How do flavivirus-infected cells resist arsenite-induced stress granule formation?

Mausumi Basu¹ & Margo A Brinton^{*,1}

First draft submitted: 8 March 2017; Accepted for publication: 9 March 2017; Published online: 8 June 2017

West Nile virus (WNV), dengue virus, yellow fever virus, Japanese encephalitis virus and Zika virus (ZIKV) are mosquito-borne members of the genus *Flavivirus*. These positive-strand RNA viruses are emerging human pathogens that cause a significant amount of human disease and mortality in ever-widening regions of the world.

The cellular stress granule (SG) response is an ancient cellular response counteracting multiple types of stress. SGs are dynamic cytoplasmic aggregates of stalled translation preinitiation complexes that are composed of mRNAs, eukaryotic translation initiation factors (including eIF4E, eIF4G, eIF4A, eIF3 and eIF2), the 40S ribosomal subunit and numerous RNA-binding proteins, including PABP, TIA 1, TIAR and ras G3BP1/2 [1]. SG formation is typically triggered by phosphorylation of the alpha subunit of the translation factor eIF2 by one of four eIF2 kinases, PKR, PERK, HRI, or GCN2. PKR is activated by viral dsRNA in infected cells, PERK is activated by ER stress, HRI is activated by oxidative stress and GCN2 is activated by nutrient starvation. We previously showed that WNV infections do not induce SGs due to the lack of PKR activation [2]. At early

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times after infection, WNV RNA levels are kept low and the viral RNA is membrane-associated, and at later times, exponential viral RNA replication takes place within virus-induced invaginations in the ER membrane, which hides the dsRNA from PKR [3]. We also showed that WNV-, dengue virus- and ZIKV-infected cells develop resistance with time after infection to the induction of SGs by the oxidative stress inducer arsenite (Ars) [4,5] even though previous data paradoxically indicated that flavivirus infections induce the production of reactive oxygen species (ROS) in the cells they infect [6–8]. ROS include superoxide, singlet O₂, H₂O₂ and hydroxyl radicals. Low levels of ROS have positive functions such as acting as second messengers in a variety of cellular processes, including activation of the antioxidant pathway, but high levels of intracellular ROS cause oxidative stress [9]. Ars treatment of uninfected cells rapidly induces high levels of ROS which cause oxidative stress, mitochondrial damage and finally cell death.

The upregulation of ROS by a WNV infection was confirmed by incubation of infected cells with CellROX[®] Green (Thermo Fisher

KEYWORDS

- flaviviruses • redox state
- stress granules

¹Department of Biology, Georgia State University, Atlanta, GA 30303, USA

*Author for correspondence: mbrinton@gsu.edu

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Scientific Inc., Waltham, MA, USA), which is nonfluorescent in the reduced state, but when oxidized by ROS fluoresces, providing an indication of the level of ROS in live cells. An increase in the CellROX Green signal was detected by 8 hpi, increased by 16 hpi and remained high thereafter. However, virus-infected cells did not display characteristic effects of oxidative stress as indicated by the maintenance of normal mitochondrial morphology and of normal mitochondrial membrane potential (detected by comparing MitoTracker® Green and MitoTracker® Red [Thermo Fisher Scientific Inc.] colocalization) and also the lack of Drp1-phosphorylation, which is indicative of increased mitochondrial fission.

Other types of viruses inhibit SG formation in infected cells either by viral protein sequestration or cleavage of a critical nucleating SG protein [10,11]. These virus-mediated strategies disable SG assembly and are effective at inhibiting SG formation in response to eIF2 α phosphorylation by all four of the eIF2 α kinases. Interestingly, treatment of WNV- or ZIKV-infected cells with the stressor DTT, which activates eIF2 α -phosphorylation through the eIF2 α kinase PERK, induced high levels of eIF2 α -phosphorylation and SG formation. The Ars-specificity of the flavivirus-mediated inhibition of SG induction indicated that the inhibitory effect occurs upstream of eIF2 α -phosphorylation [5].

Reduced glutathione (GSH) is the primary cellular electron scavenger and protects cells from endogenous and exogenous oxidative stress by donating electrons to ROS [12]. Cellular GSH levels are regulated by the rate of synthesis of GSH by antioxidant pathway genes and the rate of GSH regeneration from oxidized glutathione (GSSG). The cellular transcription factors, ATF4 and Nrf2 activate the expression of antioxidant pathway genes involved in GSH synthesis and regeneration [13–17]. AIF, an FAD-dependent flavoenzyme located in the mitochondrial intermembrane space in mammalian cells, acts as an NAD (P) H-dependent oxidoreductase to regenerate GSH from GSSG [18,19]. Under normal conditions, cells maintain low and balanced levels of ROS and GSH and SG formation is not induced. The effect of a WNV infection on intracellular GSH levels was analyzed by a colorimetric assay and by ThiolTracker™ Violet (Thermo Fisher Scientific Inc.), which fluoresces when it reacts with reduced thiols in intact cells.

GSH levels increased by 8 hpi, further increased by 16 hpi and remained high throughout the infection [5]. Consistent with the increased GSH levels, rapid upregulation and nuclear localization of the transcription factors ATF4 and Nrf2 as well as upregulation of antioxidant pathway genes were detected in response to the infection. Knockdown of Nrf2 or ATF4 decreased intracellular GSH levels and increased SG production in WNV-infected cells and Ars treatment of these cells further decreased GSH levels and further increased the number of infected cells with SGs. Treatment of infected cells with the oxidative stress inducer, buthionine sulfoximine, which inhibits GSH synthesis, also decreased intracellular GSH levels and increased the number of SG-positive infected cells. Upregulation of AIF was observed in WNV-infected cells but AIF was not upregulated by Ars treatment of cells. Knockdown of AIF decreased intracellular GSH levels and increased the number of SG-positive, infected cells after Ars treatment.

The results of our study indicate that flavivirus infections not only upregulate intracellular ROS levels but also upregulate the expression of antioxidant pathway genes, creating a new homeostasis state characterized by higher levels of ROS that are counteracted by even higher levels of GSH. The excess antioxidant capacity of the virus-infected cells is sufficient to balance the ROS induced by both the infection and Ars treatment, prevent Ars-induced SG formation, protect mitochondria from Ars-induced damage and prolong cell survival. The ability of stressors that activate eIF2 α phosphorylation through mechanisms other than oxidative stress is not inhibited in infected cells. The virus-induced alterations in the cellular redox status may provide benefits for the virus during its life cycle.

Financial & competing interests disclosure

M Basu is a senior scientist in the laboratory of Margo Brinton in the Department of Biology at Georgia State University. Brinton is a Regents' Professor and received support from grants R01AI48088 and R01AI045135 from the National Institute of Allergy and Infectious Diseases, NIH. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

No writing assistance was utilized in the production of this manuscript.

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• of interest; •• of considerable interest

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