, but had no effect on the level of IRF-3 mRNA (Fig. 4). Perhaps, nsp1 inhibited the translation of IRF-3 or the degradation of IRF-3 protein or inhibited the activation of kinase to phosphorylate IRF-3. SARS-CoV nsp3 inhibited phosphorylation of IRF-3 through its papain-like protease domain to be interacted with IRF-3 but did not down-regulate the protein level of IRF-3 (Devaraj et al., 2007), while the Npro protein of Classical Swine Fever Virus, similar to the group of papain-like proteases (Stark et al., 1993), could antagonize IRF-3 activity by inducing its proteasomal degradation (Bauhofer et al., 2007). PRRSV nsp1 also contains papain-like protease domain (den Boon et al., 1995) and PRRSV nsp1 down-regulated the protein level of IRF-3. So it is possible that PRRSV nsp1 inhibits the IRF-3 activation by inducing IRF-3 degradation or inhibits the activation of kinase to phosphorylate IRF-3. And this suppositional mechanism deserves a further study.

Based on the experimental data discussed above, we propose a model to illustrate how PRRSV nsp1 negatively regulates IFN-β signaling pathways (Fig. 5). First, PRRSV could be sensed by the professional beta interferon-producing system, although it is unclear that PRRSV could be recognized by TLRs or RIG-I-like receptors (RLRs) or by the both. Second, PRRSV nsp1 could negatively regulate IFN-β production by inhibiting the phosphorylation of IRF-3.

It is known that type I IFN system (IFN-α/β) provides an important first-host response to viral intruders (Weber et al., 2004), and type I IFN is essential to the initial control of virus infection and the establishment of an adaptive immune response (Muller et al., 1994). Many viruses developed defensive mechanisms to inhibit IFN-β production (Bowie and Unterholzner, 2008; Devaraj et al., 2007; Weber et al., 2004). The influenza virus N1 protein, a multifunctional facilitator of virus replication, could antagonize the
IFN-β production (Hale et al., 2008). Deletion of the IFN-β antagonist NS1 could produce a novel type of influenza vaccine (Wacheck et al., 2010). Novel antagonists against influenza virus NS1, only significantly and specifically inhibiting influenza A replication in cell capable of interferon production, could be a potential drug for influenza virus-treatment (Basu et al., 2009). Previous studies also showed intriguing observations that recombinant IFN-β not only protected swine alveolar macrophages and MARC-145 cells from infection with PRRSV (Overend et al., 2007), but also could reduce the yield of PRRSV in vivo (Buddaert et al., 1998). Perhaps, inhibition of IFN-β production leads to the persistent infection of PRRSV. Our data demonstrated that PRRSV could inhibit the IFN-β production by PRRSV nsP1. In the future, it will be interesting to research on the function or characterization of PRRSV nsP1 in virus-induced immunosuppression and virus replication, and PRRSV nsP1 may be used as a potent target for exploiting new drugs for PRRSV treatment or PRRSV vaccine.

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