Biochemical and Biophysical Research Communications xxx (2018) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Diphenyleneiodonium enhances oxidative stress and inhibits Japanese encephalitis virus induced autophagy and ER stress pathways

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ARTICLE INFO

Article history:
Received 15 May 2018
Accepted 20 May 2018
Available online xxx

Keywords:
Japanese encephalitis virus
Diphenyleneiodonium
N-Acetyl-L- cysteine
Reactive oxygen species
Autophagy
ER stress

ABSTRACT

Diphenyleneiodonium (DPI) and N-acetyl-L-cysteine (NAC), two widely used anti-oxidants, were employed to evaluate the role of oxidative stress in Japanese encephalitis virus (JEV) induced autophagy, stress responses and replication. DPI and NAC exerted opposite effects on ROS levels in JEV infected mouse neuronal cells (Neuro2a), mouse embryonic fibroblasts (MEFs) and human epithelial cells (HeLa). While NAC effectively quenched ROS, DPI enhanced ROS levels, suggesting that DPI induces oxidative stress in JEV infected cells. DPI treatment of JEV infected Neuro2a cells further blocked autophagy induction and activation of all three arms of the ER stress pathway, and, inhibited virus particle release. Autophagy induction in JEV infection has been previously shown to be linked to the activation of XBP1 and ATF6 ER stress sensors. Our data suggests that DPI mediated block of autophagy is a result of inhibition of ER stress responses and is not associated with an anti-oxidative effect. Since DPI has a wide inhibitory potential for all Flavin dependent enzymes, it is likely that the signalling pathways for ER stress and autophagy during JEV infection are modulated by DPI sensitive enzymes.

1. Introduction

Japanese encephalitis virus (JEV) belongs to the *Flaviviridae* family that includes several arthropod borne medically important viruses like West Nile virus (WNV), Dengue virus (DENV), and Zika virus [1,2]. JEV is the leading cause of virus induced encephalitis in endemic regions of south-east Asia and western Pacific [3].

Virus infection results in subversion of host cellular pathways to favour virus replication, and this leads to activation of several stress responses in the infected cell. The three major effects seen in flavivirus infections are induction of oxidative stress, ER stress leading to activation of the UPR and autophagy [4–8]. Autophagy induction in a virus infected cell further influences innate immune responses, inflammation, metabolic pathways, cell adhesion and cytoskeletal organization, survival, and thus directly impacts pathogenesis [9,10].

Both oxidative stress and ER stress have been shown to be the major contributors of autophagy induction in RNA virus infections [7,11–13]. Recent studies from our laboratory have established an anti-viral role of autophagy for JEV replication, and a crucial role of the XBP1 and ATF6 ER stress sensors for autophagy activation in JEV infected neuronal cells [6,7].

Oxidative stress caused by free radical generation is a major contributor to JEV induced cell death, neuroinflammation and pathogenesis [14–17]. To evaluate the contribution of oxidative stress in JEV induced autophagy we employed two widely used anti-oxidants- DPI and NAC, and checked their effect on levels of ROS and autophagy induction in the context of JEV infection. DPI, an inhibitor of flavoenzymes, including NADPH oxidase [18,19], has been extensively used in several studies to inhibit ROS production. NAC is a powerful scavenger of free-radicals that enhances glutathione biosynthesis [20,21]. We observed that while NAC reduced ROS levels in several different cell types subjected to pharmacological oxidative stress or infected with JEV, DPI increased ROS levels and thus, enhanced oxidative stress. Further, though DPI

Abbreviations: DPI, Diphenyleneiodonium; NAC, N-acetyl-L-cysteine; JEV, Japanese encephalitis virus; ROS, reactive oxygen species; UPR, unfolded protein response.

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https://doi.org/10.1016/j.bbrc.2018.05.149
0006-291X/

treatment prevented autophagy induction, as monitored by LC3-II conversion in JEV infected Neuro2a cells, and this effect was mediated by block of the ER stress pathways and not due to reduction of oxidative stress. Our data suggest that DPI sensitive enzymes are involved in the activation of ER stress sensors during JEV infection.

2. Material and methods

2.1. Cells and virus

Mouse neuroblastoma (Neuro2a) and porcine stable kidney (PS) cells were obtained from Cell Repository, National Centre for Cell Sciences, Pune, India. Mouse embryonic fibroblasts (MEF) were a gift from Prof. Mizushima and obtained through RIKEN-BioResource Cell Bank (RCB 2710). HeLa (ATCC-CCL2) cells were purchased from ATCC. For all studies JEV isolate Vellore P20778 generated in PS cells was used. JEV was titrated by plaque assay formation on PS monolayers as described earlier [22]. Plaque assay results are presented as Mean ± standard deviation (SD) of three independent experiments.

2.2. Reagents, antibodies and plasmids

Antibodies against CHOP/GADD153 (ab11419), SQSTM1 (ab56416) were from Abcam; GAPDH (2118), LC3 (3868) were from Cell Signalling Technology. Thapsigargin (T9033), DMF (242926), DPI (D2926) and NAC (A9165) were from Sigma. JEV-NS3 rabbit polyclonal antibody has been described before [6]. The plasmid-p5xATF6-GL3 (#11976) was obtained from Addgene (deposited by Ron Prywes) [23]. The plasmid pCI-Neo-hRluc was a gift from Dr. Witold Filipowicz (FMI, Basel, Switzerland).

2.3. Cell treatment and virus infection

Cells were treated with vehicle control/Thapsigargin/DMF/NAC/DPI, mock-or JEV-infected and processed for western blotting, or RNA extraction. JEV infection was done at MOI 5 for 1 h at 37 °C which results in an infection efficiency of ~90%. Following infection, cells were washed twice with PBS and complete medium was added. ER stress inducer Thapsigargin was added to cells at 1 μM for 8 h. DMF and DPI were dissolved in DMSO and used at 70 μM and 1 μM respectively. NAC was dissolved in water and used at 3 mM. DPI and NAC were added to JEV infected cells at 16 h post infection (pi) and maintained till harvest (24 hpi). After infection/treatment cells were washed twice with PBS and processed. Culture supernatants was collected for plaque assays at 24 hpi.

2.4. Assay for Xbp1 splicing

The *Xbp1* splicing assay was done as described previously [24]. The *Xbp1* transcript was amplified using the following primers Xbp1-F (5-AAACAGAGTAGCAGCGCAGACTGC-3) and Xbp1-R (5-TCCTTCTGGGTAGACCTCTGGGAG-3).

2.5. ATF6 promotor activation

Neuro2a cells were co-transfected with 1 μg of p5XATF6-GL3 and 100 ng of pCI-Neo-hRluc. After 24 h, cells were mock/JEV (5MOI) infected for 24 h. DPI/NAC was added to JEV infected cells at 16 hpi and maintained till harvest. Quantification of Firefly and Renilla luciferase activities in the samples was done using Dual-Luciferase Reporter Assay System, Promega and analyzed on Orion II microplate Luminometer (MPL4, Berthold Detection System, Germany). Results were plotted as relative luciferase activity (firefly/renilla) normalized to mock infection/treatment.

2.6. Determination of oxidative stress

The oxidative stress indicator CM-H_2DCFDA (Life Technologies) was used to quantify ROS levels. Neuro2a cells were infected with JEV at 5 MOI for 24 h, washed with pre-warmed PBS and incubated with 5 μM CM-H_2DCFDA in PBS for 15 min at 37 °C. Dye was removed by two washes in PBS. DMF (70 μM, 8 h) was used as a positive control for ROS production. DPI (1 μM) and NAC (3 mM) were added to cells at 16 hpi and maintained till harvest. Fluorescence intensity was quantified by flow cytometry (BD FACS-Canto II) and data was analyzed by FlowJo software.

2.7. Statistical analysis

Student *t*-test was used for statistical analysis. Differences were considered significant at values of $^{*}P < 0.05$; $^{**}P < 0.01$.

3. Results

3.1. DPI enhances oxidative stress in JEV infected cells

Production of ROS is the leading cause of oxidative stress in RNA virus infections. To assay oxidative stress in JEV-infected cells we measured the oxidation of H_2DCFDA, an indicator of production of intracellular ROS. This was tested in three cell lines- Neuro2a (mouse neuronal), MEF (mouse embryonic fibroblasts) and HeLa (human epithelial). The pharmacological reagent DMF (ROS inducer) was used as a positive control along with JEV infection, and, the anti-oxidants NAC and DPI were tested for their effect on levels of ROS generation. JEV infection, and DMF treatment lead to an increase in ROS levels in all the three cell types as compared to mock-infected cells (Fig. 1). NAC treatment was effective in reducing ROS levels comparable to those of control cells or even lower. DPI treatment however, further increased ROS levels in both mock- and JEV- infected cells. This was seen in all the three cell types suggesting that DPI treatment does not inhibit but rather enhances ROS levels during JEV infection (Fig. 1).

3.2. DPI blocks activation of autophagy and ER stress pathways in JEV infected Neuro2a cells

Since autophagy has been linked to levels of oxidative stress in cells we tested the effect of both DPI and NAC on the induction of autophagy and activation of ER stress pathways during JEV infection. Levels of LC3-II and SQSTM1, which are the markers of autophagy induction and autophagosome substrate respectively were estimated by western blotting [25,26]. We observed that DPI completely and NAC partially blocked JEV induced autophagy as seen by decreased levels of the autophagy marker LC3-II and a corresponding increase in levels of SQSTM1 (Fig. 2A). However since DPI treatment was enhancing oxidative stress in JEV- infected Neuro2a cells, its effect on autophagy inhibition was not due to any effect on ROS levels.

Recent studies from our laboratory have demonstrated a crucial role of the ER stress responses for induction of autophagy during JEV infection [7]. Therefore, we tested the effect of DPI and NAC treatment on the activation of ER stress pathways in JEV- infected cells. As a readout of PKR-like ER kinase (PERK) activation we looked at expression levels of the transcription factor CHOP (GADD153), which is a well characterized readout of persistent ER stress [27]. As expected, expression of CHOP was observed in JEV-infected cells [7,28], however, in DPI- treated JEV- infected cells expression of CHOP was significantly suppressed (Fig. 2A).

Please cite this article in press as: M. Sharma, et al., Diphenyleneiodonium enhances oxidative stress and inhibits Japanese encephalitis virus induced autophagy and ER stress pathways, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.05.149