SINV induces oxidative stress in baby hamster kidney host cells

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ABSTRACT

Viruses are cellular parasites that invade and manipulate cellular energy pathways to support viral production. Alphaviruses belong to the *Togaviridae* family that has a species of virus known as the Sindbis virus. Sindbis virus shares characteristics with other alphaviruses that are currently causing worldwide infection and disease (i.e., Chikungunya virus, Ross River Virus and Western and Eastern Equine Encephalitis virus). Oxidizing conditions are assumed to play a role in alphavirus replication. In this study we evaluated the previously unstudied relationship between oxidative stress and viral replication. Using a modified Sindbis virus with a duplicated subgenomic promoter driving the expression of a fluorescent reporter was used in this analysis. An oxidative fluorescent probe was also used to measure relative levels of oxidation within cells. Cells were infected with Sindbis virus then incubated in media that contained pro-oxidative conditions or anti-oxidizing conditions. Fluorescence intensity was recorded with a flow cytometer to determine the intensity of replication in an infected cell. We have found that an oxidizing environment increased viral replication, where an antioxidant environment reduced virus replication. Treatments with oxidants or antioxidants significantly altered viral replication, indicating that an intricate redox balance must be maintained for successful viral replication.

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BACKGROUND

Alphaviruses have caused persistent endemic infections throughout the world, especially in tropical regions where their mosquito vectors are highly abundant.[1] Alphaviruses are transferred by mosquitos which enables their rapid spread.[2] In the past decade, several strains of alphaviruses have made their way through South America, Mexico, and North America. Reported cases in these countries are common to tropical regions with high humidity levels. Outbreaks of CHIKV cause more than 1.8 million infections each year in America.[3] Similarly, Dengue virus, from the Flaviviridae family, infects 300-400 million people each year.[4] With little-known interventions or treatments, symptoms are treated while the infections maintain their virulence and control over the cellular machinery.

Alphaviruses contain a single-stranded positive-sense RNA genome that is about 11kB in size and packaged into an enveloped virion that is about 70nm.[5] Alphaviruses replicate in the cytoplasm of the infected host cell. Transcription of the viral RNA results in the expression of the viral nonstructural proteins. These viral enzymes replicate the genome and start the process of infection. During infection, a subgenomic promoter on the viral genome is used to transcribe a small 26S subgenomic RNA. This subgenomic RNA contains the genes for the structural proteins that the virus needs for packaging and release of new virions. Alphaviruses are especially prone to genetic modifications because the subgenomic promoter can be duplicated, which allows for additional genes to be packaged and transcribed in infected cells.[6,7,8] Through genetic modifications the Sindbis virus can be equipped with a green fluorescent protein (GFP) that is expressed during infection. GFP expression serves as an indicator for viral replication within a host cell. The fluorescent indicator GFP has allowed us to track viral infections in BHK cells during treatments with an oxidant and antioxidant compound. Double subgenomic alphavirus reporters have been used for several years in alphavirus research and are great reporter viruses used for gathering data on virus replication.

Viruses are entirely dependent on the host cell for energy and metabolic pathways.[9,10] Viruses have been shown to actively modify the host cell's normal physiology to produce an environment that is optimal for viral replication. These modifications can include increases in glycolytic flux, glucose uptake, mitochondrial manipulation, electron transport changes, and oxidative stress.[11,12] Understanding critical changes to cellular pathways during viral infection can help to determine potential interventions or target points to inhibit the virus. In a previous study with a flavivirus, it was determined that successful replication is dependent on elevated oxidative conditions.[13,14,15] The opposite was shown in an antioxidant environment, that lowered the number of virion particles.[16] Therefore, the question was presented if other arboviruses, in closely related genera, do they have a similar dependence on oxidative conditions during infection? Alphaviruses, despite distinct differences in their replication cycles, are reasonably similar to flaviviruses in their genome type, size, and replication in the host cell cytoplasm.[17] We hypothesized that alphaviruses might require similar cellular conditions to support viral replication.

METHODS AND MATERIALS

Cells and Cell Culture

Baby Hamster Kidney (BHK) cells are commonly used in viral infections due to their suscepti-

bility of infection and permissibility of viral replication. BHK cells were cultured using DMEM media containing streptomycin/penicillin and FBS (10%). BHK cells are adherent and were cultured in T75 flasks or 24-well culture plates in an incubator at 37 degrees Celsius and 5% CO2. T-75 flasks were used to culture cells before plating out for analysis and 24-well plates were used to subject various treatments and ran for analysis. Cells were monitored daily, and at ~75% confluence in a T-75 flask, the cells were passaged by washing with PBS and treating with 0.25% Trypsin. Cells were counted with a hemocytometer and seeded into the 24 -well plates 24 hours before an experiment so that the cells would be nearly 70% confluent at the time of infection/ treatment.

Virus and Viral Infection

SINV is the prototype alphavirus and was used as the model virus for all experiments. Sindbis virus (SINV) belongs to the *Togaviridae* family and alphavirus genus of viruses. We used a double subgenomic promoter Sindbis virus (dsSINV) expressing a green fluorescent protein (GFP) at the 3' end of the viral genome. The GFP reporter was inserted into the viral genome, and GFP fluorescence serves as an indicator for viral replication in infected cells.[6,7,8] To infect BHK cells, the cells were removed from the T-75 flask using trypsin, resuspended, and counted with Trypan Blue staining and a hemocytometer. The resulting cell concentration (cells/well) was used to calculate how much virus to add to each well. Cells were infected with a multiplicity of infection (MOI) of 1 or otherwise stated, which is the ratio of infectious virus to a cell, of 1 or otherwise stated. Virus was taken from a stock vial and added to culture media and then placed on the cells and incubated for 1 hour at 37°C, which is sufficient time for the virus to enter the host cells. After 1 hour of infection, the virus media was replaced with fresh media. This fresh media could contain the oxidant/antioxidant treatment. The infected cells were incubated for 24 hours, to allow the virus to gain control over the host cell and begin production of virion particles.

Oxidant/ Antioxidant Treatment

Hydrogen peroxide (HP) and butylated hydroxyanisole (BHA) were purchased from Sigma Aldrich. The compounds were resuspended in DMSO at a 500mM stock concentration. The 100mM stock was serial diluted with solvent to make stock concentrations at 50mM, 25mM, 12.5mM and 6.25mM. Cells were treated with (mM) stocks diluted at 1:1000, resulting in μM concentrations. In cell culture, media concentrations of HP or BHA was added directly to the infected or mock-infected cells. A mock/control treatment was prepared by diluting only solvent (DMSO) 1:1000 in cell culture media. From a cytotoxicity curve generated, concentrations were selected and sequentially chosen at 12.5uM that were viable for oxidant or antioxidant treatments. The dilutions of HP or BHA for treatment were always prepared fresh.

Quantifying Virus Replication, Oxidative Probe, and Flow Cytometry

Virus replication/infection was measured by monitoring GFP protein fluorescence. Measuring oxidative stress was done with 10uM of CM-H2DCFDA (green fluorescent probe) from Life Technologies, Invitrogen ™ Catalog # C6827 by placing the oxidative probe onto cells for one hour before analyzing. Cells were analyzed using a Guava easyCyte® flow cytometer from Millipore. Briefly, cells were trypsinized, suspended, and run over the flow cytometer to quantify the number and percent of cells infected (expressing GFP) and the relative levels of GFP fluo-

rescence per cell; similar steps were taken for the oxidative probe. Ten thousand cells were analyzed for each sample, and each condition was run in triplicate on the flow cytometer. The same settings (gains, parameters, regions, thresholds, and gating) were used for all analysis to quantify and measure dsSINV-GFP positive cells accurately. Similar steps to create standard settings for the oxidative stress probe were used in quantifying stressed conditions. SINV GFP and green oxidative stress probe were not used together due to both the probe being green and the virus replication indicator also being green. For experiments with green oxidative stress probes, an untagged (nonfluorescent) WT SINV was used. Data from the flow cytometer allowed specific analysis of GFP fluorescence per cell, the number of cells infected and percent infected.

Statistics

Sample sizes were of n=4 and shown as a percentage change from the control and is represented as mean ± SEM. All statistics were done using a Welch's t-test. Assumptions were met by having normal distribution but unequal variances and were compared to each other in a welches t-test. R-code was used to compute the statistical analysis. P-values of $p<0.05$, were considered significant and are present in text as the level of significance.

RESULTS

Sindbis virus infection induced oxidative stress in infected BHK cells (Figure 1). As shown in Figure 1, nearly half of the cells had high levels of oxidative stress, whereas less than 5% of cells had a detectable amount of oxidative stress. Treating the cells with either an oxidant, such as Hydrogen Peroxide (H2O2), or with an antioxidant like butylated hydroxyanisole (BHA) had severe impacts on virus replication. Treatment with 12.5uM of H2O2 resulted in almost a 20% increase in viral replication, whereas treatment with 12.5uM of BHA inhibited the virus by more than 50% (Figure 2A). At both of these concentrations, cell viability was maintained (Figure 2B). This indicates that virus replication is sensitive to oxidative conditions and that viral replication is enhanced during an oxidizing environment. This matches what has been seen with flaviviruses such as Kunjun virus and Dengue virus.[18,19,20,21]

Viruses are dependent on the host cell for all biomolecules and energy. If cells are grown without nutrients, the cells become stressed. We then analyzed the stress induced by depriving cells of glucose. The impact of deficient media lacking glucose was used to measure the impact of oxidative stress. Cells grown in the absence of glucose had higher levels of oxidative stress as compared to cells grown in complete media. There was an upward trend of oxidative stress in Figure 3A, indicating a slight elevation in oxidative stress. This increased oxidative environment induced by starvation did not benefit the virus. Viral replication was significantly inhibited in cells deficient in glucose (Figure 3B). This indicates that although sindbis virus replication appears to favor an oxidizing environment, a sufficient supply of carbohydrates such as glucose are required for efficient replication.

This was confirmed by monitoring glucose uptake in Sindbis-infected BHK cells. Cells that were infected significantly increased their glucose uptake compared to mock, uninfected cells (Figure 4). Taken together, this data shows that sindbis virus replication significantly alters cellular pathways and results in an increase in glucose uptake and also in oxidative stress during infec-

tion. Altering the glucose concentrations available during infection significantly reduced viral replication. Similarly, by treating cells with an antioxidant, the virus was not able to replicate as quickly and to the same levels as untreated cells. Treatment with the oxidant hydrogen peroxide increased the virus replication.

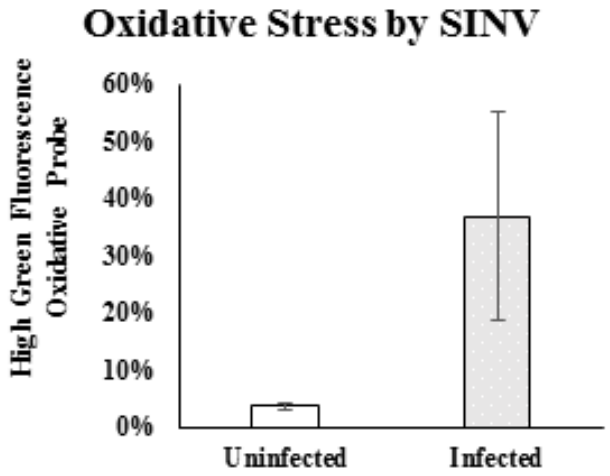

Figure 1: Baby hamster kidney cells (BHK) were cultured for 24 hours then infected with sindbis virus for 24hrs then analyzed using flow cytometry. Regional parameters were used to detect high green fluorescence present from the oxidative stress probe. Sindbis did not contain the GFP marker in this analysis. The sample size was of n=3 wells and 10,000 cells were analyzed using flow cytometry. A $p<0.01$ was indicated by rcode using a welches t-test for statistical analysis.

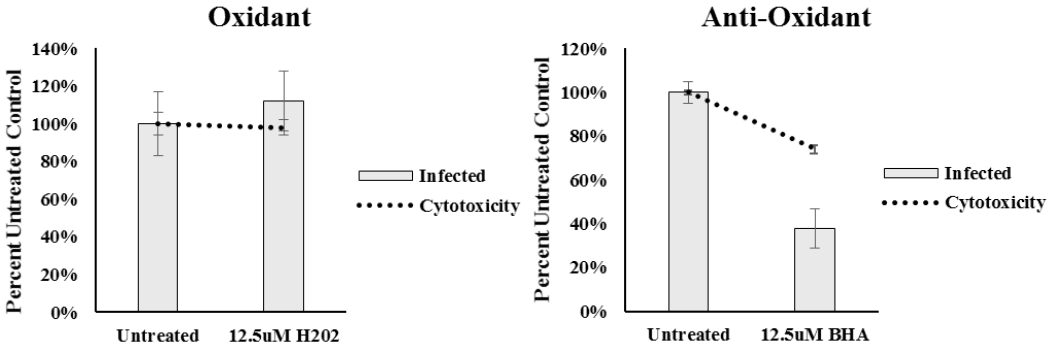

Figure 2A/2B: BHK cells in 2A were plated out and incubated for 24 hours before infected with dsSINV-GFP. dsSINV-GFP was allowed to infect cells for one hour than received 12.5uM H2O2 (oxidant). 24 hours post treatment cells were analyzed using flow cytometry and were normalized to the percent of untreated control. A cytotoxicity curve was done and is indicated by the dotted line. This represents the level of cell viability post treatment and infection. Here it is represented by an n=3 and was close to the level of significance required, however was not significant. Therefore, the 20% increase is reported. BHK cells in 2B were subjected to the same procedure as 2A however, the difference is BHA (anti-oxidant) is used. Here the sample size was an n=3 and had a $p<0.01$ indicated by rcode using a welches t-test for statistical analysis.

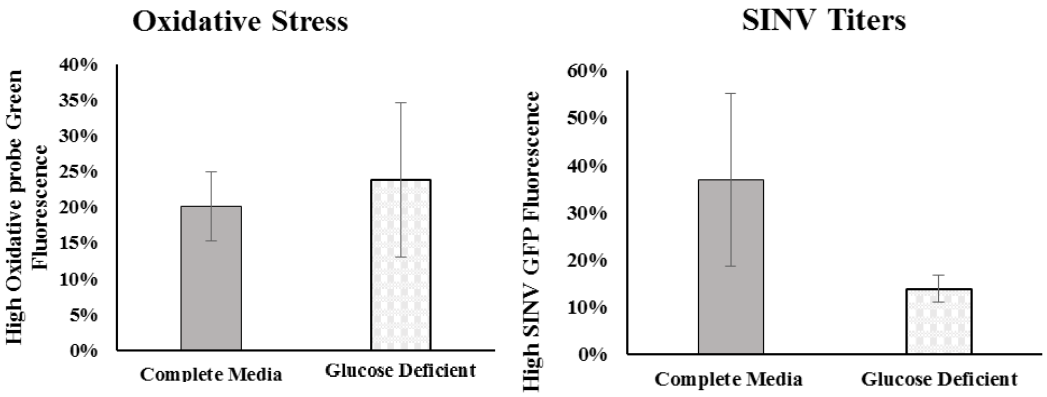

Figure 3A/3B: BHK cells in 3A were plated out and incubated with complete media containing 25mM glucose or without glucose, indicated by glucose deficient for 24 hours. Cells were than incubated with oxidative stress probe as previously explained and then ran on flow cytometry. A sample size of n=3. BHK cells in 3B were plated out in normal media and allowed to incubate for 24 hours then were subjected to SINV infection in complete media or glucose deficient for another 24 hours before analyzing on flow cytometry. The sample size was an n=3 with a $p<0.05$ indicated by rcode using a welches t-test for statistical analysis.

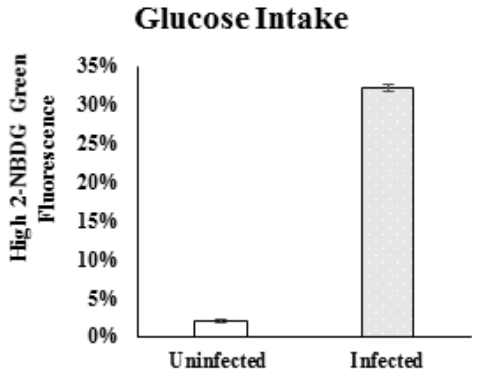

Figure 4: BHK cells were plated out and incubated for 24 hours before being infected with SINV. Infected cells were allowed to incubate for another 24 hours than a rate of glucose intake was measured by using a green 2-NBDG reporter that was analyzed using flow cytometry. The sample size was of an n=3 with a $p<0.01$ as indicated by a welches t-test using rcode.

DISCUSSION

Alphaviruses are causing common infections with limited drug treatments or interventions. Evidence suggests that viruses are sensitive to oxidative conditions. Previous work has looked at other viruses, including flaviviruses such as Kunjin virus and Dengue virus, and this work is confirming that alphaviruses, such as Sindbis virus, also induce oxidative stress and are enhanced by the resulting oxidizing environment (Figure 1 and Figure 2A). There is an increase in GFP fluorescents when treated with H_2O_2 indicating more viral replication is taking place in an oxidant rich environment (Figure 2A). Sindbis virus is able to modulate the efficiency of protons moving across the electron transport chain located in the inter-mitochondrial membrane. Specifically complex I and III of the electron transport chain, which has been linked to the manipulation by sindbis virus infection. The response associated with the elevated inhibitory activity from

complex I and III is elevated oxidative stress.[11] What we have determined is the level of oxidative stress helps viral replication. We detected significant increases in oxidative stress during infection and amplified viral replication when the cells were treated with an oxidant such as hydrogen peroxide. This clearly indicates that sindbis virus purposely induces oxidative stress to favor its own replication.

Interestingly, starvation-induced oxidative stress, by culturing cells in glucose-deficient media, resulted in increased levels of oxidative stress (Figure 3A), but the cells were not as susceptible to producing high levels of virus replication (Figure 3B). Sindbis virus is utterly dependent on the host cell metabolism, and previous literature has shown that sindbis virus requires glycolysis for replication.[22] We showed that Sindbis virus infection resulted in a significant increase in glucose uptake after 24 hours (Figure 4). This implies that Sindbis viruses, though it favors an oxidizing environment, it also requires significant carbon sources for fueling the metabolic processes during infection. At 24 hours post infection elevated levels of oxidative stress have shown to cause mitochondrial dysfunction leading to lowered ATP production.[11] The direct impact of elevating glucose consumption has not been further evaluated. It has been speculated that glucose is used in a Warburg like effect resulting in glucose fermentation in the presence of oxygen. In a few reports lactate production has been observed during sindbis infection inferring the Warburg like effect. It has not been confirmed that this process of lactate production in the presence of oxygen is occurring during sindbis infection. Evaluating at 24 hours has elicited a response in oxidative stress and increase in glucose consumption. What is not demonstrated and is of future interest is when do theses occurrences begin and when do they spike? Early onset of viral replication could potentially upregulate activity from the mitochondria to support oxidative stress while later in infection the mitochondria activity is downregulated to maintain an anabolic state. The anabolic state would help support viral genetic replication while also supporting packaging and release of the newly produced viral particles. Future work will investigate the impacts of viral replication on the host mitochondrion, as oxidative stress is closely related to this organelle.[23] We also plan on treating virally infected cells with different antioxidants (e.g., Vitamin E and NAC) and oxidants to investigate different exposure times of treatment, cell lines, and doses, to determine the potential use of antioxidants as a viral inhibitor.

We have determined that sindbis virus replication induce oxidative stress in the infected cells. During treatments with an antioxidant, viral replication was reduced by 62%. These results indicate that Sindbis virus, like other viruses, requires an oxidizing environment for optimal replication. Reducing the oxidizing environment results in less viral replication. This study confirms our hypothesis that sindbis virus replication is dependent on oxidative stress for successful viral replication.

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