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# Cell-type specific variation in the induction of ER stress and downstream events in chikungunya virus infection

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ABSTRACT

Over the last decade infections with the mosquito transmitted chikungunya virus (CHIKV) have become a major worldwide concern, and considerable efforts have been made in understanding the interaction of this virus with the host cell machinery. Studies have documented the induction of the unfolded protein response (UPR), as well as the induction of apoptosis and autophagy in response to CHIKV infection. This study comparatively analysed these three processes in two cell lines, Hela and HepG2. Infection of Hela cells was characterized by activation of the PERK/eIF2α branch of the UPR, the induction of autophagy and early apoptosis, while infection of HepG2 cells was characterized by activation of the IRE/XBP1 branch of the UPR, limited or no activation of autophagy and comparatively later apoptosis. These results show that the specific cell context is an important mediator of the host cell response to CHIKV infection.


## 1. Introduction

Chikungunya virus (CHIKV) is an important *Alphavirus* belonging to the family *Togaviridae* that is mainly transmitted by *Aedes aegypti* and *Ae. albopictus* mosquitoes. The virus genome consists of a linear, positive sense single-stranded RNA molecule of approximately 11.8 kb [1,2] that is 5′-capped and 3′-polyadenylated encoding two open reading frames (ORFs). The first ORF encodes four non-structural proteins (nsP1-nsP4), while the second ORF encodes the three structural proteins, including capsid protein (C) and two envelope proteins (E2 and E1) as well as two small accessory proteins, E3 and 6k [3].

CHIKV is the causative agent of Chikungunya fever (CHIKF), which was first formally described in 1955 following an outbreak in Tanzania, Africa in 1952 [4] and CHIKF is characterized by high fever, rash, muscle pain and joint pain (arthralgia). In severe cases the joint pain may persist for weeks or months [5–8].

There are three distinct CHIKV lineages, the Asian, West African and East, Central and South African (ECSA) lineages [9]. In the last decade the ECSA lineage cause a significant outbreak in the countries around the Indian Ocean which affected millions of people [10]. More recently, CHIKV has emerged in the Americas with numerous reports of autochthonous transmission [11], although somewhat surprisingly these cases are apparently caused by the Asian lineage, and not by the ECSA lineage which was responsible for the Indian Ocean outbreak [12].

CHIKV is believed to enter susceptible cells by receptor mediated endocytosis which is clathrin-independent, but Eps15 dependent [13]. After membrane fusion, the genomic RNA is directly translated to generate the replication complex (nsP1-nsP4) which directs synthesis of the negative strand which acts as a template to produce both the full length positive sense genome as well as a sub-genomic positive sense RNA that serves as the template for translation of the second open reading frame producing the structural and accessory proteins [3].

As with many RNA viruses [14], the process of CHIKV infection results in disturbances of endoplasmic reticulum (ER) homeostasis, probably through the influx of nascent unfolded protein, leading to the activation of various stress response pathways including the unfolded protein response (UPR) pathway [15,16]. The ER resident UPR is a prosurvival signal [17,18] that restores ER homeostasis and reduces the accumulation of unfolded proteins by inducing genes involved in protein folding, inhibiting the translation of protein to

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reduce the influx of new protein into the ER lumen and removing unfolded proteins by promoting ER-associated protein degradation (ERAD) [19].

The UPR is primarily mediated by the chaperone glucose regulated protein 78 (GRP78), and the accumulation of unfolded proteins in the ER leads to the disassociation of GRP78 from the three transmembrane signaling proteins, protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring protein 1 (IRE1) allowing their activation [17]. Activated PERK phosphorylates eukaryotic translation initiation factor 2 on the alpha subunit (eIF2α) at Ser51, and *p*-eIF2α decreases the load of proteins entering into the ER lumen by blocking general protein translation [20,21]. Activated ATF6 is a transcription factor which increases the transcription of a number of ER chaperones, the X box-binding protein 1 (XBP1) and other transcription factors. Activation of IRE1 results in the IRE1 mediated splicing of the XBP1 mRNA and spliced XBP1 generates a transcription factor to activate the expression of downstream genes like chaperones and proteins involved in protein degradation [22,23].

If ER homeostasis cannot be reinstated leading to prolonged ER stress, the expression of the C/EBP homologous protein (CHOP), which is induced by both ATF6 and PERK, leads to the induction of apoptosis through its interaction with the anti-apoptotic B-cell lymphoma 2 protein (Bcl-2), leading to the activation of the intrinsic apoptosis pathway [24,25]. In addition prolonged ER stress can lead to the p53 mediated induction of Noxa and the p53 upregulated modulator of apoptosis (PUMA), which induce the activation of BAK/BAX, resulting in cytochrome *c* release from mitochondria and the subsequent induction of intrinsic apoptosis [26].

CHIKV infection has been shown to induce apoptosis in a number of different cell types [27], and it has been proposed that apoptotic blebs resulting from the apoptosis of a CHIKV infected cell may serve to hide CHIKV and promote its distribution around the body [28]. There are two main pathways (intrinsic and extrinsic) of apoptosis induction in mammalian cells, although a number of alternate pathways are known [29]. Intrinsic apoptosis is broadly characterized by the activation of caspase 9 through events occurring in mitochondria, while extrinsic apoptosis occurs after activation of a cell surface death receptor leading to the subsequent activation of caspase 8 [30,31], There is crosstalk between the two pathways, as caspase 8 cleaves the Bcl-2 homology domain 3 (BH3)-only protein Bcl-2 interacting domain (Bid), promoting cytochrome *c* release from the mitochondria resulting in the activation of caspase 9 [32].

Studies have shown that activation of the UPR can induce autophagy in an attempt to reduce ER stress from an accumulation of unfolded or misfolded proteins which cannot be degraded by the proteasome [33,34]. Autophagy is a catabolic process that is important for maintaining cellular homeostasis by removing excess or damaged cellular organelles as well as long-lived and aggregated proteins [35]. It is induced during organelle turnover, cell differentiation, hormone response and as a consequence of a wide variety of stresses stimuli, including starvation, growth factor withdrawal, oxidative stress, hypoxia, redox stress and viral infection [36–38]. Viruses have been shown to have a complex interaction with the host cell autophagic machinery, with the interactions broadly being characterized as one of defense, evasion or subversion [39].

Several studies have documented the induction of autophagy in response to CHIKV infection [40–42], and it has been proposed that CHIKV subverts autophagic induction to promote its own replication, as inhibiting autophagy reduces viral output [42], although this interaction is species specific [41]. It has also been proposed that the induction of autophagy delays the onset of caspase-dependent cell death [40] and thus CHIKV infection is proposed to result in the early induction of autophagy to increase virus replication and the later induction of apoptosis to promote viral spread [43]. While a number of studies have looked at the induction of ER stress [15,16], apoptosis [27,28,44,45] and autophagy [40–42] in a number of different cell lines, few studies have compared these processes simultaneously in two different cell lines to look at the role of cell type in the response to CHIKV infection. The cell lines selected for investigation were the human cervical epithelial cell line Hela, and the human hepatocarcinoma cell line HepG2, both of which have been shown to be susceptible to infection [27]. The results show clear differences in the induction of ER stress, autophagy and apoptosis in response to CHIKV infection, and highlight a role for cell context in mediating the interaction between CHIKV and the host cell machinery.

## 2. Materials and methods

### 2.1. Cells, virus and infection

Hela (human cervical epithelial, ATCC CCL-2) and HepG2 (human hepatocarcinoma, ATCC HB-8065) cells were cultured at 37 °C, 5% $CO_2$ in Dulbecco's modified eagle's medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen) (DMEM/FBS) and 100x penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria). The CHIKV East, Central and South African (ECSA genotype, Thai isolate E1:226V) was propagated as described previously [27].

For infection of Hela and HepG2 cells, cell were seeded into cell culture dishes and grown until cells reach approximately 80% confluence, after which cells were infected with CHIKV at the selected multiplicity of infection (MOI) diluted in ice-cold serum free medium for 2 h. After 2 h of viral adsorption at 37 °C, the medium containing the virus was removed, fresh medium containing serum was added and the cells were incubated at 37 °C under 5% $CO_2$ until the day of the experiment. As appropriate, cells were treated with 2 μg/ml of tunicamycin (TM), 2.5% and 5% (v/v) DMSO or 100 nM rapamycin (Sigma-Aldrich, St. Louis, MO).

### 2.2. Cell viability

Mock infected or Hela and HepG2 cells infected with CHIKV at m.o.i 10 and m.o.i. 1, respectively were collected at the indicated time points. On days 1–3 p.i., cells were stained with 0.4% trypan blue (Invitrogen) and were counted on a hemocytometer under an inverted light microscope to determine the percentage of viable cells. The experiments were performed independently in triplicate for each day as three biological replicates.

### 2.3. Determination of infectious CHIKV titer by using standard plaque assay

The infectious CHIKV titer was determined by standard plaque-forming unit assay on Vero cells. Vero cells were grown in 6 well-tissue culture plates until a confluent monolayer was reached within 24 h. The culture medium was removed from the 6-well plate and the cells were inoculated with 200 μl of culture supernatant containing infectious viruses which was prepared as 10 fold serial dilutions in BA-1 medium. After incubating cells with virus at 37 °C for 2 h with constant agitation, the cells were gently overlaid with 4 ml per well of 0.8% Seakem Le agarose (Cambrex, USA) mixed with nutrient overlay (Earle's Balanced Salts supplemented with 0.033% (w/v) yeast extract, 0.165% lactalbumin hydrolysate, 3% FBS). The plates were left at room temperature for approximately an hour until the overlayer completely set follow by incubation