Japanese encephalitis virus activates autophagy through XBP1 and ATF6 ER stress sensors in neuronal cells

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Abstract

Endoplasmic reticulum (ER) stress and autophagy are key cellular responses to RNA virus infection. Recent studies have shown that Japanese encephalitis virus (JEV)-induced autophagy negatively influences virus replication in mouse neuronal cells and embryonic fibroblasts, and delays virus-induced cell death. Here, we evaluated the role of ER stress pathways in inducing autophagy during JEV infection. We observed that JEV infection of neuronal cells led to activation of all three sensors of ER stress mediated by eIF2α/PERK, IRE1/XBP1 and ATF6. The kinetics of autophagy induction as monitored by levels of SQSTM1 and LC3-II paralleled activation of ER stress. Inhibition of the eIF2α/PERK pathway by siRNA-mediated depletion of proteins and by the PERK inhibitor had no effect on autophagy and JEV replication. However, depletion of XBP1 and ATF6, alone or in combination, prevented autophagy induction and significantly enhanced JEV-induced cell death. JEV-infected cells depleted of XBP1 or ATF6 showed reduced transcription of ER chaperones, ERAD components and autophagy genes, resulting in reduced protein levels of the crucial autophagy effectors ATG3 and BECLIN-1. Conversely, pharmacological induction of ER stress in JEV-infected cells further enhanced autophagy and reduced virus titres. Our study thus demonstrates that a crucial link exists between the ER stress pathways and autophagy in virus-infected cells, and that these processes are highly regulated during virus infection.

INTRODUCTION

Japanese encephalitis virus (JEV) is a member of the family Flaviviridae that includes such viruses as West Nile virus (WNV), Dengue virus (DENV), tick-borne encephalitis virus (TBEV) and Zika virus. These viruses are arthropod-borne and a global health concern [1–3]. JEV is endemic in large parts of the Indian subcontinent and causes encephalitis in a predominantly paediatric population with a high mortality rate [4, 5]. With the ever-increasing threat and impact of these viruses on human health, a detailed understanding of the host–virus interaction is imperative for the development of novel and effective anti-virals. Vaccination is an available preventive measure and has been effectively employed by some countries. However, limitations of poor efficacy and lack of long-term protection persist [6, 7].

Generation of endoplasmic reticulum (ER) stress and induction of the unfolded protein response (UPR) are generic host responses to flavivirus infection as virus replication occurs in close association with ER-derived membranes [8–11]. The mammalian cell has three ER stress sensors that lead to activation of mechanistically distinct pathways. Protein kinase R (PKR)-like ER kinase (PERK) is a well-characterized regulator of the ER stress-induced translational control pathways [12, 13]. Under ER stress, PERK dissociates from GRP78, is activated by dimerization and phosphorylation and, further, phosphorylates eIF2α leading to global translation arrest [12]. This further leads to expression of transcription factor GADD153 (CHOP), which causes cell-cycle arrest and induces apoptosis [14, 15]. The IRE1 sensor produces the transcriptional factor XBP1s, activates JUN N-terminal kinase (JNK) and degrades selective mRNAs through the regulated IRE1-dependent decay (RIDD) pathway [16–19]. The ATF6 pathway activation leads to translocation of ATF6 to the Golgi, and its cleavage to produce the N-terminal fragment of ATF6 which functions as a transcription factor [20]. XBP1...
and ATF6 together lead to transcription of distinct but overlapping sets of genes that attempt to restore ER homeostasis by modulating protein folding and ER-associated degradation (ERAD) [17, 21, 22]. The three UPR sensors provide the cell with the flexibility to respond to a large variety of cellular stressors. By arm-selective UPR activation, the cell can thus remodel the ER proteostasis network between enhanced protein folding and trafficking versus enhanced ERAD [23]. UPR signalling also intersects with and influences other host pathways such as lipid metabolism, autophagy, innate immunity and differentiation [24].

Autophagy is a highly regulated degradation pathway that also maintains cellular homeostasis [25]. Under conditions of starvation and stress, such as during virus infection, the pathway is upregulated and several studies have established the role of autophagy/autophagy proteins in influencing virus replication and pathogenesis. The significance of autophagy in RNA-virus infections is complex and not completely understood, with studies supporting both pro- and anti-viral roles for the pathway [26–32]. Studies from our group and others have shown that JEV infection leads to upregulation of autophagy in several cell types [27, 31, 33]. Our recent studies have shown that autophagy negatively regulates virus replication in mouse neuronal cells and mouse embryonic fibroblasts and functions as a pro-survival pathway during JEV infection. Interestingly, the LC3 protein, a marker of autophagosomes (as lipidated LC3-II) associates with virus replication complexes in its non-lipidated form (LC3-I) [31].

Several studies on Flaviviruses have reported the induction of ER stress and activation of one or more ER stress sensor [8, 9, 11, 34–37]. JEV infection has been shown to activate expression of CHOP [10] and the XBP1 pathway of ER stress [38]. We have previously shown activation of the RIDD pathway that benefits JEV replication in neuronal cells [39].

Activation of ER stress pathways has been implicated in autophagy induction for the flaviviruses hepatitis C virus (HCV) and Dengue [36, 40, 41], and other viruses such as encephalomyocarditis virus, bluetongue virus and varicella-zoster virus [42–44]. In the present study, we have characterized the ER stress response and autophagy in JEV-infected neuronal cells. We observe that JEV infection leads to activation of all three ER stress pathways mediated by eIF2α/PERK, XBP1 and ATF6. While the eIF2α/PERK pathway does not influence autophagy, depletion of the proteins XBP1 and ATF6 curbed autophagy and enhanced cell death during JEV infection. These proteins directly impact the RNA and protein levels of crucial autophagy modulators like ATG3 and BECLIN 1 in JEV-infected cells. While RNAi-mediated depletion of XBP1 did not affect virus replication, a similar depletion of ATF6 enhanced virus replication and titres, suggesting an important role of ATF6 in the virus life cycle. We demonstrate that the ER stress sensors of XBP1 and ATF6 are crucial for JEV-induced autophagy in neuronal cells, which directly impacts cell survival during virus infection. Pharmacological activation of ER stress in JEV-infected cells further enhanced autophagy and reduced virus titres, suggesting that UPR and autophagy are highly regulated in the virus-infected cell.

RESULTS

JEV-induced autophagy is independent of the PERK/eIF2α pathway

To investigate the link between ER stress pathways and autophagy, we first analysed the phosphorylation of PERK and eIF2α over a 24 h time course of JEV-infected Neuro2a cells (Fig. 1a, left panel). As a positive control for ER stress, cells were treated with thapsigargin for 8 h (Fig. 1a, right panel). In JEV infection, phosphorylation levels of PERK increased from 14 h post-infection (p.i.) and remained high until 24 h p.i. (Fig. 1a). A transient increase in levels of phosphorylated eIF2α was seen from 14 h p.i., which then returned to basal levels by 24 h p.i. (Fig. 1a). Expression of GADD153 was also observed in JEV-infected cells, which is consistent with earlier reports [10]. Finally, we analysed autophagy in JEV-infected cells by monitoring levels of LC3-II and SQSTM1, which are widely used markers of autophagy induction and autophagosome substrate, respectively [45]. Levels of LC3-II increased in JEV-infected neuronal cells from 14 h p.i. Similarly, levels of SQSTM1 decreased, which is indicative of a functional autophagy pathway that culminates in degradation in the lysosomes. Collectively, these data indicate that the PERK/eIF2α pathway of UPR is activated in JEV infection, along with the induction of autophagy.

To examine the possibility of a functional link between PERK activation and induction of autophagy, we targeted the PERK protein by both pharmacological inhibition (Fig. 1b) and RNA interference (Fig. 1c). The PERK inhibitor blocked phosphorylation of PERK in response to both thapsigargin treatment and JEV infection. This treatment also led to a decrease in levels of SQSTM1; however, the levels of LC3-II did not change significantly in JEV-infected cells (Fig. 1b). The siRNA treatment resulted in a 70% decrease in PERK RNA levels (data not shown) and a significant decrease in protein levels (Fig. 1c). However, the levels of SQSTM1 and LC3-II remained unchanged following siRNA treatment as compared to non-targeting (NT) siRNA (Fig. 1c). This clearly demonstrated that PERK activation was not involved in JEV-induced autophagy. We further targeted eIF2α by RNA interference (Fig. 1d). Here, a significant block in GADD153 production was observed. However, there was no effect on autophagy induction as seen by decreased levels of SQSTM1 and high levels of LC3-II (Fig. 1d). Collectively, these data suggest that the PERK/eIF2α pathway does not play a role in JEV-induced autophagy in neuronal cells.

JEV-induced autophagy requires XBP1 but is independent of JNK activation

Activation of the IRE1-XBP1 pathway during flavivirus (including JEV) infection has been reported in earlier studies [9, 11, 35, 38]. To reproduce the activation of the IRE1-XBP1 pathway in JEV-infected Neuro2a cells, we performed a time course of JEV infection and extracted total RNA at the
indicated times p.i. Activation of IRE1 leads to an unconventional post-transcriptional splicing of Xbp1 mRNA \((uXbp1)\) to produce a spliced form of Xbp1 \((sXbp1)\) mRNA that leads to the translation of the XBP1s protein. We followed a standard reverse transcription PCR protocol that detects \(uXbp1\) as a 442 bp fragment and \(sXbp1\) as a 416 bp product [46]. The \(uXbp1\) retains a \(Pst\) 1 digestion site and can be cleaved into fragments of 295 and 147 bp, while the \(sXbp1\) loses that

\[ \text{PERK} \]

\[ \text{P-eIF2} \alpha \]

\[ \text{eIF2} \alpha \]

\[ \text{GADD153} \]

\[ \text{SQSTM1} \]

\[ \text{LC3-I} \]

\[ \text{LC3-II} \]

\[ \text{JEV-NS1} \]

\[ \text{J EV-NS3} \]

\[ \text{GAPDH} \]

\[ \text{siRNA: } \text{NT} \quad \text{NT} \]

\[ \text{PERK} \]

\[ \text{S QST M1} \]

\[ \text{LC3-I} \]

\[ \text{LC3-II} \]

\[ \text{J E V - N S 3} \]

\[ \text{GAPDH} \]

\[ \text{siRNA: } \text{NT} \quad \text{eIF2} \alpha \]

\[ \text{PERK} \]

\[ \text{GADD153} \]

\[ \text{SQ STM1} \]

\[ \text{LC3-I} \]

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\[ \text{JEV-NS3} \]

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\[ \text{siRNA: } \text{NT} \quad \text{NT} \]

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\[ \text{siRNA: } \text{NT} \quad \text{eIF2} \alpha \]

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\[ \text{siRNA: } \text{NT} \quad \text{NT} \]

\[ \text{PERK} \]

\[ \text{S QST M1} \]

\[ \text{LC3-I} \]

\[ \text{LC3-II} \]

\[ \text{J E V - N S 3} \]

\[ \text{GAPDH} \]

\[ \text{siRNA: } \text{NT} \quad \text{eIF2} \alpha \]

\[ \text{PERK} \]

\[ \text{GADD153} \]

\[ \text{SQSTM1} \]

\[ \text{LC3-I} \]

\[ \text{LC3-II} \]

\[ \text{JEV-NS3} \]

\[ \text{GAPDH} \]
restriction site and remains undigested. We observed the generation of the sXbp1 mRNA in JEV-infected cells from 14 h p.i. (Fig. 2a, left panel). By 20–24 h p.i. and in thapsigargin-treated cells (Fig. 2a, right panel), a major fraction of the Xbp1 mRNA was in the spliced form, indicating the activation of the IRE1/XBP1 pathway of ER stress.

To evaluate the role of XBP1, we depleted the protein by RNA interference (Fig. 2b) and studied its effect on levels of LC3-II and SQSTM1 in JEV-infected cells. The siRNA treatment resulted in a 70–80% decrease in the RNA levels of the gene and a significant decrease in protein levels (Fig. 2b). XBP1 depletion attenuated the JEV-induced autophagy, as seen by the increased SQSTM1 levels and a significant decrease in conversion of LC3-I to LC3-II, suggesting that autophagy in response to JEV infection requires XBP1.

Under ER stress, IRE1 has been shown to lead to activation of JNK [47]. This serves as a pro-apoptotic signal and can also induce autophagy in response to starvation and ER stress by Beclin-1 activation [18]. We observed that JEV infection also leads to JNK phosphorylation (Fig. 2c). To check whether JNK inhibition had any effect on autophagy, the JNK inhibitor SP600125 was added to cells after JEV infection. While the

Fig. 2. JEV-induced autophagy requires XBP1 but is independent of JNK activation. (a) JEV activates the IRE1/XBP1 pathway. Total RNA was isolated from mock- or JEV-infected (m.o.i. of 5) cells (left panel; DMSO/thapsigargin (right panel)-treated Neuro2a cells, and amplified using Xbp1 and Gapdh primers and separated by electrophoresis on 2% agarose gels. The unspliced Xbp1 (uXbp1) is amplified as a 442 bp fragment while the spliced Xbp1 (sXbp1) gives a 416 bp fragment. The PCR products from Xbp1 amplification (upper panel) were digested with Pst I. The uXbp1 retains the Pst I site and is digested into the 295 and 147 bp fragments, while the sXbp1 loses the site and is seen as a 416 bp fragment. (b) Neuro2a cells transfected with NT/Xbp1 siRNA were mock-/JEV-infected. At 24 h p.i., cell lysates were blotted with the indicated antibodies. Ratio of LC3-II:GAPDH is represented below the blots. (c) To mock-/JEV-infected Neuro2a cells, vehicle control/JNK inhibitor was added. Cell lysates were prepared at 24 h p.i. and Western blotting was done with the indicated antibodies. The JEV-NS3 blot is infection control and the GAPDH blot is a loading control. Similar results were obtained in three independent experiments.
inhibitor blocked JNK phosphorylation, it had no effect on autophagy induction, suggesting that JEV-induced autophagy is independent of JNK activation (Fig. 2c).

**ATF6 is activated during JEV infection and is essential for autophagy**

The third sensor of ER stress is the 90 kDa protein ATF6, which on its dissociation from GRP78 is targeted to the Golgi, where it is proteolytically cleaved and its 50 kDa DNA-binding domain is targeted to the nucleus to activate gene expression [20, 48, 49]. Activation of the ATF6 pathway of ER stress has not been reported for JEV, but has been shown for the related flaviviruses WNV, DENV and TBEV [8, 9, 11, 34, 37]. Western blot analysis of a time course of JEV infection and thapsigargin treatment showed an increase in levels of the 50 kDa-cleaved ATF6 fragment (Fig. 3a). For further validation, we performed an ATF6 promoter activation assay. Neuro2a cells were co-transfected with plasmids p5xATF6-GL3 and pCI-Neo-hRluc. In response to JEV infection and thapsigargin treatment, significant activation of the ATF6 promoter was observed as measured by relative increase in luciferase activity (Fig. 3b). Collectively, these data demonstrate that ATF6 is activated in Neuro2a cells during JEV infection.

To examine the role of ATF6 in autophagy, we depleted the protein either alone (Fig. 3c) or in combination with XBP1 (Fig. 3d) and examined the levels of LC3-II and SQSTM1. The siRNA treatments resulted in a significant decrease in the protein levels (Fig. 3c, d). Both ATF6 depletion alone and in combination with XBP1 curbed JEV-induced autophagy, as seen by increased SQSTM1 levels and a significant decrease in the levels of LC3-II, suggesting that autophagy in response to JEV infection requires both XBP1 and ATF6.

**Effect of depletion of ER stress sensors on virus replication and virus-induced cell death**

We further tested the direct impact of the depletion of these ER stress sensors on virus replication and virus-induced cell death. The siRNA-mediated depletion of the ER stress sensors did not impact cell viability as seen by MTT assay (Fig. 4a). Depletion of the proteins PERK and XBP1 had no impact on JEV RNA levels and titres (Fig. 4b, c). However, ATF6 depletion significantly enhanced both virus RNA levels and titres, suggesting that ATF6 controls critical replication node(s) of the virus life cycle. Since we earlier observed that autophagy-deficient cells are highly susceptible to cell death, we analysed the cell viability in the background where autophagy has been reduced by depletion of XBP1 and ATF6 (Fig. 4d). We observed significantly enhanced cell death in JEV-infected cells depleted of XBP1 and ATF6, thus further validating our earlier study [31] that demonstrated a critical role of autophagy as a pro-survival pathway during virus infection.

**Effects of XBP1 and ATF6 depletion on autophagy effectors**

XBP1 and ATF6 govern the expression of a large range of partially overlapping target genes. XBP1 controls gene transcription of protein-folding, ERAD and phospholipid synthesis programs, while ATF6 has been shown to influence ER chaperones, ERAD components and also XBP1 [12, 17, 22, 24, 50]. To probe for a mechanistic link between autophagy induction during JEV infection and the ER stress pathways, we evaluated the effect of XBP1 and ATF6 depletion on transcription of a subset of ER chaperones, and ERAD and autophagy-related genes that are significantly upregulated during JEV infection (Fig. 5a). Depletion of Atf6 had no effect on either mRNA levels of Xbp1 or Xbp1 splicing (Fig. 5a, b). Consistent with the current literature, we found that XBP1 depletion impacted transcription of ERAD effectors while ATF6 depletion had a greater influence on ER chaperones. We also examined the transcript levels of certain crucial autophagy genes: Atg3, Atg5 and Beclin1. Transcription of Atg3 was reduced by depletion of both XBP1 and ATF6, while that of Beclin1 was influenced by XBP1 alone. In JEV-infected cells depleted of XBP1, significantly reduced protein levels of BECLIN 1 were observed, while in ATF6-depleted cells the levels of ATG3 were reduced (Fig. 5c). These data thus show the direct role of the ER stress sensors in modulating autophagy during JEV infection.

**Effects of pharmacological ER stress activators on JEV-induced autophagy and replication**

We further activated specific arms of the UPR pharmacologically and tested their effect on autophagy in JEV-infected cells. The PERK inducer, CCT020312, activated PERK-initiated signalling [51] and showed phosphorylation of PERK (Fig. 6a). For IRE1 activation, we employed the flavanol quercetin [52], which demonstrated PERK-initiated signalling [51] and showed phosphorylation of IRE1 (Fig. 6b). Treatment of JEV-infected cells with the PERK and IRE1 activators and thapsigargin led to a significant enhancement in levels of LC3-II compared to the DMSO control (Fig. 6c). This suggests that the extent of autophagy induction in JEV-infected cells is highly regulated via the extent of UPR activation. Interestingly, while the PERK activator showed an increase in levels of LC3-II, a corresponding decrease in levels of SQSTM1 was not observed, suggesting its potential effect on autophagy flux. While the PERK and IRE1 activators did not affect JEV RNA levels (Fig. 6d), a significant decrease was seen in the translation of viral protein (Fig. 6c) and production of infectious virus particles was inhibited by over 90% (Fig. 6e). Thapsigargin treatment completely abolished JEV replication (~99% block), suggesting that a global activation of UPR is detrimental to virus infection (data not shown).

**DISCUSSION**

JEV is a neurotropic virus that can breach the blood–brain barrier and infect neuronal cells, leading to severe outcomes such as encephalitis, neurological sequelae and death. Hence, understanding how the virus interacts with neuronal cell processes is crucial to better understanding the disease process and designing novel therapies. Autophagy is one such cellular pathway that plays an important role in the replication and
pathogenesis of viruses. Host responses to infection, such as oxidative and ER stress, activation of innate immune sensors and signalling downstream of virus–receptor interactions have, been linked to autophagy induction. While there are reports that describe the induction of autophagy [27, 31, 33], how the pathway is regulated during JEV infection is poorly understood. Studies from our laboratory have shown that autophagy negatively impacts JEV replication in neuronal cells and functions as a pro-survival pathway. While autophagy flux is functional at the early stages of infection (24 h), the pathway becomes dysfunctional as infection progresses (48 h), leading to accumulation of misfolded proteins and cell death [31]. Here we performed a detailed characterization of the ER stress activation in response to JEV infection and show that autophagy in neuronal cells is controlled by the XBP1 and ATF6 ER stress sensors. This has a direct impact on virus-induced cell death.

ER stress leads to the activation of an adaptive mechanism in the cell known as the UPR, which is regulated by signalling molecules PERK/elf2α, IRE1/XPB1 and ATF6 [24, 53, 54]. The UPR is an integrated signal transduction pathway that tries to restore homeostasis by expansion of the ER membrane, enhancement of the protein-folding capacity,
attenuation of protein influx in the ER and/or upregulation
of ERAD [17, 21, 46, 54]. It can also have other physiological
outcomes that modulate autophagy, innate immunity,
lipid synthesis and cell differentiation. If stress remains
unresolved, apoptosis is triggered [24]. Thus, depending
on the nature of the insult, ER stress can result in distinct
and contrasting outputs that are tightly controlled by the
amplitude and kinetics of signalling via the three UPR
sensors.

Activation of the IRE1 protein leads to its dimerization fol-
lowed by phosphorylation, which triggers its RNase activity
to produce XBP1s [17, 19]. XBP1s controls transcriptional
activation of genes involved in protein-folding, ERAD, pro-
tein quality control and phospholipid synthesis [21, 24, 50].
IRE1 also induces JNK-driven stress pathways and degrada-
tion of selective-host mRNAs through the RIDD pathway
[16, 18]. Cleaved ATF6 controls the upregulation of genes
encoding ER chaperones and ERAD [17, 22, 23].

Flaviviruses are known to induce ER stress in the infected
cell as a result of their replication in membranes closely
associated with/derived from the ER [8–11, 35, 38]. Addi-
tionally, accumulation of viral proteins floods the protein-
folding capacity of the ER. Studies have shown that JEV
induces ER stress in fibroblasts and neuronal cells, as seen
by enhanced expression of ER chaperones and activation of
GADD153, ultimately leading to apoptosis [10]. Further
more, JEV- and DENV-2-triggered IRE1-XBP1 pathway
activation in neuronal cells and mouse brains has been
reported previously [38]. In addition to Xbp1 splicing, IRE1
selectively degrades other cellular mRNAs through the
RIDD pathway [16]. The RIDD pathway is activated during
JEV infection and enhances virus replication [39].

Studies have shown the contribution of one or more UPR sen-
sors in the induction of virus-induced autophagy. eIF2α is
essential to inducing autophagy in response to herpes simplex
virus and bluetongue virus infection [44, 55]. eIF2α and ATF6

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**Fig. 4.** Effect of depletion of ER stress markers on JEV replication and cell death. (a) Neuro2a cells either mock-transfected or trans-
fected with NT/Perk/eIF2α/Xbp1/Atf6/Xbp1+Atf6 siRNAs. The viability of cells at 48 h of siRNA treatment was tested by MTT assay.
(b–d) Neuro2a cells treated as above were infected with JEV (m.o.i. of 5). (b) At 24 h p.i., total RNA was isolated and JEV RNA levels
were determined by qRT-PCR. (c) JEV titres in supernatants at 24 h p.i. were determined by plaque assays. (d) Percentage of cell death
in JEV-infected cells. Values represent mean ± SD of three independent experiments. Student’s t-test was used to calculate P values.
*P<0.05, **P<0.01.
have been shown to be essential for HCV-activated autophagy through MAP1LC3B and ATG12 expression in Huh7 hepatoma cells [36]. PERK-deficient mouse embryo fibroblasts have demonstrated an important role of PERK in DENV-induced autophagy [40]. An essential role for IRE1 has been demonstrated for Chikungunya virus-induced autophagy [56].

Fig. 5. Effects of XBP1 and ATF6 depletion on autophagy effectors. (a) Neuro2a cells were treated with Xbp1 or Atf6 siRNA and infected 48 h later with JEV. The levels of ER chaperone and ERAD and autophagy gene transcripts 24 h pi were studied by qRT-PCR. The graph shows the relative expression of gene transcripts normalized to mock-infected samples. Values represent mean±SD of three independent experiments. (b) Xbp1 amplification from Neuro2a cells transfected with NT/Atf6 siRNA that were mock-/JEV-infected for 24 h. (c) Neuro2a cells transfected with NT/Xbp1/Atf6 siRNA were mock-/JEV-infected, and at 24 h p.i. cell lysates were blotted with the indicated autophagy protein antibodies and GAPDH (loading control). Ratio of autophagy protein : GAPDH is represented in the table on the right. Similar results were seen in three independent experiments. Student’s t-test was used to calculate P values. *P<0.05, **P<0.01.
In our present study, we have performed a detailed characterization of all three arms of the UPR in the context of JEV-induced autophagy. We observed that JEV infection leads to activation of the PERK/eIF2α pathway as seen by enhanced phosphorylation of PERK, transient phosphorylation of eIF2α and synthesis of GADD153. A reversal of eIF2α phosphorylation is likely mediated by the activation of GADD34 that catalyses dephosphorylation of eIF2α [57]. A similar transient phosphorylation of eIF2α has also been reported for DENV [9]. We also observed robust activation of IRE1 as seen by increased Xbp1 splicing and activation of JNK, although cleavage of ATF6 and activation of ATF6 promoter activity was also observed. Upregulation of GADD153 and activation of the XBP1 pathway in response to JEV infection has previously been reported [10, 38], but this is the first report of ATF6 activation in response to JEV infection.

We observed that JEV-induced autophagy in neuronal cells is completely independent of the PERK/eIF2α axis, as PERK inhibition and siRNA-mediated depletion of PERK or eIF2α had no effect on LC3-II production and SQSTM1 degradation. While JNK activation does not appear to be essential for JEV-induced autophagy, we observed that both XBP1 and ATF6 are crucial for autophagy and also directly impact cell survival during infection. Depletion of the two proteins, either alone or together, completely blocked LC3-II conversion and SQSTM1 degradation in JEV-infected neuronal cells. Studies have shown that XBP1 and ATF6 govern the expression of a large range of partially overlapping target genes [17, 22, 24]. ATF6α and XBP1 have also been shown to heterodimerize for the induction of ERAD components [22]. Indeed, we observed that XBP1 and ATF6 impact the transcription and protein levels of ATG3 and BECLIN 1 in JEV-infected cells. Further more, both XBP1 and ATF6 play a crucial role in determining virus-induced cell death, most likely through their role in autophagy induction.

The anti-viral role of ATF6 on JEV replication could also be potentially mediated by its effect on innate immune responses. Several studies have suggested that UPR activation can have a synergistic effect on the induction of innate immune responses.
immune response genes [58, 59]. ATF6 has been shown to play a role in the expression of pro-inflammatory cytokines such as TNFα and IL-6 [60]. The impact of UPR sensors on these anti-viral mechanisms needs to be explored further.

Pharmacological activation of PERK, IRE1 and global UPR activation by thapsigargin further enhanced autophagy in JEV-infected cells. This manifested in decreased virus titres, emphasizing the anti-viral role of autophagy in JEV infection. Our data clearly suggest that the extent of UPR activation and autophagy is fine-tuned in the infected cell to maintain a balance between virus replication and cell survival. Further dissection of the regulatory check-points that specifically control these processes could provide further insights to better understand JEV pathogenesis.

METHODS

Cells and virus

Mouse neuroblastoma (Neuro2a) and porcine stable kidney (PS) cells were obtained from the Cell Repository at the National Centre for Cell Sciences, Pune, India. Neuro2a cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10 % fetal bovine serum (FBS). PS cells were grown in Eagle’s minimal essential medium (MEM) with 10 % FBS. All media were additionally supplemented with 100 µg ml⁻¹ penicillin/streptomycin and 2 mM l-glutamine. For all studies, JEV isolate P20778 grown in PS cells was used. JEV was titrated by plaque formation on PS monolayers as described previously [31]. HRP-coupled secondary antibodies were produced in our laboratory and have been described previously [61]. Plaque assay results are presented as means ± SD of three independent experiments.

Reagents, antibodies and plasmids

Antibodies against ATF6 (ab11909), eIF2α (ab5369), phospho-eIF2α (ab32157), GADD153 (ab11419) and SQSTM1 (ab56416) were obtained from Abcam. Antibodies against ATG3 (3415), ATG5 (12 994), BECLIN 1 (3495), GAPDH (2118), LC3 (3868), PERK (C33E10) and phospho-PERK (T980) were purchased from Cell Signalling Technology. XBP1 antibody (SAB2102720), thapsigargin (T9033), IRE1 activator Quercetin (Q4951) and JNK inhibitor SP600125 (10 µM), IRE1 activator (50 µM) and thapsigargin (1 µM) were added to JEV-infected cells at 10h p.i. and maintained till harvest. All drug treatments were tested for toxicity by MTT assays and did not affect cell viability at the indicated concentrations and duration of treatment. After infection/treatment, cells were washed twice with PBS and processed. In all experiments, culture supernatant was collected for plaque assays at 24 h p.i. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Transfections were typically allowed to proceed for 18–24 h before infection/analysis.

siRNA depletion experiments

Mouse-specific Atf6, eIF2α, Perk, Xbp1 targeting siRNA and non-targeting (NT) siRNA were purchased from Dharmacon (ON-TARGET plus SMART pool). Cells were transfected with siRNAs according to the manufacturer’s recommendations and harvested 24 and 48 h later to check for mRNA and protein depletion. All siRNA treatments resulted in a significant decrease in mRNA levels (70–80 %). To check for protein depletion, cell lysates were run on SDS-PAGE and Western blotting was done for ATF6, eIF2α, PERK, XBPI and GAPDH (loading control). For infection experiments, cells were transfected with siRNAs for 48 h, following which they were infected with JEV. The siRNA treatments did not affect cell viability till 72 h, as tested by MTT assay.

Quantitative real-time (qRT)-PCR for JEV and other genes

Cells were given the appropriate treatment/infection and RNA was extracted by lysis in Trizol reagent. The cDNA was prepared with random hexamers. To determine JEV RNA levels, qPCR was done using Taqman probes and Gapdh amplification served as the internal control. JEV was amplified using the following probes – Taqman probe: CCACGCACCTCGACCCATAGACTG (5’ end FAM, 3’ end TAMRA); 5’ primer: AGAGCCAAGGGAATGAA TAGT, 3’ primer: AATAAGTTGTAGTGGGCGACTCTG. Gapdh Taqman probe sequence: ACAACCTTGCTCCTCAGTGAAGC (5’ end FAM, 3’ end TAMRA); 5’ primer: AGGCGCCAAAAAGGATAAGTAGT, 3’ primer: AATAAGTTGTAGTGGGCGACTCTG. The PCR conditions were as follows: 94°C for 2 min (1 cycle), 94°C for 15 s, 55°C for 30 s and 72°C for 1 min (40 cycles). For all ER chaperones, ERAD and autophagy genes, qRT-PCR was performed using SYBR green reagents. Primers for all these genes were designed from sequences available from the Harvard qPCR primer
bank. Gapdh served as the endogenous house-keeping control. The qRT-PCR was done on the Applied Biosystems ABI 7500 instrument. All experiments had biological duplicates and were performed independently three or more times. The fold-change in the expression level of genes and JEV RNA levels is represented as mean ± SD of three or more independent experiments.

**Western blots**

Treated/mock or JEV-infected cells were washed in PBS and lysed in buffer containing 1% Triton-X-100 in 50 mM Tris HCl, pH 7.5, 150 mM NaCl and protease inhibitor cocktail (Sigma). Equal amounts of protein extracts were separated on polyacrylamide gels and transferred to PVDF membranes for immunoblotting. Band intensities were quantified with ImageJ software. Similar results were seen in three independent experiments.

**Assay for Xbp1 splicing**

The Xbp1 splicing assay was done as described previously [46]. Total RNA was isolated and cDNA was prepared using random hexamers. The Xbp1 transcript was amplified using the following primers: Xbp1-F (5′-AACAGAGTAGCAGGCCGACTGC-3′) and Xbp1-R (5′-TCCCTCTGGG TAGACCTCTGGGAG-3′). The purified PCR product was digested with the restriction enzyme Pst I (NEB) and resolved on a 2% agarose gel. Similar results were seen in three independent experiments.

**ATF6 promoter activation**

Neuro2a cells were co-transfected with 1 µg p5XATF6-GL3 and 100 ng pC1-Neo-hRluC. At 24 h post-transfection, cells were either mock/JEV infected (m.o.i. of 5) for 24 h or treated with vehicle control/thapsigargin (8 h). Quantification of firefly and renilla luciferase activities in the samples was done following the manufacturer’s recommendations (Dual-Luciferase Reporter Assay System, Promega). Samples were analysed on an Orion II microplate Luminometer (MPL4, Berthold Detection System, Germany). Results were plotted as relative luciferase activity (firefly/renilla) normalised to mock infection from four biological repeats.

**MTT assay**

Cells were seeded in 96-well plates. After 24 h, siRNA treatment was given in triplicate. At 72 h post-transfection, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Amresco, 0793-1G] was added to the cells at a final concentration of 0.5 mg ml⁻¹. After 3 h of incubation with MTT solution, the entire medium was removed and the assay was terminated by adding 100 µl DMSO per well. Readings were taken at 570 nm using an ELISA plate reader. The percentage cell viability was calculated as [(absorbance for siRNA treated cells)−(absorbance for mock-treated cells)] × 100.

**Cell death assay**

Apoptosis in JEV-infected cells was assayed using the Annexin-V-FITC Apoptosis kit (KA0714) from Abnova. Briefly, cells were stained with Annexin-V-FITC and PI and were analysed by flow cytometry using a Becton-Dickinson FACSCantoII flow cytometer. All cell death experiments were performed with biological duplicates and results shown are means ± SD of three independent experiments.

**Statistical analysis**

Student’s t-test was used for statistical analysis. Differences were considered significant at values of *P*<0.05; **P**<0.01.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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