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Tick-borne encephalitis virus triggers inositol-requiring enzyme 1 (IRE1) and transcription factor 6 (ATF6) pathways of unfolded protein response☆

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ABSTRACT

Tick-borne encephalitis (TBE) is a serious human neurological disease caused by TBE virus (TBEV). However, the mechanisms of TBEV-caused pathogenesis remain unclear. The endoplasmic reticulum (ER) stress response, also defined as the unfolded protein response (UPR), is an important conserved molecular signaling pathway that modulates many biological functions including innate immunity and viral pathogenesis. Here, we investigated the effects of the two UPR signaling pathways upon TBEV infection in Vero E6 cells. We showed that the amount of heat shock protein 72 (Hsp72) increased in the course of TBEV infection. We then confirmed that TBEV infection activates the IRE1 pathway, leading to RNA and protein expression of the spliced X box binding protein 1 (sXBP1). Furthermore, we observed the translocation of ATF6 during TBEV infection and expression of cleaved transcription factor 6 (ATF6) which suggest activation of ATF6 pathway. Finally, we examined whether inhibition of the IRE1 pathway has an effect on TBEV infection. Cell treatment with 3,5-Dibromosalicylaldehyde (IRE1 inhibitor) and tauroursodeoxycholic acid (TUDCA) showed that TBEV replication was significantly limited. These findings provide the first evidence that TBEV infection activates the two UPR signaling pathways. Moreover, inhibition of TBEV replication by UPR inhibitors may provide a novel therapeutic strategy against TBE.

1. Introduction

Tick-borne encephalitis virus, within the genus *Flavivirus* of the family *Flaviviridae*, is an emerging zoonotic virus transmitted by ticks in Europe, the Far East and Asia (Gritsun et al., 2003; Mansfield et al., 2009). It can cause severe infection in humans with a variety of neurological symptoms and diseases (Lindquist and Vapalahti, 2008). In recent years, thousands of diagnosed TBE cases were reported annually, although TBEV infection can be efficiently prevented by vaccination (Demicheli et al., 2009; Donoso Mantke et al., 2011; Suss, 2008). The genome of TBEV consists of a single-stranded positive-sense RNA of about 11 kb in length. It has a single open reading frame which translates for a polyprotein consisting of three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Heinz and Allison, 2003). Translation, secretion and modification of the viral proteins as well as virus RNA replication and virus assembly take place at the membranes of the endoplasmic reticulum (ER) and Golgi-derived membranes called vesicle packets (VP). Therefore, TBEV and other flaviviruses modify the ER architecture, resulting in membrane proliferation and hypertrophy of ER, which is beneficial for flavivirus protein secretion and modification (Fernandez-Garcia et al., 2009; Gillespie et al., 2010). Furthermore, reorganized ER protects and maintains newly replicated RNA for the step of virus replication (Miorin et al., 2013).

ER stress is one of the most important cellular reactions caused by virus infection due to high amounts of viral proteins in the ER (He, 2006). The cell reacts to ER stress by activating the unfolded protein response (UPR) pathway which removes misfolded proteins either by attenuating general translation or by enhancing ER folding capacity and ER-associated degradation (Lin et al., 2008). In the ER stress situation, the UPR signaling pathway is mediated by three major transmembrane ER-resident proteins, namely protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) (Ron and Walter, 2007). In the PERK pathway, PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α), resulting in a decline of protein synthesis (Harding et al., 1999). Activation of the ATF6

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pathway generates an active ATF6 fragment which translocates to the nucleus and up-regulates transcription of UPR genes (Ye et al., 2000). The IRE1 pathway is initiated by IRE1α and controlled by a series of regulators termed as the UPRosome. The UPRosome consists of a complex protein assembled at the ER membrane, such as heat shock protein 72 (Hsp72), apoptosis signal-regulating kinase 1 (ASK1)-interacting protein 1 and the pro-apoptotic proteins Bcl-2-associated X protein (BAX) and Bcl-2 homologous antagonist/killer (BAK) (Hetz, 2012). However, modulation of the IRE1 pathway signaling by these regulators, which associate or dissociate with IRE1α, is cell type dependent. Upon activation of the IRE1 pathway, active IRE1 processes the X box binding protein 1 (XBP1) mRNA by shifting the open reading frame. This results in the expression of the active transcription factor spliced XBP1 (sXBP1), which then up-regulates its target genes (Yoshida et al., 2001).

Recent studies have shown that several flaviviruses preferentially use different UPR pathways to facilitate their replication. Studies with Japanese encephalitis virus (JEV) and Dengue virus (DENV) have shown activation of the IRE1 pathway during infection (Umareddy et al., 2007; Yu et al., 2006). West Nile virus (WNV) modulates all three pathways of the UPR which results in up-regulation of the production of viral RNA and protein (Ambrose and Mackenzie, 2011). Moreover, WNV or JEV infections trigger cell death processes by enhancing the expression of CCAAT/-enhancer-binding protein homologous protein (CHOP) which is a transcription factor induced by the UPR (Medigeshi et al., 2007; Su et al., 2002). Although the effect of these flaviviruses on different UPR pathways has been unveiled, the role of TBEV infection in cellular UPR is still unknown. The goal of our study was to analyze the role of the UPR, in particular regarding the IRE1 pathway and ATF6 pathway in the course of TBEV infection. We first showed that TBEV infection enhanced Hsp72 protein expression which regulates and enhances the IRE1 pathway. We then analyzed that TBEV infection induced the IRE1 pathway, which resulted in high expression of sXBP1. Moreover, we demonstrated that the translocation and expression of the cleaved ATF6, which indicated that ATF6 pathway activation during TBEV infection. Finally, we found that 3,5-Dibromosalicylaldehyde (IRE1 inhibitor) and tauroursodeoxycholic acid (TUDCA), two inhibitors of the UPR, impair TBEV replication. These findings provide new insights into the molecular mechanism of TBEV pathogenesis and may offer a new therapeutic approach to treat TBEV-induced diseases.

2. Materials and methods

2.1. Cell lines and virus strains

Vero E6 cells (ATCC CRL-1586) and A549 cells (ATTC CCL-185) were cultured in 24-well plates and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% L-glutamine, 1% penicillin and 1% streptomycin. Both cell types were incubated at 37 °C with 5% CO_2. The propagation of the TBEV strain K23 (GenBank accession no. AM600965) was performed in Vero E6 cells (Achazi et al., 2012). Viral titer was determined by plaque assay as described below. Unless stated otherwise, an MOI of 1 was used for the TBEV infection experiments.

2.2. RNA extraction and RT-PCR

The total RNA was extracted from cells using QIAshredder/RNeasy RNA purification columns (Qiagen, Hilden, Germany) following the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA using Superscript II (Invitrogen, Karlsruhe, Germany) and random hexamer primers (Invitrogen). PCR was performed with the following primer pair: forward primer TTACGAGAGAAAACTCATGGCC and reverse primer GGGTCCAAGTTGTCCAGAATGC (Samali et al., 2010). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control with the following primer pair: forward primer CCCATGTTCGTCATGGGTGT and reverse primer: TGGTCATGAGTCCTTCCACGATA (Kurisaki et al., 2003). Cells treated with 1 μg/mL of Tunicamycin (TM) (Sigma–Aldrich, Munich, Germany) for 12 h were used as positive control. TM is an ER stress inducer which inhibits the N-linked protein glycosylation. The products of amplification were separated by electrophoresis on a 3% agarose gel and visualized by ethidium bromide staining. Images were photographed using the Chemidoc system (Bio-Rad, Munich, Germany) and analyzed by the ImageJ 1.42 software.

2.3. ER stress inhibition experiments

To inhibit the ER stress response, the UPR inhibitors IRE1 and TUDCA were used. The IRE1 inhibitor has the salicylaldehyde form of the salicylaldimine and inhibits the IRE1 endoribonuclease activity specifically (Volkmann et al., 2011). TUDCA is a derivative of an endogenous bile acid that alleviates ER stress (Berger and Haller, 2011). Vero E6 cells were pre-treated for 1 h with 60 μM IRE1 inhibitor (Sigma–Aldrich) or 500 μg/mL TUDCA (Calbiochem, Darmstadt, Germany), respectively, and inoculated with TBEV for another hour. Subsequently, cells were washed with PBS to remove the unbound virus particles and were further incubated in the presence of the inhibitors. After 24 and 48 h post infection, respectively, virus-containing cell culture supernatant was analyzed by plaque assay and viral protein from lysed cells was detected by western blotting, respectively, as described below.

2.4. Plaque assay

Virus titers of cell culture supernatants were measured by plaque assay. Briefly, A549 cells were grown overnight in a 24-well plate at 37 °C in 5% CO_2. Serial dilutions of the cell culture supernatant were added to the wells. After an incubation period of 1 h, carboxymethylcellulose (CMC) overlay medium (1.6% CMC in DMEM) was added to each well. The plates were incubated under the same conditions for four days. Each well was fixed by 3.7% formaldehyde for 60 min and stained with Naphthalene Black (1 g of naphthol blue black, 13.6 g of sodium acetate, 60 mL of glacial acetic acid and up to 1 L of ddH_2O). After 1 h, the Naphthalene Black was decanted, the plaques were counted and the calculation of plaque-forming units (PFU) was carried out according to Reed and Muench (1938). Data were shown as PFU/mL.

2.5. Western blotting

Cells were washed twice with ice-cold PBS and lysed using RIPA buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% NP40, 150 mM NaCl, 20% glycerol, 2 mM dithiothreitol and 0.5% deoxycholate acid). Nuclear proteins were harvested using the NE-PER nuclear extraction kit (Thermo Scientific). Equivalent amounts of cellular lysates or nuclear proteins were electrophoretically separated by 4–20% Tris-HEPES gels (Thermo Fisher Scientific, Schwerte, Germany). After electrophoresis, proteins were transferred onto a PVDF membrane by using a semi-dry blotter (Thermo Scientific). Then membranes were incubated with blocking buffer (5% non-fat milk in PBS solution with the detergent Tween 20) for 1 h and subsequently incubated with primary antibodies (diluted 1:500 to1:1000) at 4 °C overnight. Anti-TBEV E protein antibody (MAB 1367) was used for detecting TBE virus (Niedrig et al., 1994). Anti-actin and anti-PCNA (Proliferating cell nuclear antigen) antibody was obtained from Cell Signaling Technology (Cell Signaling Technology, Frankfurt am Main, Germany). Anti-XBP1 was obtained