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Effect of modulation of unfolded protein response pathway on dengue virus infection

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Abstract

The unfolded protein response (UPR) is a cascade of events that helps restoring cellular homeostasis under stressful conditions. It is activated when there is an imbalance in the protein load and protein folding capacity of the endoplasmic reticulum (ER) as a result of an increase in the naïve, unfolded, or misfolded protein content of the cell. Dengue virus (DENV) utilizes the host machinery to synthesize viral proteins and replicates in the cell. During DENV infection, up-regulation of viral proteins increases the protein pool of the cell, resulting in the induction of UPR pathway. In this study, we have tried to understand the consequence of UPR induction during DENV infection in human monocytic cells. To fulfill this objective, we have used VER-155008 (VER), a known inhibitor of the 78 kDa glucose-regulated protein (GRP78), which is the master regulator of the UPR pathway. After VER treatment, cells were infected with DENV, and the induction of the UPR elements and their downstream activation was studied by western blotting and RT-PCR analysis. Interestingly, inhibition of GRP78 via VER treatment led to the decreased expression of DENV envelope protein through the activation of the UPR elements, protein kinase-like ER resident kinase, activating transcription factor 6, and inositol-requiring enzyme 1 (IRE1), and then led to the activation of innate immune factors such as double-stranded RNA-activated protein kinase (PKR), interferon regulated factor 3 (IRF3), nuclear factor-κB (NF-κB) and interleukin 1β (IL-1β). This strategy may be used to decrease viral infection transiently. Thus UPR elements could be important therapeutic targets for decreasing DENV multiplication.

Key words: UPR, GRP78, dengue virus, PKR, interferon

Introduction

Dengue virus (DENV) is the causative agent of mild dengue fever, severe dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), affecting millions of people annually. It is predominant in the tropical and sub-tropical regions of the world [1]. The virus is transmitted through the bite of the arthropod vector *Aedes* spp. Dengue virus is a flavivirus with positive sense, single stranded, 10.7 kb long RNA genome coding for three structural (capsid, C; premembrane, prM; and envelope, Env) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) [2,3]. The DENV genome has a 5′-cap (m7GpppAmpN2) but lacks a 3′-poly(A) tail. It is translated into a large polyprotein that is processed co- and post-translationally by viral and host proteases into 10 viral proteins [4]. While the structural proteins prM and Env are cleaved by the cellular signalase, the C protein is cleaved at the hydrophobic signal sequence on its C-terminal end by the viral NS2B-NS3 protease. The non-structural proteins are mostly processed by the viral protease except NS1 which is cleaved by an unidentified protease [5].

Monocytes/macrophages (Mo/Mϕ) are the primary targets of infection of DENV [6–8]. Despite the progress in understanding the flavivirus translation strategies, the complete understanding of how DENV interacts with the host cell translational machinery is inadequate. A protective target against this virus is not yet known. Therefore, there is an urgent need to develop anti-DENV therapeutic agent, which requires the elucidation of various aspects of DENV infection in human Mo/Mϕ such as the strategies employed by viral proteins in manipulating the host cellular machinery through their interactions with cellular proteins.

The immunopathogenesis of DHF and DSS involves antibody-dependent enhancement of infection and detrimental host responses [9,10]. Several factors govern the severity of the disease. There are four serotypes of DENV, types 1–4. Differences in DENV serotypes, host immune response, environmental factors, arthropod vector, and viral titer in circulation tend to determine the disease severity. During DENV infection, interaction of the host and virus is a crucial factor which skews the condition to either clearance of the virus or worsening of infection. Recognition of the virus and appropriate activation of anti-viral responses is essential for clearing the virus; however, viruses tend to evolve over time and undergo mutation favorable to them which helps in evading the host immune recognition systems. The unfolded protein response (UPR) is one such host cellular pathway which primarily maintains homeostasis. During DENV infection, synthesis of large amount of viral proteins leads to accumulation of unfolded or misfolded proteins and thus the protein processing load on the endoplasmic reticulum (ER) increases, leading to activation of the UPR in the host. Moreover, the induction of the UPR pathway may provide additional 'danger' signal to the cell besides activation of specific microbial pattern recognition receptors during DENV infection, which may help in mounting an effective immune response to curb virus multiplication [11].

The cellular mechanism to counteract an unprecedented increase in the level of unfolded/misfolded proteins leading to ER stress involves sensing of these proteins by three transmembrane elements of the ER: protein kinase-like ER resident kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) [12–14]. The UPR is regulated closely by the 78 kDa glucose-regulated protein (GRP78) or immunoglobulin heavy chain binding protein (BiP) [15]. BiP remains bound to the three UPR elements and maintains their inactive state. During the ER stress, BiP dissociates from the UPR element(s) resulting in the activation of the element(s) and induction of UPR. Activation of PERK occurs by means of the oligomerization and autophosphorylation of the transmembrane kinase PERK. Following PERK activation, the eukaryotic translation initiation factor-2 (eIF2α) gets phosphorylated and results in attenuation of translation, thereby providing some relief from the protein stress on the ER [16]. However, DENV has been shown to trigger PERK-mediated eIF2α phosphorylation early during infection and suppress it subsequently [17]. Similarly, oligomerization of IRE1 leads to splicing of XBP1 mRNA and subsequent activation of genes involved in protein folding, ER-associated degradation pathway (ERAD), and phospholipid biosynthesis. IRE1 may also degrade ER-bound mRNA through the regulated IRE1-dependent decay pathway (RIDD) in order to decrease protein translation. Whereas, during ER stress, ATF6 gets cleaved by proteases and the DNA binding domain containing part of ATF6 moves towards the nucleus to activate genes involved in protein folding [18,19].

This study demonstrated that inhibition of GRP78 led to the decreased DENV envelope protein expression through the activation of the UPR elements, PERK, ATF6, and IRE1α, and then led to the activation of innate immune factors such as PKR, IRF3, IL-1β, and NF-κB. This strategy may be used to decrease viral infection transiently. Thus UPR elements could be important therapeutic targets for decreasing DENV multiplication.

Materials and Methods

Cells and virus

Human monocytic cell line THP-1 was obtained from National Centre for Cell Science (Pune, India) and maintained in RPMI (Sigma, St Louis, USA) supplemented with 10% fetal bovine serum. Dengue virus type-2 New Guinea C strain grown in C6/36 insect cell line with viral titer of 10^6 pfu/ml was obtained from International Centre for Genetic Engineering and Biotechnology (New Delhi, India).

MTT assay

THP-1 cells at a concentration of 1×10^6 cells/ml were treated with 10, 20, 30, 40, and 50 μM VER-155008 (VER, a GRP78 inhibitor) (Sigma) for 72 h. Five milligrams of VER lyophilized powder was reconstituted in 1 ml of DMSO, generating a stock solution of 9 mM which was diluted in PBS to form the working solution. Assay procedure using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazoliumbromide (MTT) (Sigma) was described previously [20]. The optical density was measured at 570 nm using a spectrophotometer (Biotek Instruments, Winooski, USA) to calculate the cytotoxic effect of VER treatment in cells.

VER treatment and DENV infection in THP-1 cells

THP-1 cells were harvested and resuspended in fresh 2% complete RPMI medium (RPMI supplemented with 2% FBS) at a concentration of 2×10^6 cells/ml. Cell suspension (2 ml) was cultured in a six-well culture plate dish and treated with 20 μM VER for 24 h. Then, cells were infected with DENV at a multiplicity of infection (MOI) of three for 24 h, and finally the cells were harvested for reverse transcriptase polymerase chain reaction (RT-PCR) analysis and western blot analysis.

RNA extraction and reverse transcriptase polymerase chain reaction

Total cellular RNA was extracted from THP-1 cells using the RNeasy Protect mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Amplification of target genes was done using One-step RT-PCR kit (Qiagen) by using specific primers, following the manufacturer's protocol. The PCR product obtained was resolved on 1%–3% agarose gel. The primer pairs and their product sizes were: GRP78 forward primer (5′-GTTCTTGCCGTTCAAGGTGG-3′), reverse primer (5′-TGGTACAGTAACAACTGCATG-3′), 181 bp; XBP1 forward primer (5′-CTGGAACAGCAAGTGGTAGA-3′), reverse primer (5′-CTGGGTCCTTCTGGGTAGAC-3′), 424 bp; CHOP forward primer (5′-ATGAGGACCTGCAAGAGGTCC-3′), reverse primer (5′-TCCTCCTCAGTCAGCCAAGC-3′), 136 bp; β-actin forward primer (5′-GAGACCTTCAACACCCCAGCC-3′), reverse primer (5′-GGATCTTCATGAGGTAGTCAG-3′), 207 bp.

Immunoblotting

THP-1 cells untreated or treated with VER, infected with DENV or mock infected as described above, were harvested, and cytoplasmic or nuclear extract was prepared as per the standard protocol [21]. Lysates containing 30 μg of protein were separated on 12% sodium dodecyl sulfate-polyacrylamide gel following the method described by Laemmli [22] and transferred to polyvinylidene difluoride membranes, as described by Towbin *et al.* [23]. The membranes were incubated for 1 h with 3% BSA in TBS buffer (0.1 M Tris–HCl, pH 7.4, and 0.9% NaCl) to block non-specific binding followed by washing with $TBST_{20}$ (0.1% Tween-20 in TBS) and incubated with primary antibodies. Antibodies against GRP78, DENV Env, PERK, ATF6, and pIRE1α were from Thermo Fisher Scientific (Waltham, USA). Antibody against NF-κB was from BioVision (Milpitas, USA). Antibodies against PKR, IRF3, and β-actin were from Sigma. Specially, NF-κB was primarily detected with the NF-κB p-65 subunit.

Subsequently, the membranes were washed thrice for 10 min with $TBST_{20}$, and then incubated with horseradish peroxidase-conjugated IgG secondary antibody (AbD Serotec, Kidlington, UK) against the corresponding host primary antibody, respectively. The protein bands were detected by using chemiluminescence kit (Sigma).

Cytokine quantification by cytofluorimetry-based ELISA

The expression level of the cytokine IL-1β was quantified by means of a FlowCytomix cytofluorimetry-based ELISA system (eBioscience, San Diego, USA). THP-1 cells were treated either with or without 20 μM VER for 24 h. Subsequently, the cells were either mock or DENV (MOI 3) infected and cultured for 24 h. Supernatant was aspirated from each well and kept at −80°C until assay. Cytokine estimation was done according to the manufacturer's instructions. Data were acquired on a flow cytometer (BD FACS Calibur, San Jose, USA) using Cell Quest Pro software (BD Biosciences).

Intracellular staining for DENV

THP-1 cells mock infected and DENV infected with or without VER treatment were harvested and washed twice with 0.01 M PBS. Cells were fixed and permeabilized with Cytofix and cytoperm buffer for 30 min in FACS tubes (BD Falcon, Franklin Lakes, USA). Furthermore, cells were washed twice and incubated with FITC-conjugated anti-DENV Moabs (Biorbyt, UK) at 1:100 dilution as a final concentration for 60 min. After being washed with PBS, cells were resuspended in 0.5 ml of PBS and 10,000 cells were acquired on the flow cytometer using Cell Quest Pro software.

Statistical analysis

The results are expressed as the mean ± SEM and all the statistical comparisons were carried out using Student's *t*-test for repeated measurements wherever applicable. Experiments were repeated at least thrice. Significance level was set at $P < 0.05$.

Results

The cytotoxicity of GRP78 inhibition via VER treatment on THP-1 cells

The chemical compound VER or 5′-O-[(4-cyanophenyl)methyl]-8-[[(3,4-dichlorophenyl)methyl]amino]-adenosine is an inhibitor of protein isoforms of the Hsp70 family, in particular, having similar potencies for GRP78 and Hsp70 [24]. Cytotoxic effect of VER was measured using the MTT assay at the doses of 10, 20, 30, 40, and 50 μM in THP-1 cells for up to 72 h. The VER doses from 10 to 40 μM showed more than 80% viability. The cell cytotoxicities of VER at doses of 10, 20, 30, 40, and 50 μM were found to be 14.0% ± 2.6%, 15.0% ± 1.3%, 15.0% ± 3.3%, 18.0% ± 2.1%, and 38.0% ± 7.5%, respectively (Fig. 1). The optimum dose of VER was determined as 20 μM, as most of the changes in UPR element activation were observable at this dose (data not shown).

GRP78 inhibition via VER treatment decreased the expression of GRP78

The inhibition of GRP78 was performed by using the chemical inhibitor of the ATPase domain, VER [24]. The mRNA and protein expression levels of GRP78 were measured. GRP78 was found to be significantly increased on DENV infection at both the mRNA and protein levels (Fig. 2A,B). The results suggested that the mRNA and protein levels of GRP78 expression were decreased in VER-treated cells when compared with DENV-infected cells alone (Fig. 2A,B).

GRP78 inhibition via VER treatment decreases DENV-induced Env protein expression

Moreover, the inhibition of GRP78 in VER-treated cells infected with DENV significantly decreased the expression of the viral Env protein when compared with the DENV-infected cells alone (Fig. 3A,B). We further measured the effect of GRP78 inhibition on the UPR elements and some of the downstream genes and proteins to account for the decrease in DENV protein expression.

The effects of GRP78 inhibition via VER treatment on the UPR elements

The VER treatment activated the UPR element PERK. It was shown that DENV infection significantly increased PERK expression, which was further increased on VER treatment (Fig. 3A,C). Thus, GRP78 inhibition was able to induce the host UPR element PERK during DENV infection, facilitating the host to restore ER homeostasis.

The UPR element ATF6 was not induced by VER treatment or DENV infection alone when compared with control. However, the DENV infection on VER-treated THP-1 cells induced a significant increase in ATF6 level when compared with control (Fig. 4A,B). This result indicated that on inhibiting GRP78, there was an enhanced host response during DENV infection through the activation of the UPR element ATF6. Induction of the UPR element IRE1α was also found to be significantly induced in all the three treatment groups when compared with control (Fig. 4A,C). Interestingly, inhibition of GRP78 showed maximum induction of IRE1α in the absence of DENV infection.

Splicing of XBP1, which is downstream of IRE1α in the UPR pathway, was measured by RT-PCR (**Fig. 5**A,B). The ratio of XBP1 spliced (XBP1-s) to total (XBP1 total) mRNA was found to be increased in DENV-infected cells when compared with control, and was further enhanced in DENV-infected cells treated with VER. However, GRP78 inhibition alone was found to show significant increase in XBP1 splicing when compared with other groups. This directly showed that inhibition of GRP78 during DENV infection is able to cause effective induction of the IRE1α pathway of the UPR. During continued stress in the ER, the host tends to activate either the

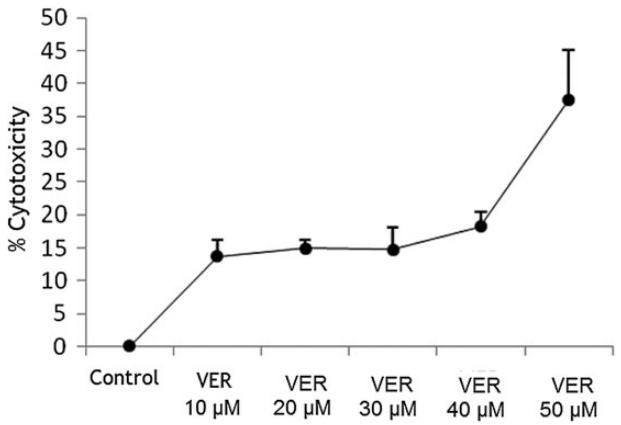

Figure 1. Effect of VER treatment on THP-1 cell viability The effect of VER treatment in cells did not show cytotoxicity at doses of 10, 20, 30, and 40 μM, but showed 38% cytotoxicity at the dose of 50 μM. THP-1 cells were treated with different doses of VER for 72 h and viability of the cells was measured using MTT assay. Percent cytotoxicity in different treatment groups of THP-1 cells was measured. Control, untreated cells. Cells treated with different doses of VER: 10, 20, 30, 40, and 50 μM. Data were presented as the mean ± SEM of three independent experiments.

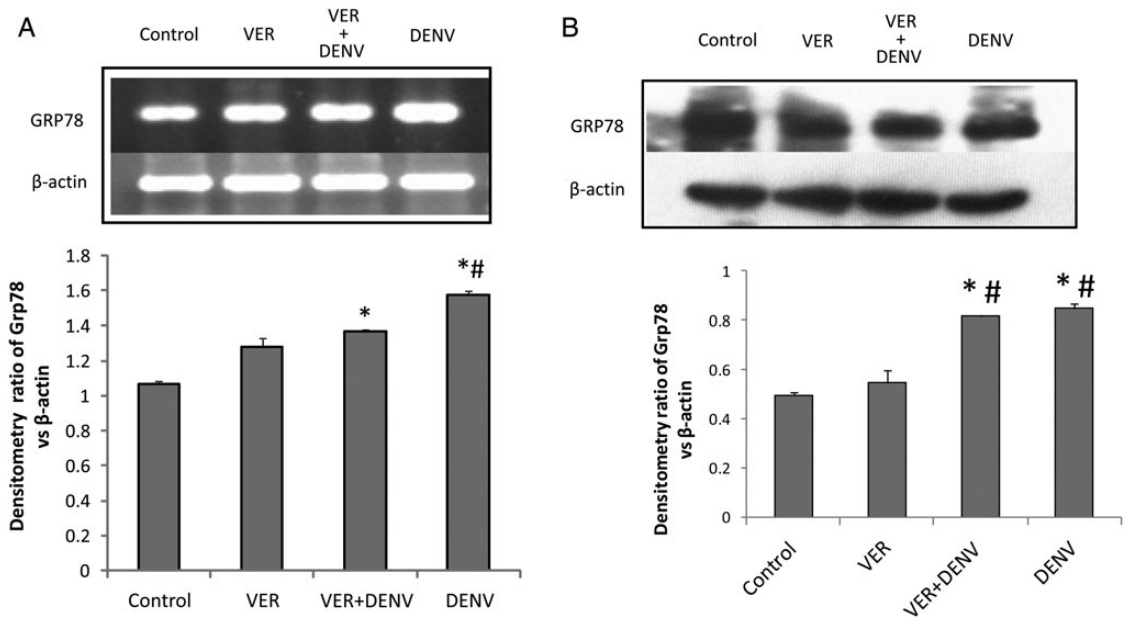

Figure 2. DENV increased the expression of GRP78 (A) The mRNA expression levels in THP-1 cells were analyzed by RT-PCR. GRP78 mRNA expression was increased in DENV-infected cells; however, it was decreased by VER treatment. (B) Immunoblotting was done using lysates of THP-1 cells as described in the section 'Materials and Methods'. Immunoblot shows the expression of GRP78 and β-actin. GRP78 protein expression was increased in DENV-infected cells; however, it was decreased by VER treatment. Control, untreated cells; VER, cells treated with 20 μM VER; VER + DENV, 20 μM VER-treated cells infected with DENV; DENV, DENV-infected cells. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. VER. Data were presented as the mean ± SEM of three independent experiments.

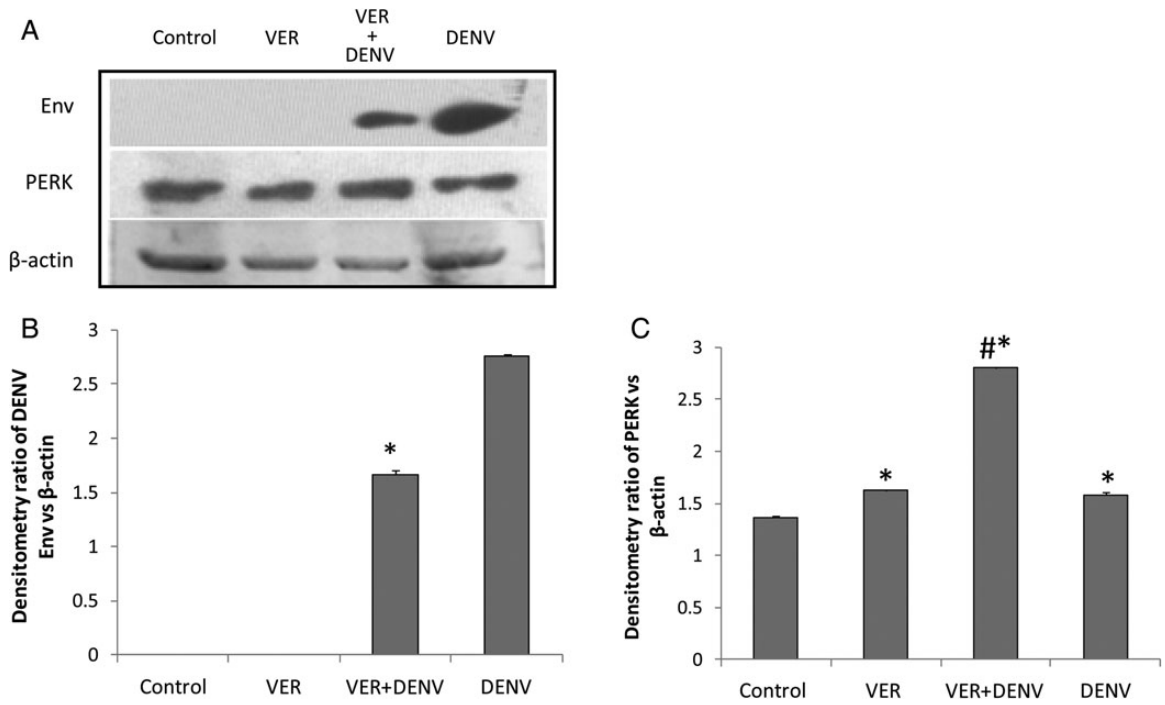

Figure 3. Western blot analysis of DENV Env protein and host protein PERK (A) Immunoblot showing DENV Env protein and host UPR element PERK expression, along with β-actin. Immunoblotting was done using lysates of THP-1 cells as described in the section 'Materials and Methods'. (B) Densitometry of DENV Env protein expression. DENV Env expression was significantly decreased in VER-treated DENV-infected cells when compared with DENV-infected cells alone. $^{*}P < 0.05$ vs. DENV. (C) Densitometry of UPR element PERK expression. PERK was found to be significantly increased in DENV-infected cells with or without VER treatment when compared with control. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. DENV. Control, untreated cells; VER, cells treated with 20 μM VER; VER + DENV, 20 μM VER-treated cells infected with DENV; DENV, DENV-infected cells. Data were presented as the mean ± SEM of three independent experiments.

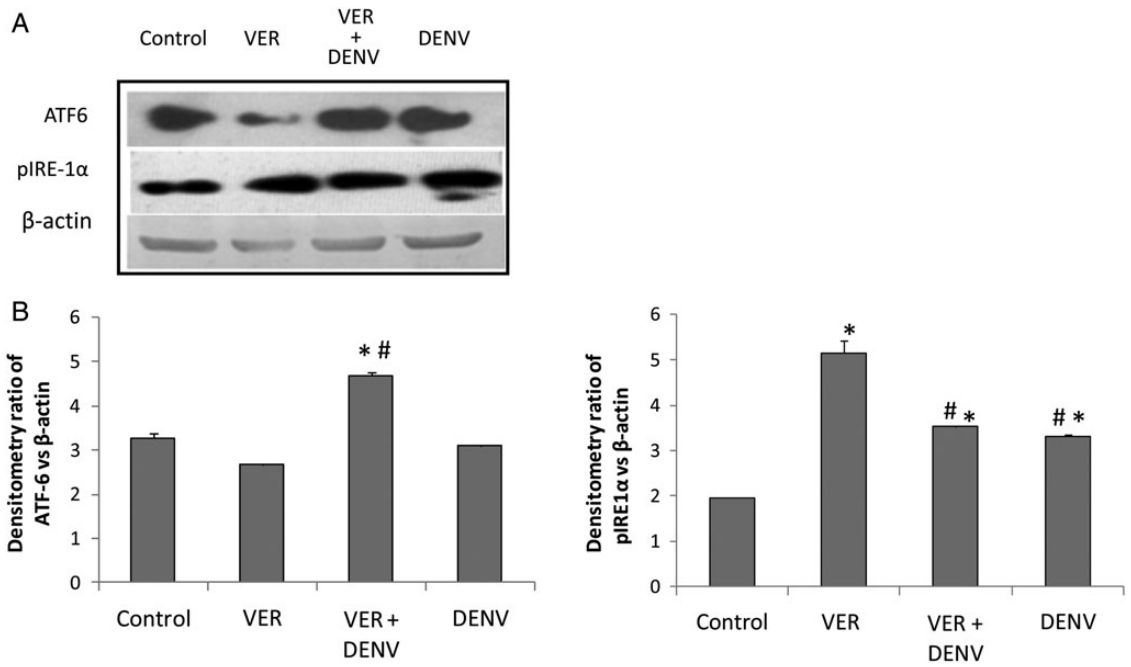

Figure 4. Western blot analysis for ATF6 and pIRE1α (A) Immunoblot showing UPR elements ATF6 and pIRE1α expressions, along with β-actin. Immunoblotting was done using lysates of THP-1 cells as described in the section 'Materials and Methods'. (B) ATF6 was found to be significantly increased in DENV-infected cells treated with VER when compared with control and DENV-infected cells alone. *P < 0.05 vs. control; #P < 0.05 vs. DENV. (C) pIRE1α was found to be significantly increased in DENV-infected cells treated with or without VER when compared with control. pIRE1α expression in DENV-infected cells with or without VER treatment showed reduced expression when compared with VER-treated cells alone. *P < 0.05 vs. control; #P < 0.05 vs. VER. Control, untreated cells; VER, cells treated with 20 μM VER; VER + DENV, 20 μM VER-treated cells infected with DENV; DENV, DENV-infected cells. Data were presented as the mean ± SEM of three independent experiments.

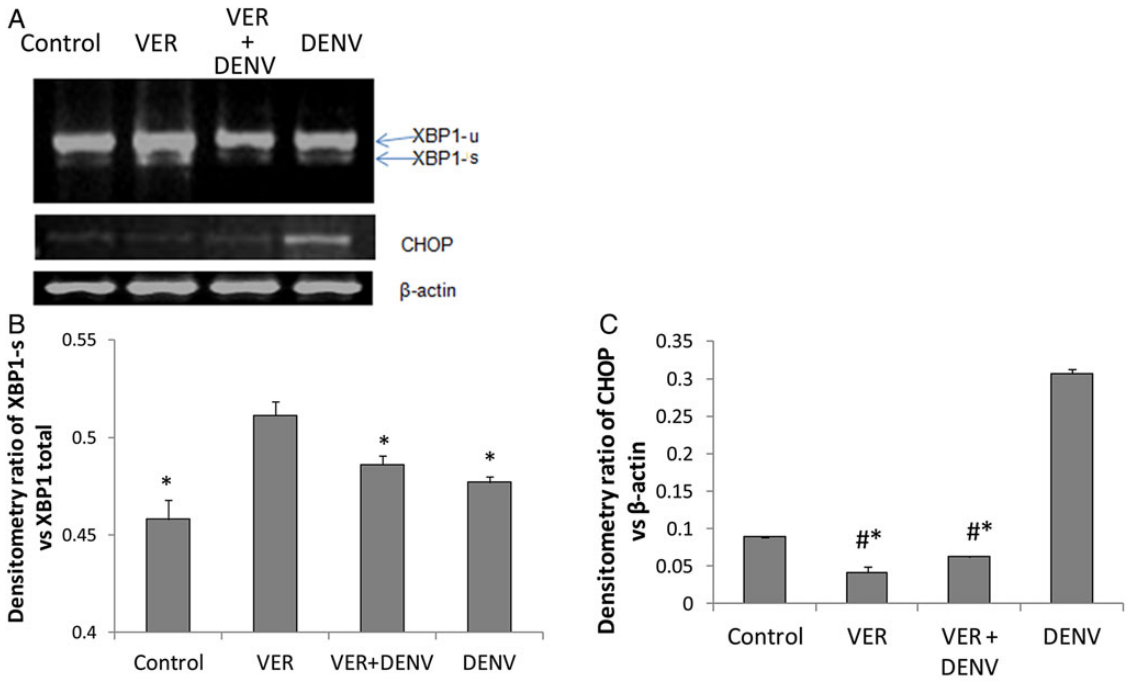

Figure 5. Expression of XBP1 and CHOP (A) The mRNA expression levels of XBP1 and CHOP in THP-1 cells were analyzed by RT-PCR. (B) The densitometry ratio of XBP1-s to XBP1 total was significantly higher in VER-treated cells alone when compared with the other groups, *P < 0.05 vs. VER. (C) Densitometry analysis showed that there was a significant increase in CHOP in DENV-infected cells alone and GRP78 inhibition significantly decreased CHOP expression. *P < 0.05 vs. DENV; #P < 0.05 vs. control. Control, untreated cells; VER, cells treated with 20 μM VER; VER + DENV, 20 μM VER-treated cells infected with DENV; DENV, DENV-infected cells. Data were presented as the mean ± SEM of three independent experiments.

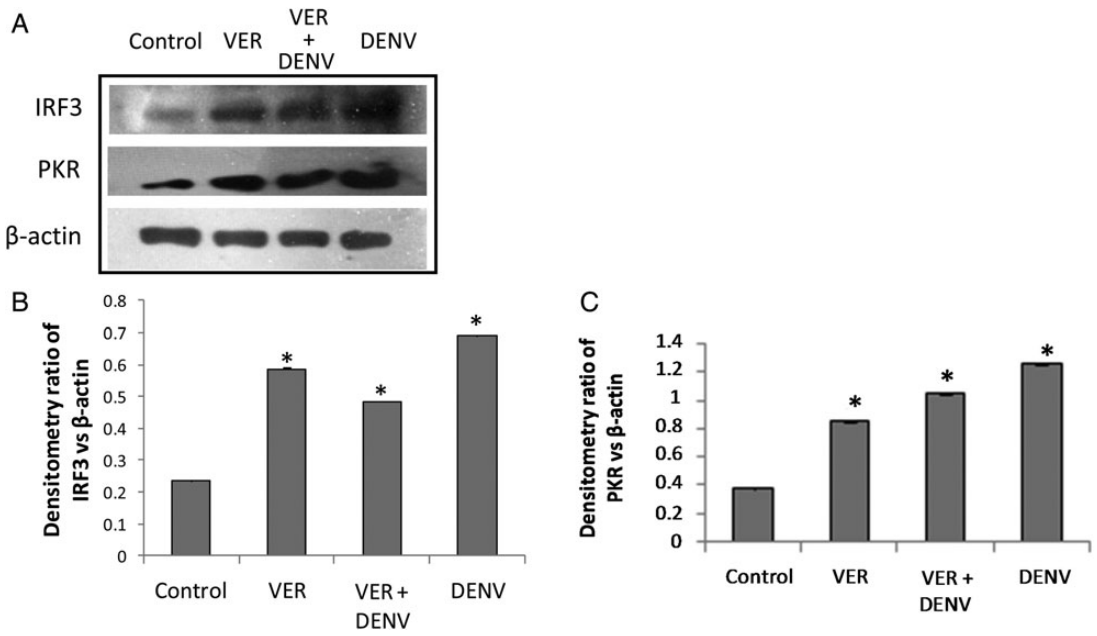

Figure 6. IRF3 and PKR were increased on VER treatment and DENV infection (A) Immunoblotting was done using lysates of THP-1 cells as described in the section 'Materials and Methods'. Immunoblot shows the expression of IRF3, PKR, and β-actin. (B,C) Densitometry analysis showed that both IRF3 and PKR were increased in VER-treated cells alone and in DENV-infected cells treated with or without VER treatment when compared with control. Control, untreated cells; VER, cells treated with 20 μM VER; VER + DENV, 20 μM VER-treated cells infected with DENV; DENV, DENV-infected cells. *$P < 0.05$ vs. control. Data were presented as the mean ± SEM of three independent experiments.

IRE1-XBP1 or ATF6 pathway or both [17]. In this study, we observed that during DENV infection the host cell responded by activating the IRE1-XBP1 pathway more than the ATF6 pathway. DENV was also found to significantly increase the expression of the downstream protein CHOP when compared with the other groups (**Fig. 5**A,C). Inhibition of GRP78 decreased CHOP expression in mock and DENV-infected cells when compared with control.

Activation of innate immune factors upon GRP78 inhibition via VER treatment

Interferons (IFNs) are the cytokines required for activating an effective anti-viral response in the host. Interferon regulatory factor such as IRF3 activates the type I IFNs, which helps in clearing the viral infection. Furthermore, the activation of IFNs is known to cause the induction of dsRNA-dependent protein kinase (PKR). In this study, we found that IRF3 and PKR were increased on VER treatment, DENV infection, and VER-treated DENV infection (**Fig. 6**). This shows that the activation of UPR on GRP78 inhibition via VER treatment also activates the IFN arm of innate immunity.

Activation of pro-inflammatory factors upon GRP78 inhibition via VER treatment

The induction of NF-κB is necessary for the activation of various genes in response to the UPR during DENV infection. The translocation of NF-κB p-65 subunit was found to be significantly increased in VER-treated groups when compared with control. While DENV-infected groups showed significant increase in NF-κB activation when compared with VER-treated cells as well as the control group; and VER-treated DENV infection group showed maximum translocation of NF-κB (**Fig. 7**). Thus, DENV infection increased NF-κB activation; however, VER treatment further enhanced the NF-κB activation

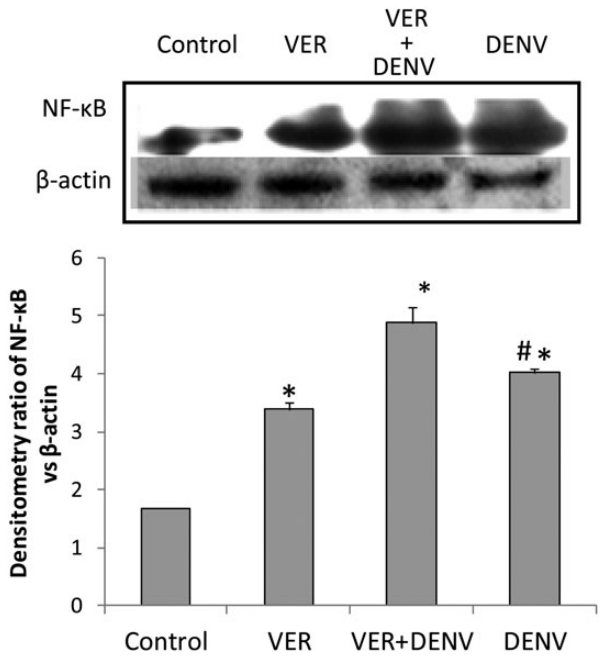

Figure 7. GRP78 inhibition and DENV infection increased NF-κB Immunoblotting was done using nuclear extract of THP-1 cells as described in the section 'Materials and Methods'. Immunoblot shows the expression of NF-κB and β-actin. Densitometry analysis showed that DENV infection increased NF-κB p-65 translocation into the nucleus; however, VER treatment in DENV-infected cells further enhanced its expression. Control, untreated cells; VER, cells treated with 20 μM VER; VER + DENV, 20 μM VER-treated cells infected with DENV; DENV, DENV-infected cells. *$P < 0.05$ vs. control; #$P < 0.05$ vs. VER. Data were presented as the mean ± SEM of three independent experiments.

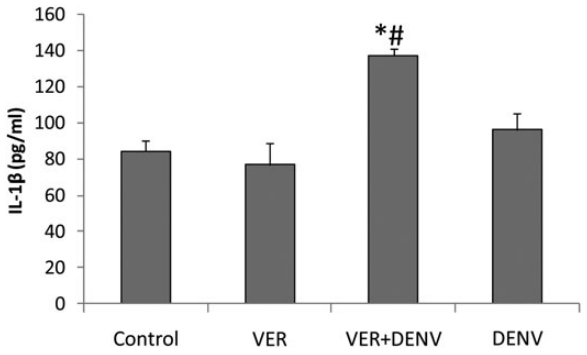

Figure 8. VER treatment increased the pro-inflammatory cytokine IL-1β expression The expression of the cytokine IL-1β was measured in THP-1 cell supernatants as described in the section 'Materials and Methods'. IL-1β was visibly increased in DENV-infected cells when compared with control, but was further increased significantly on VER treatment. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. DENV. Data were presented as the mean ± SEM of three independent experiments.

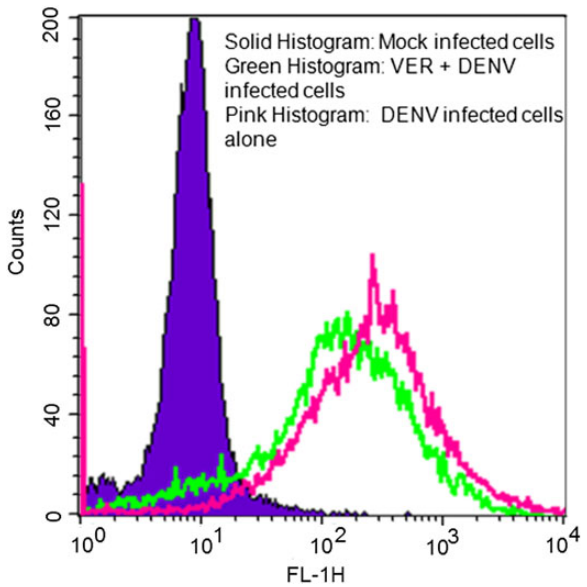

Figure 9. Intracellular viral load in DENV-infected cells treated with VER VER treatment decreased the intracellular DENV load in THP-1 cells. DENV-infected cells were treated with VER for 24 h and intracellular viral load was determined as described in the section 'Materials and Methods'. The intracellular viral load was decreased in the DENV-infected VER-treated cells when compared with DENV-infected cells alone.

induced by DENV infection. The expression of IL-1β was found to be slightly increased in DENV-infected cells when compared with control. However, the VER-treated DENV infection induced a potent and significant increase in the IL-1β level when compared with DENV infection alone (**Fig.** 8).

GRP78 inhibition via VER treatment decreased intracellular DENV load

The activation of UPR elements on GRP78 inhibition via VER treatment seems to have an impact on the viral multiplication. Flow cytometry analysis for intracellular DENV load showed a mean fluorescence intensity of 7.15 in mock infected cells, 116.56 in VER-treated DENV-infected cells, and 213.16 in DENV-infected cells. The intracellular DENV load was decreased in THP-1 cells treated with VER prior to DENV infection when compared with DENV-infected cells alone (**Fig.** 9).

Discussion

Dengue virus infection has been known to cause ER stress. Signaling pathways associated with ER stress during viral infections lead to the activation of pro-survival genes and anti-viral factors. During viral infections, the host machinery, especially the ER, is exploited by viruses to make mature viral progenies and thus propagate itself. In response to the stress brought about by the changes in the protein load, the ER triggers the UPR pathway in order to maintain the homeostasis of the ER. However, viruses often suppress certain genes and proteins of the UPR and continue to disrupt the ER homeostasis. In such a scenario, agents that can enhance the UPR pathway genes and proteins may aid in the successful restoration of ER homeostasis and additional anti-viral responses to disrupt the activities of the virus. In this study, the modulation of UPR pathway during DENV infection was found to evoke the innate immune and anti-viral responses (**Fig.** 10).

Inhibition of GRP78 can occur by means of its binding to a substrate or protein leading to UPR activation [25]. DENV activates PERK and IRE1α but not ATF6 in human monocytic cells. Activation of the three transmembrane UPR elements is required to induce a potent adaptive response. Inhibition of GRP78 using VER in DENV-infected cells led to the activation of PERK, ATF6, and IRE1α. Furthermore, it also significantly enhanced the splicing of XBP-1 mRNA when compared with DENV-infected cells alone. Splicing of XBP1 transcription factor is induced by IRE1α activation, which in turn activates genes involved in protein folding, phospholipid biosynthesis, and ERAD [26,27]. PERK activation alone is not enough to resolve ER stress, so the host cell activates the IRE1-XBP1 pathway rather than ATF6 during DENV infection. However, VER treatment improves the host response by activating all the three arms of the UPR pathway during DENV infection. Moreover, the protein load on the ER can be directly decreased by IRE1α through the RIDD, wherein, the ER-bound mRNA gets degraded [28]. A significant increase in the expression of CHOP was observed in DENV-infected cells alone. CHOP is induced during persistent stress [29,30]. CHOP can function either as a pro or anti-apoptotic factor [25,31,32]. Expression of CHOP was not altered in GRP78-inhibited cells infected with or without DENV when compared with control. Moreover, expression of CHOP was significantly decreased in GRP78-inhibited cells infected with or without DENV when compared with control. VER treatment was found to decrease DENV replication, as it decreased the intracellular viral load. This suggests that inhibition of GRP78 decreased the overall level of ER stress by activating a potent and protective UPR response in favor of the host and in the direction of decreasing viral replication.

The cellular response to viral infection is evoked when the host is able to recognize molecular patterns associated with the virus. The host may respond by causing the induction of several innate immune response factors, which often results in increased interferon levels and facilitates clearance of the virus. Induction of the transcription factor IRF, in turn activates PKR which is central to evoke a variety of cellular responses, bringing the host into an anti-viral state. Inhibition of GRP78 not only activates UPR but also IRF3 and PKR, showing that an effective anti-viral response is generated by the host.

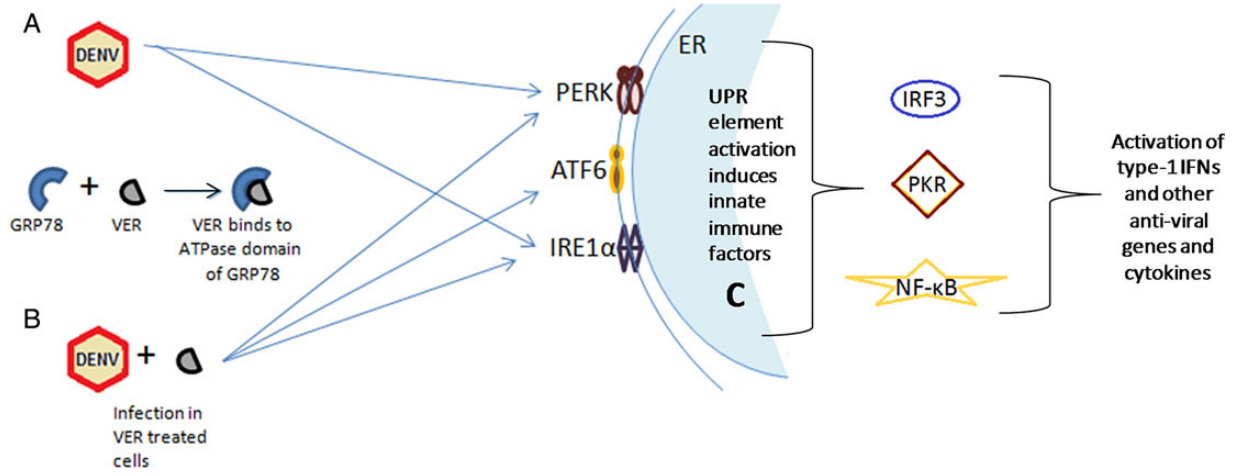

Figure 10. Effect of UPR pathway modulation on DENV infection (A) DENV infection in THP-1 cells activates PERK and IRE1α. (B) DENV infection in VER-treated THP-1 cells activates all the three UPR elements, PERK, ATF6, and IRE1α. (C) Activation of the UPR elements in DENV-infected and VER-treated cells was found to activate the innate immune factors such as IRF3, PKR, and NF-κB, which can mediate the induction of type-1 IFNs and other anti-viral genes and cytokines. Key: blue arrow represents activation.

Activation of PKR in turn facilitates the translocation of NF-κB, which further induces pro-inflammatory and anti-viral cytokines [33]. Interestingly, IRE1 is a key mediator of anti-microbial responses, as it can cause the induction of NF-κB and thus evoke a pro-inflammatory response [34]. Moreover, IRE1-mediated RIDD pathway activation purportedly generates small RNA fragments which are sensed by RIG-1, leading to NF-κB activation [11]. Therefore, the activation of the innate immune factor IRF3 and PKR leads to the transloaction of NF-κB and increased expression of the pro-inflammatory cytokine IL-1β, resulting in effective anti-viral response and a decrease in viral propagation.

This study therefore suggests that VER is effective in enhancing the host response during DENV infection by activating the UPR pathway. On similar lines, other studies have shown anti-viral agents against DENV that exhibit the ability to modulate the UPR pathway. For instance, Fraser *et al.* [35] have shown that N-(4-hydroxyphenyl) retinamide (4-HPR) can specifically up-regulate the PERK arm of the UPR and provide protection against a lethal DENV mice model. In another study by Rathore *et al.* [36], Celgosivir was found to modulate the UPR for its anti-DENV action. Thus, modulating the UPR can be an effective strategy to evoke an effective anti-DENV response in the host. The dose of the GRP78 inhibitor employed in this study decreased the viral protein expression without causing toxicity to the cells. Furthermore, the effect of GRP78 inhibition can be modulated using higher doses or increased exposure time to the inhibitor or similar strategies of inhibiting GRP78 could be used for optimizing the maximum host response to decrease viral multiplication.

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