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Triggering unfolded protein response by 2-Deoxy-D-glucose inhibits porcine epidemic diarrhea virus propagation

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ABSTRACT

The unfolded protein response (UPR) is cyto-protective machinery elicited towards an influx of large amount of protein synthesis in the endoplasmic reticulum (ER). Extensive studies suggest that the UPR can also be activated during virus infection. In the present studies, we first evaluated if porcine epidemic diarrhea virus (PEDV) infection activated the UPR pathways. Electron microscopy analysis demonstrated the morphology changes of ER post-PEDV infection. Western blot and real-time PCR identified the differences of UPR genes in response to PEDV infection. The results suggested that PEDV infection induced UPR in Vero cells. Meanwhile, we silenced the expression of PKR-like ER kinase (PERK) by shRNA, we found that the knockdown of PERK increased virus loads in the cells, which was consistent with the result on 4-phenylbutyrate (4-PBA) treatment. We next determined whether 2-Deoxy-D-glucose (2-DG), an ER stress inducer, possessed antiviral activity against PEDV infection. Plaque formation assay, RT-PCR and Western blot analysis suggested that 2-DG might inhibit virus infection by affecting viral protein translation during the early stage of virus infection. Interestingly, we also found that 2-DG treatment could affect virus assembly, which is similar to previous studies on influenza virus. All these results support the therapeutic potential of using 2-DG or glucose/mannose analogs to induce the UPR to block virus replication.

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1. Introduction

Porcine epidemic diarrhea (PED) is first found among English feeder and fattening pigs in 1971 (Wood, 1977). It is a devastating enteric disease that manifests as sporadic outbreaks during winter. PEDV is the causative agent of PED and causes severe enteritis, vomiting and watery diarrhea, resulting in a high mortality in piglets. Since 2013, PEDV spreads rapidly in swine farms in the United States, causing significant economic loss (Huang et al., 2013; Mole, 2013). PED is now becoming one of the most important swine diseases causing great economic loss and public health concerns.

PEDV was first reported in Belgium and the United Kingdom (Pensaert and De Bouck, 1978). PEDV belongs to the family of coronavirus and is classified into group I of the genus *Coronavirus*. Its size ranges in diameter from 95 nm to 190 nm, including its projection. The viral replicase is encoded by ORF1a and ORF1b (Kocherhans et al., 2001). The viral structural proteins include spike protein, envelope protein, membrane protein and nucleocapsid protein (Song and Park, 2012). The virus also encodes an accessory protein, ORF3a, which has been suggested as an important virulent determinant of this virus (Park et al., 2011).

The unfolded protein response (UPR) is a cellular stress response induced by the accumulation of misfolded or unfolded proteins within the endoplasmic reticulum (ER) in order to initiates a number of cellular responses to restore ER homeostasis. The primary function of UPR is for stress adaptation and cell survival. The UPR can also be induced through glycosylation inhibitors treatment (e.g., tunicamycin), calcium homeostasis changes, nutrient depletion, overexpression of abnormal proteins, or virus infection (Dorner et al., 1989). UPR is represented by a marked increase of ER-localized proteins such as glucose-regulated protein 78 (GRP78/BiP) or 94 (GRP94). UPR is controlled by three main signaling pathways, including inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like ER kinase (PERK). In non-stressed cells, GRP78 binds to PERK, IRE1 and ATF6 to keep them in an inactive form (Bertolotti et al., 2000).

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IRE1 is an ER transmembrane protein that contains endoribonuclease and cytoplasmic protein kinase domains. ER stress can induce the activation of IRE1. Activated IRE1 removes 26 nucleotides from the mRNA of X-box binding protein 1 (XBP-1) to generate a spliced mRNA encoding the functional XBP-1(s) protein (Yoshida et al., 2003). PERK, an ER resident serine/threonine protein kinase, can phosphorylate the -subunit of eukaryotic translation initiation factor 2 (eIF2α). During UPR, the release of GRP78 from PERK results in the homodimerization of PERK, causing phosphorylation of eIF2α to impair protein translation (Fernandez et al., 2002; Liu et al., 2009). ATF6 normally anchors in the ER membrane. GRP78 release exposes a Golgi localization signal on ATF6. After translocation to the Golgi, ATF6 precursor (p75ATF6) is further processed into its active form (p50ATF6), which enters the nucleus to activate the transcription of UPR target genes (Ye et al., 2000).

Many enveloped DNA and RNA viruses infection can induce the UPR in mammalian cells (Zhang and Wang, 2012). For example, studies on hepatitis C virus (HCV) show that HCV infection induces the UPR, which can activate the autophagic pathway to promote HCV RNA replication in human hepatoma cells (Ke and Chen, 2011). Some viruses regulate the UPR to initiate an environment more favorable for their replication (Buchkovich et al., 2009, 2008). African swine fever virus (ASFV) uses the endoplasmic reticulum (ER) as a site of replication. ASFV infection triggers ER stress and the unfolded protein response (UPR) in the host cells by inducing ATF6 signaling pathway of the UPR, but not PERK or IRE1 pathway. UPR regulation by ASFV might prevent early apoptosis to facilitate viral replication (Galindo et al., 2012). Tick-borne encephalitis virus infection activates the IRE1 and ATF6 signal pathways (Yu et al., 2013). A study on severe acute respiratory syndrome associated coronavirus (SARS-CoV) identified one of accessory proteins of SARS-CoV (8ab protein) binds directly to the luminal domain of ATF6, suggesting 8ab protein might facilitate protein folding and processing by modulating UPR (Sung et al., 2009). This finding suggests that some viruses might use their own protein(s) to regulate UPR response.

The glucose analog, 2-Deoxy-D-glucose (2-DG), can be used to block or probe sugar metabolism in cancer cells (Kurtoglu et al., 2007a). Because of its mannose-like structure, 2-DG can compete with mannose in the growing oligosaccharide chain during the initial steps of N-linked glycosylation occurring in the ER (Kurtoglu et al., 2007b). Oligosaccharide chains incorporated with 2-DG cannot form the functional $glucose_3$ $mannose_9$ moiety for proper protein glycosylation. Abnormal N-linked glycosylation interferes with protein folding and induces ER stress to activate UPR, which results in the inhibition of protein synthesis. It has been reported that 2-DG can inhibit the replication and gene expression of Kaposi's Sarcoma-Associated Herpesvirus (Leung et al., 2012). 2-DG also inhibits influenza virus infection (Nakamura and Compans, 1978). Thus, we proposed that UPR induced by 2-DG would counter the ability of PEDV to circumvent UPR-mediated blockage of protein synthesis, thereby impairing PEDV gene expression and resulting in inhibition of viral replication.

In these studies, we demonstrated that PEDV infection can induce UPR. We showed an interaction between UPR and the replication of PEDV. As expected, we revealed that 2-DG induced UPR leads to the inhibition of viral replication in Vero cells. Our results provide new antiviral insights that may be applicable to inhibit the replication of PEDV in clinic.

2. Materials and methods

2.1. Cell and virus

Porcine epidemic diarrhea virus (strain HLJBY) was propagated in Vero cells in the presence of 60 μg/ml trypsin cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, China). To obtain replication-incompetent PEDV, 10 ml aliquots of virus were dispersed in culture medium (10 cm tissue culture dish) and exposed UV light (254 nm) for 2 h on ice. Following the exposure, the samples were harvested and stored at −80 °C. The absence of virus infectivity after UV irradiation was confirmed by plaque formation assay and Q-PCR as described below (Jheng et al., 2010).

2.2. Chemicals, antibodies and other reagents

2-Deoxy-D-glucose (2-DG), tunicamycin (Tu), 4-phenylbutyrate (4-PBA), anti-β-actin antibody were purchased from Sigma–Aldrich. The following rabbit primary antibodies were purchased from Cell Signaling: PERK, phospho-PERK, eIF2α, phospho-eIF2α, GRP78. Rabbit primary antibody ATF6 was purchased from Santa Cruz. The polyclonal antibody for PEDV N was generated previously in our lab. The PERK shRNA designed specifically to knockdown PERK expression was purchased from Genepharma (Shanghai, China) along with the control shRNA.

2.3. Cell culture, virus infection and plaque formation assay

According to the requirement of different experiments, Vero cells were either infected with PEDV (MOI of 0.01, 0.1 or 1) or mock-infected with phosphate-buffered saline (PBS). After 1 h incubation in 37 °C, unbounded viruses were removed by washing the cells thrice with PBS and the cells were cultured in DMEM supplemented with 2% FBS at 37 °C for different time. For 2-DG treatment experiments, Vero cells were pretreated with varying concentrations of 2-DG for 24 h before virus infection (MOI = 0.01). The cells were infected with PEDV at 37 °C for 2 h, the unbounded viruses were removed by washing thrice with PBS and cultured in DMEM supplemented with 2% FBS and varying concentrations of 2-DG at 37 °C for 24 h. For 4-PBA treatment experiments, Vero cells were first infected with PEDV (MOI = 0.01) for 2 h at 37 °C, unbounded viruses were removed by washing thrice with PBS. Then cells were cultured in DMEM supplemented with 2% FBS and varying concentrations of 4-PBA at 37 °C for 48 h.

For virus entry study, the cells were incubated with PEDV for 1 h (MOI = 1) at 4 °C. The cells were washed three times with PBS, then incubated with DMEM supplemented with 2% FBS at 37 °C. 30 min later, the cells were washed twice with a low pH buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 30 s to inactivate or remove the bound viruses that did not penetrated the cells. The cells were then washed twice with PBS for further experiments (Hancock et al., 2010).

For plaque formation assay, viral culture supernatants with 10-fold dilutions (from 10^2 to 10^5) were added into 6-well plate with confluent monolayer of Vero cells. The plate was then incubated at 37 °C for 2 h with gentle agitation at every 15-min interval. The excess virus inocula were removed by rinsing the wells with PBS for three times. Subsequently, overlay medium (2% low melting-point agarose with DMEM medium containing 2% FBS) was added to each well and the plates were further incubated at 37 °C with 5% CO_2 for 3 days. The cells were stained with 0.5% crystal violet.

2.4. Western blot analysis

The cells in 6-well plate were washed with PBS for three times, scraped from the culture plate, and lysed with the cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS, 5 mM sodium orthovanadate) containing a protease inhibitor cocktail (Roche Molecular Biochemicals) and 0.1 mM PMSF for 2 h. The cell lysates were centrifuged at 14,000*g* for 20 min at 4 °C. Protein concentration was determined using the

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