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Dengue virus induced changes in Ca^{2+} homeostasis in human hepatic cells that favor the viral replicative cycle

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ABSTRACT

The role of Ca^{2+} during dengue virus (DENV) replication is unknown; thus, changes in Ca^{2+} homeostasis in DENV infected human hepatic HepG2 and Huh-7 cells were analyzed. Infected HepG2 cells, but not Huh-7 cells, showed a significant increase in plasma membrane permeability to Ca^{2+}, while both cell lines showed marked reduced levels of Ca^{2+} stored in the endoplasmic reticulum. While the expression levels of STIM1 and ORAI1 showed no changes, STIM1 and ORAI1 were shown to co-localized in infected cells, indicating activation of the store-operated Ca^{2+} entry (SOCE) pathway. Finally, manipulation in the infected cells of the intra and extracellular Ca^{2+} levels by chelators (BAPTA-AM and EGTA), SOC inhibitor (SKF96365), IP3 Receptor antagonist (2APB) or increase of extracellular [Ca^{2+}], significantly reduced DENV yield, but not vesicular stomatitis virus yield, used as a control. These results show that DENV infection alters cell Ca^{2+} homeostasis and that such changes favor viral replication.

1. Introduction

Dengue is the most prevalent mosquito borne viral disease to humans. The global incidence of dengue has grown dramatically in the recent decades and nowadays about half of the world's population is living in risk areas. One recent estimate indicates over 390 million dengue infections per year and another study estimates that 3.9 billion people, in 128 countries, are at risk of infection with dengue viruses (Bhatt et al., 2013; Brady et al., 2012; WHO, 2016b). Four serotypes (DENV-1 to DENV-4) have been described and the infection with each of them can produce a wide range of clinical conditions (Dengue Fever – Severe Dengue) (Gubler, 1998; WHO, 2016a). Despite the great disease burden associated with dengue, currently there are neither highly protective vaccines nor specific treatment for the disease (Simmons et al., 2012)

The dengue virus (DENV) belongs to the genus *Flavivirus* within the family *Flaviviridae*. The virion is enveloped and the genome consists of a single stranded RNA molecule of positive polarity of approximately 11 Kb. The genome of DENV encodes for 3 structural proteins (capsid, C; precursor membrane and membrane prM/M; envelope, E) and for 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5), which are all derived from the proteolytic processing of a polyprotein of approximately 3400 amino acids (Clyde et al., 2006)

Ionic calcium (Ca^{2+}) is a crucial second messenger that control many intracellular processes in mammalian cells (Berridge et al., 2000; Carafoli et al., 2001; Jaiswal, 2001). Thus, intracellular [Ca^{2+}] is finely regulated by a number of proteins that maintain Ca^{2+} intracellular homeostasis in different compartments to regulate spatial–temporal Ca^{2+} signaling (Berridge et al., 2003). The transmembrane Ca^{2+} – sensor protein resident in the ER, STIM1 and the plasma membrane resident protein ORAI1 (CRAC channel; Ca^{2+} – release – activated Ca^{2+} channel) are both key components involved in the store-operated Ca^{2+} entry (SOCE) pathway that maintain intracellular Ca^{2+} levels that regulate Ca^{2+} store in the endoplasmic reticulum (ER), especially in non- excitable cells (Barritt et al., 2009; Rosado, 2016).

DENV replication occurs in the cytoplasm in close association with the rough endoplasmic reticulum (RER) and involves the NS proteins as well as the assistance of cellular proteins, for replication, translation

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and encapsidation of the genome, and for the proper folding of the viral proteins (Clyde et al., 2006) and as observed for other positive sense RNA viruses, extensive ER membrane remodeling occurs in DENV infected cells (Romero-Brey and Bartenschlager, 2016). Since the ER is the main Ca^{2+} store in mammalian cells, alterations in ER Ca^{2+} homeostasis may be expected in DENV infected cells. Indeed, cellular mechanisms known to be activated by alterations in ER Ca^{2+} homeostasis, such as the unfolded protein response (UPR) (Datan et al., 2016; Ke and Chen, 2011; Medigeshi et al., 2007; Mohl et al., 2012; Su et al., 2002; Umareddy et al., 2007) or cellular processes responsive to increases in cytoplasm $[Ca^{2+}]_i$ such as autophagy (Datan et al., 2016; Dreux et al., 2009; Ke and Chen, 2011; Lee et al., 2008; Li et al., 2012; McLean et al., 2011; Mohl et al., 2012; Panyasrivanit et al., 2009), apoptosis (Medigeshi et al., 2007; Su et al., 2002; Thepparit et al., 2013; Thongtan et al., 2004) and cytoskeleton remodeling have been described in flavivirus infected cells. Disturbances in Ca^{2+} homeostasis in flavivirus infected cells have been shown depending on the virus, to favor the viral replicative cycle or to be related to pathogenesis. Early events during West Nile Virus (WNV) infection, cause an increase Ca^{2+} influx into the cytosol by disturbing plasma membrane permeability. The use of Ca^{2+} chelators and plasma membrane channel blockers resulted in a significant reduction in viral yield, suggesting that the observed increased in Ca^{2+} influx was required for an efficient viral replication by activating Ca^{2+} signaling that extends the survival of the infected cells (Scherbik and Brinton, 2010). On the other hand, HCV replication of hepatic cells produces ER Ca^{2+} depletion and an increase in Ca^{2+} uptake by mitochondria, which activates Bax, depolarization of the mitochondrial membrane, release of cytochrome *c*, apoptosis, and eventually liver damage (Benali-Furet et al., 2005; Ferri and Kroemer, 2001).

The effects of DENV replication on cellular Ca^{2+} homeostasis have not been studied. Given the close association of DENV replication with the ER and the alterations in membranes architecture induced, and the importance of such changes for the viral replicative cycle and pathogenesis, we sought to investigate if changes in Ca^{2+} regulation or homeostasis occur in DENV infected human hepatic cells. The study of intracellular processes activated during DENV replication in the ER and to understand how the virus may use these processes to favor viral replication may help in the design of therapeutic strategies for dengue. The results indicate that DENV infection increased plasma membrane permeability to Ca^{2+} and diminished the ion pool releasable to agonist stored in the ER and that such changes appear to favor an efficient production of infectious viral particles.

2. Materials and methods

2.1. Cell cultures and viral strains

Human hepatoma cell lines Huh7 and HepG2 and rhesus monkey Vero cells all were grown at 37 °C and a 5% CO_2 atmosphere using advanced DMEM medium (Gibco) supplemented with 2 mM glutamine, penicillin (5×10^4 U/ml), streptomycin (50 μg/ml) (InVitro S.A., Mexico), and 5% fetal bovine serum (FBS, Gibco). Propagation of DENV serotype 4, H241 strain, was done in CD1 suckling mice brains as previously described (Gould and Clegg, 1991) and viral titers were determined by focus assays in Vero-E6 cells (a kind gift of Dr. Susana López; ATCC# CRL-1586), as described below. CD1 suckling mice brains from mock infected animals were used as controls.

The Indiana strain of Vesicular Stomatitis Virus (VSV) was propagated in Vero-E6 cells and viral titers were determined by plaque assays in BHK – 21 cells.

2.2. Drugs treatments effect on DENV infection

The following drugs were used to treat DENV infected cells: The Ca^{2+} intracellular chelator BAPTA-AM (Invitrogen; cat: B6769), the Ca^{2+} extracellular chelator EGTA (USB; cat:15703), the IP3R antagonist 2-APB (Sigma Aldrich; cat: D9754), and the SOC inhibitor SKF 96365 (Sigma Aldrich; cat: S7809); in addition, $CaCl_2$ was used to the increase extracellular Ca^{2+} in the culture medium. Drugs were all diluted in DMSO and/or following manufacturer's instructions.

Hepatic cells were grown in 24-well culture cluster plates (Corning Incorporated, Corning, NY) at 37 °C in 5% CO_2. Confluent cell monolayers were infected with DENV4 (MOI = 3) for 1 h at 37 °C, washed with acid glycine (pH 3) to inactivate non-internalized viruses and washed 2 times with serum free DMEM. Followed by addition of 500 μl of Advance DMEM supplemented with 5% FBS, with or without the drug of interest, was added to each well and the infection was permitted to proceed for up to 24 h at 37 °C in 5% CO_2. At that time, supernatants were collected and virus yield determined by focus forming unit assay. Finally, viral titers of aliquots of DENV stocks treated or not with different concentrations of EGTA were also analyzed by focus forming assay.

2.3. Focus forming units assay

Focus forming unit assays as described by Ludert et al. (2008). Vero cells were seeded in 96-well cell culture cluster plates (Corning Incorporated, Corning, NY) at a density of 2.0×10^4 cells per well and incubated at 37 °C in 5% CO_2 until cells reached confluency. Cell monolayers were inoculated with 10-fold serial dilutions of virus in a final volume of 50 μl. Viral adsorption was allowed for 1 h at 37 °C and unbound virus removed by washing two times with medium. One hundred microliters of Advance DMEM (Gibco) medium supplemented with 5% FBS were added to each well. At 24hpi, the culture medium was removed and the cells fixed with 50 μl/well of cold methanol (−20 °C) for 20 min and washed twice with 150 μl/well of PBS (Sigma, cat: P4417) before addition of antibodies. The mouse mAb anti-DENV E protein, 4G2 and a goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Jackson Immuno Research, cat: 115-035-003), were used as primary and secondary antibodies, respectively. Both antibodies were diluted in PBS–FBS 1% and incubated with the monolayers for 1.5 h at 37 °C. The excess of antibody was removed by washing the monolayers 5 times with PBS 1x. Diaminobenzidine (DAB, Thermo-Fisher Scientific, cat: 34065) was used as a chromogenic substrate and color development was monitored at room temperature until optimal development. The reaction was stopped by rinsing the cells with 100 μl/well of PBS and the number of DENV infected cells determined using a light inverted microscope (Nikon Eclipse Ti; Japan) and results are reported as percentage (%) of viral yield, taking the control condition (DMSO treatment alone; 0.25% final concentration) as 100%.

2.4. Immunofluorescence assays

The number of hepatic infected cells was determined by immunofluorescence microscopy. Cells grown on 96-well plates were infected with DENV4 using a MOI = 3. At 24 hpi cells were fixed with paraformaldehyde, permeabilized with cold acetone and infected cells revealed using an anti-NS3 mouse Mab (a generous gift of Dr. Eva Harris) as a primary antibody and an Alexa Fluor 488–conjugated goat anti-mouse IgG (Life Technologies; cat: A11001) as a secondary antibody; cell nuclei were counterstained with DAPI (4′,6′-diamidino-2-phenylindole). The total number of cells in 3 fields was counted under an inverted microscope (Nikon Eclipse Ti; Japan) using the immersion 40X objective and the average number of infected cells was expressed as percentage of the total number of cells.

2.5. Cell viability assays

Calcium chelators, as well as channel blockers drugs were tested for cell toxicity, in mock infected as well as DENV infected cells, using