Phospholipase C signaling is involved in porcine reproductive and respiratory syndrome virus infection in cell cultures

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Summary. – The phospholipase C (PLC) is a family of kinases that hydrolyze phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] to generate two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which stimulate distinct downstream signaling. Recently, it has been reported that PLC signaling is activated by multiple viruses for efficient replication and the virus-induced inflammatory response. In this study, we demonstrated that PLC-specific inhibitor U73122 strongly suppressed porcine reproductive and respiratory syndrome virus (PRRSV) productive infection in cell cultures. The inhibitor affected both viral post-binding cell entry and post-entry processes. The virus infection led to an early transient activation of PLCγ-1 at 0.5 h post-infection (hpi), and sustained event at a stage from 4 to 16 hpi in MARC-145 cells. In addition, U73122 inhibited the activation of p38 MAPK signaling stimulated by PRRSV infection, suggesting that PLC signaling may be associated with the virus infection-induced inflammatory response. Taken together, these studies suggested that PLC signaling played an important role in PRRSV infection or pathogenesis.

Keywords: PRRSV; U73122; phospholipase C; PLCγ-1

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-strand RNA virus belonging to the family *Arteriviridae*, and is the causative agent of porcine reproductive and respiratory syndrome (Conzelmann *et al*., 1993). Since its discovery in the late 1980s in Europe and North America, it has been one of the most important diseases in swine industry and causes significant economic losses worldwide (Neumann *et al*., 2005). In 2006, an outbreak of highly pathogenic PRRSV (HP-PRRSV) known as "pig high fever disease" occurred in China and caused disastrous losses to the farmers (Tong *et al*., 2007). Currently, HP-PRRSV is still a major concern for the swine industry in China and surrounding countries.

Phospholipase C (PLC) is a family of protein serine/threonine kinases that cleave PI(4,5)P2 into DAG and IP3, which activates the calcium-dependent protein kinase C (PKC) and Ca^{2+} release from the endoplasmic reticulum to the cytoplasm (Vines, 2012). PKC and calcium spike in turn activate downstream effectors to mediate various biological activities. A total of 13 different PLC family members, divided into six classes, have been identified in humans, including β, γ, δ, ε, η, and ζ (Vines, 2012), and each has a specific tissue distribution (Suh *et al*., 2008). PLC-γ has two isoforms, PLC-γ1 and PLC-γ2. PLC-γ1 is ubiquitously expressed (Ji *et al*., 1997). The involvement of PLC-γ1 signaling in virus infection as well as the virus-induced inflammatory

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Abbreviations: DAG = diacylglycerol; PLC = phospholipase C; hpi = post-infection; IP3 = inositol 1,4,5-trisphosphate; MOI = multiplicity of infection; PI(4,5)P2 = phosphatidylinositol 4,5-bisphosphate; PRRSV = porcine reproductive and respiratory syndrome virus

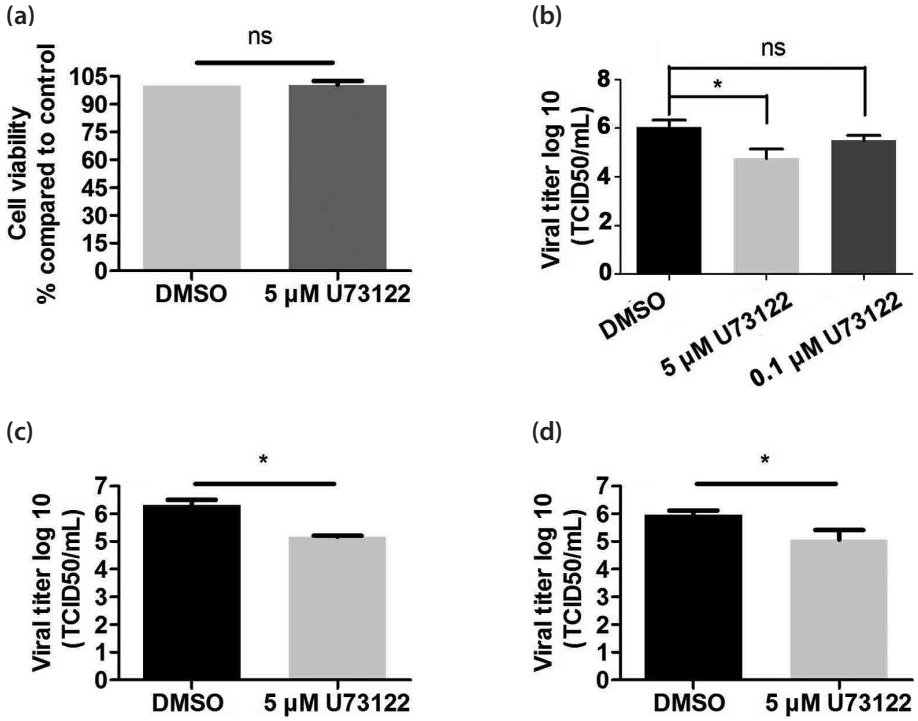

Fig. 1

The inhibitory effect of U73122 on PRRSV infection in cell cultures

(a) The cytotoxicity assay for U73122 in MARC-145 cells; **(b)**MARC-145 cells were pretreated with the inhibitor at indicated concentrations for 1 h, infected with PRRSV (MOI = 1) for 24 h along with the treatment with corresponding inhibitor. The viral titer was determined with MARC-145 cells with the results expressed as $TCID_{50}$/ml. MARC-145 cells infected with PRRSV (MOI = 1) were treated with U73122 at a concentration of 5 μmol/l; **(c)** at virus post-binding entry stage; **(d)** or at the post-entry process, respectively. At 24 hpi the viral titer was determined with results expressed as $TCID_{50}$/ml. Values represent three independent experiments. Significance was assessed with the Student's *t* test (**P* < 0.05).

responses have been recently reported, such as in influenza virus and bovine herpesvirus 1 infection (Zhu *et al*., 2016; 2017). In this study, the role of PLC signaling in PRRSV infection was investigated.

We initially tested the effects of PLC signaling inhibitor U73122 on PRRSV replication in MARC-145 cells. To identify proper concentrations for this study, the cytotoxicity of U73122 in MARC-145 cells was assessed with WST-1 cell proliferation and cytotoxicity assay kit (Beyotime Biotechnology, China) following the manufacturer's instructions. Based on our pilot experiment, U73122 at a concentration of 5 μmol/l was selected for this evaluation. As a result, the treatment of MARC-145 cells with U73122 at a concentration of 5 μmol/l showed no cytotoxicity to the cells compared to the control, as determined by the same kit (Fig. 1a). To test the effect of this compound on PRRSV infection, MARC-145 cells pretreated with U73122 at a concentration of 0.1 and 5 μmol /l for 1 h prior to infection, were infected with PRRSV (MOI = 1) for 1 h in the presence of this inhibitor, then fresh medium with inhibitor was replaced for further incubation. At 24 h post-infection the viral titer was determined and expressed as $TCID_{50}$/ml. In parallel, the infected cells treated with DMSO were used as a control. As a result, U73122 reduced the virus titer by ~1.4- and 0.6-log at concentrations of 5 and 0.1 μmol/l, respectively (Fig. 1b). These results indicate that PLC signaling is important for PRRSV infection.

The detailed process in the virus replication cycle affected by U73122 was further determined. To test whether this inhibitor affected the viral post-binding entry stages of infection, MARC-145 cells in 6-well plates were incubated with PRRSV (MOI = 1) for 1 h at 4°C. After extensive washing with ice-cold PBS, fresh medium with or without inhibitor was added, and cells were quickly shifted to 37°C for 1 h. Fresh medium without inhibitor was replaced and continuously incubated at 37°C for 24 h. The virus yield was determined and expressed as $TCID_{50}$/ml. Addition of U73122 (5 μmol/l) during virus post-binding entry stages significantly reduced virus titer (~1.1-log) when compared to control (Fig. 1c). To test whether this inhibitor affects the post-entry stage of PRRSV infection, confluent MARC-145 cells in 6-well plates were infected with PRRSV (MOI = 1) for 1 h at 37°C. After washing with PBS, fresh medium with or without inhibitor was replaced and further incubated for

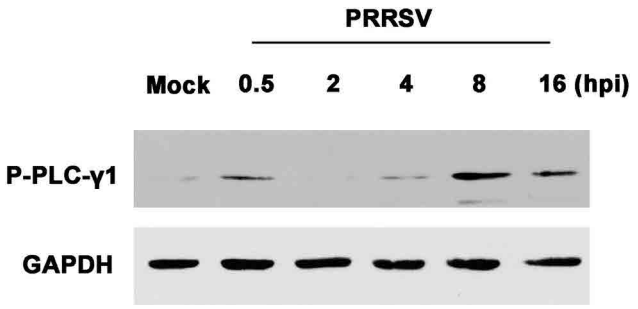

Fig. 2

The activation of PLCγ-1 signaling in PRRSV-infected MARC-145 cells

Growth arrested MARC-145 cells were mock-infected or infected with PRRSV at a MOI of 10. At the indicated time points, cells were lysed and subjected to Western blotting analysis using the indicated antibodies. Data are representative results of two independent experiments.

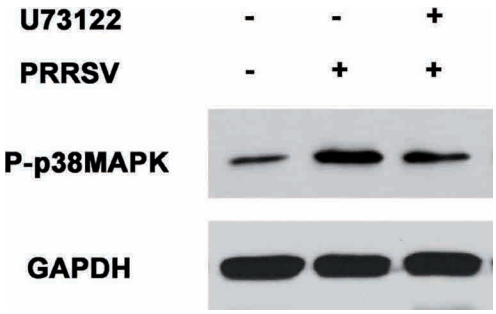

Fig. 3

Effect of U73122 on p38 MAPK signaling in response to PRRSV infection

Serum starved MARC-145 cells in 60-mm dishes were pretreated with U73122 at a concentration of 5 μmol/l for 1 h, then infected with PRRSV (MOI = 10) in the presence of the inhibitors for 0.5 h. Cell lysate was prepared and subjected to Western blotting analysis. The data are representatives of three independent experiments.

24 h at 37°C. As a result, the treatment of cells with U73122 at the post-entry stage significantly interfered with the virus production, with approximately 0.9-log decrease relative to the control (Fig. 1d). These results indicated that the PLC signaling affected the virus replication at both post-binding entry and post-entry stages.

Since PLC signaling inhibitor significantly interfered with PRRSV replication in cell cultures, we further examined whether the virus infection affected PLC signaling by testing the kinetics of phosphorylated PLCγ-1 (Ser1248) during virus infection of MARC-145 cells with Western blots using antibody provided by Cell Signaling Technology (Beverly, MA, USA). To assess the burst effects of virus infection on the detected signaling, the cells were infected with PRRSV at a high MOI of 10. At 0.5, 2, 4, 8 and 16 hpi, the cell lysates were prepared using RIPA buffer (50 mmol/l Tris-HCl, pH 8, 150 mmol/l NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors (Thermo-Scientific) and subjected to Western blots analysis. As shown in Fig. 2, the level of phospho-PLCγ-1 (Ser1248) was first detected to increase at 0.5 hpi, then it decreased to the basal level from 2 hpi, and re-increased at 4 hpi, then up to higher levels at 8 and 16 hpi. The kinetics of PLCγ-1 activation correlated with the role of PLC signaling in PRRSV replication cycle.

The lethal lung inflammation due to porcine reproductive and respiratory syndrome virus infection is mainly caused by "cytokine storms" induced by the virus infection (Lee and Lee, 2012). It is known that the inflammatory mediators released during acute and chronic diseases activate multiple intracellular signaling cascades including p38 MAPK signal transduction pathway. In addition, the functional consequences of p38 MAPK activation include oxidative burst and production of inflammatory mediators (Herlaar and Brown, 1999; Yang *et al*., 2014). It seems that there is a loop feedback between p38 MAPK signaling activation and inflammatory mediators production. It has been reported that p38 MAPK is activated in PRRSV-infected BMDMs, and p38 MAPK pathway is involved in PRRSV-induced IL-10 expression (Song *et al*., 2013). Recently it has been reported that BoHV-1 infection-activated p38 MAPK signaling is controlled by PLC signaling (Zhu *et al*., 2017). Here, the effects of U73122 on the levels of phosphorylated p38 MAPK (Thr180/Tyr182) in response to PRRSV infection were examined by Western blots using antibody provided by Cell Signaling Technology (Beverly, MA, USA). As expected, PRRSV-induced activation of p38 MAPK, was attenuated by U73122 at a concentration of 5 μmol/l (Fig. 3). Therefore, our results indicated that the activation p38 MAPK signaling by PRRSV infection was partially dependent on PLC signaling.

Ca^{2+} is a PLC downstream signaling mediator. It has been reported that calcium induces activation of p38 MAPK signaling (Wright *et al*., 2007). Based on this report and our results, we speculated that PRRSV induced activation of p38 MAPK through PLC-mediated calcium signaling, which needs further study. In addition, the p38 MAPK signaling is involved in cytokines expression induced by several viruses, including PRRSV. The activation of p38 MAPK signaling via PLC signaling suggested that PLC signaling may be involved in PRRSV infection-induced inflammatory response (Zhu *et al*., 2015, 2016).

In summary, in this study we provided for the first time the evidence that PLC signaling was required in PRRSV replication cycle. Moreover, the activation of inflammation-related signaling by p38 MAPK through PLC signaling during PRRSV infection is a potential pathogenic mechanism for the virus-induced inflammation.

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