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Reduction of porcine parvovirus infectivity in the presence of protecting osmolytes

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ABSTRACT

Osmolytes are natural compounds found in the cells of many organisms that stabilize intracellular proteins against environmental stresses. Protecting osmolytes can promote protein folding, whereas denaturing osmolytes have the opposite effect. A variety of osmolytes were tested for their antiviral activity against porcine parvovirus (PPV). PPV is a non-enveloped, icosahedral, single-strand DNA virus. We have discovered two protecting osmolytes, trimethylamine N-oxide (TMAO) and glycine that reduce the infectivity of PPV by four logs (99.99%). We hypothesize that both osmolytes stabilize viral capsid proteins and prevent them from assembling into viable virus particles. The advantage of the antiviral compounds found is that they can be applied post-infection, which increases their potential to serve as a therapeutic drug.

1. Introduction

Many diseases are caused by pathogenic virus infection. In recent decades, scientists have defined the structure and function of many different viruses. This has aided in the creation of specific antiviral compounds. Compounds that inactivate certain viruses have changed the treatment of many diseases, including respiratory syncytial virus (RSV) (Glatthaar-Saalmüller et al., 2011) and herpes simplex virus type-1 (HSV-1) and type-2 (HSV-2) (Hayashi et al., 2012). The AIDS epidemic has been treated with HIV protease inhibitors that now allow people to live decades with the virus (Antonelli and Turriziani, 2012). However, there is still a need for the continued discovery of antiviral compounds.

Many researchers are now studying natural products as a source of antiviral compounds, since viruses are starting to become resistant to current drugs (Kitazato et al., 2007). In an effort to find natural compounds that have antiviral activity, we screened the antiviral activity of a panel of osmolytes and a salt against the non-enveloped virus porcine parvovirus (PPV). Osmolytes are small organic compounds that are found in the cells of many organisms and they have the ability of stabilize intracellular proteins against environmental stress, such as extreme temperature or high osmotic pressure (Bolen, 2004). A balance between protecting and denaturing osmolytes assist in the delicate equilibrium needed for protein stabilization (Dong et al., 2009). Protecting osmolytes fold proteins by structuring water around themselves and changing the interaction between the water and the protein backbone. Denaturing osmolytes bind directly to the protein backbone, causing the protein to unfold (Street et al., 2006).

Parvoviruses, from the family *Parvoviridae*, are small, non-enveloped, icosahedral, single-stranded DNA viruses that infect vertebrates and arthropods (Halder et al., 2012). PPV infects the intestines of pigs and is the most frequent cause of swine reproductive failure (Boisvert et al., 2010). This virus is often used as a model for the human B19 parvovirus. Although different natural compounds have been studied in recent decades, osmolytes have not been previously shown to have antiviral activity. This study describes the reduction of PPV infectivity in the presence of the protecting osmolytes TMAO and glycine.

2. Materials and methods

2.1. Materials

The osmolytes trimethylamine N-oxide (TMAO) dihydrate, glycine, betaine, D-alanine, D-arginine, sucrose, trehalose dihydrate, urea, and the salt ammonium sulfate were purchased from Sigma–Aldrich (St. Louis, MO) at a minimum purity of ⩾98.0%. Poly-L-lysine, 4′,6-diamidino-2-phenylindole dihydrochloride

Abbreviations: PPV, porcine parvovirus; TMAO, trimethylamine N-oxide; RSV, respiratory syncytial virus; HSV-1, herpes simplex virus type-1; HSV-2, herpes simplex virus type-2; HIV, human immunodeficiency virus; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; IC_{50}, 50% infectious dose.

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(DAPI), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), agarose type I, low EEO, neutral red solution (0.33%), and sodium dodecyl sulfate (SDS) were purchased from Sigma–Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS, pH 7.2) and 0.25% trypsin/EDTA for cell propagation were purchased from Life Technologies (Grand Island, NY). 12.1 M hydrochloric acid (HCl) and 3.7% formaldehyde in water were purchased from VWR (Radnor, PA). The monoclonal mouse anti-PPV antibody was purchased from VMRD (Cat no. 3C9D11H11, Pullman, WA) and the polyclonal Alexa fluor 546-conjugated rabbit anti-mouse antibody was purchased from Life Technologies (Cat no. A11060, Grand Island, NY). All solutions were made with NanoPure water (Thermo Scientific, Waltham, MA, resistance >18 MΩ) and filtered with either a 0.2 μm syringe filter (Nalgene, Rochester, NY) or a Millipore 0.2 μm bottle top filter (Billerica, MA) prior to use.

2.2. Cell propagation

Porcine kidney (PK-13) cells were a gift from Dr. Ruben Carbonell at North Carolina State University and were propagated as described previously (Heldt et al., 2006). Briefly, the cells were grown in minimum essential medium (MEM) (Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Oakwood, GA) and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY).

2.3. Virus production and titration

PPV strain NADL-2 was a gift from Dr. Ruben Carbonell at North Carolina State University and were propagated in PK-13 cells, as described previously (Heldt et al., 2006). Briefly, cells were infected with 10^3 MTT_{50} of PPV, and 1.5 h later, 9 ml of supplemented media were added. After 4–6 days, the flasks were placed at −20 °C. The flasks were thawed, and the monolayer was scraped. The scraped cells and media were centrifuged at 5000 rpm in a Sorvall ST16R Centrifuge (Thermo Scientific, Pittsburgh, PA) at 4 °C for 15 min to remove the cell debris. The supernatant was stored at −80 °C.

PPV was titrated with a colorimetric cell viability assay, the MTT Assay (Heldt et al., 2006). The reduction of the MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) inside the mitochondria of metabolically active cells produces formazan crystals (Mosmann, 1983). Upon dissolving the crystals, the cell viability can be quantified by measuring the absorbance of the solution at 550 nm. This has been shown to be linearly comparable to a $TCID_{50}$ for PPV on PK-13 cells (Heldt et al., 2006). Cells were seeded in 96-well plates, as described earlier (Heldt et al., 2006). The cells were infected with PPV in quadruplicate and 5-fold serial dilutions were made across the plate. After five days, 10 μl/well of 5 mg/ml of MTT in PBS was added. Four hours later, 100 μl/well of solubilizing agent (0.01 M HCl and 10% SDS) were added. Plates were read on a Synergy Mx microplate reader (BioTek, Winooski, VT) at 550 nm between 18–24 h after addition of the solubilizing agent. The 50% infectious dose (MTT_{50}) value was determined to be the virus dilution that yielded 50% of the uninfected cell absorbance. The value was converted to a per milliliter basis and stated as the MTT_{50}/ml titer (Heldt et al., 2006).

2.4. Cytotoxicity assay

Antiviral activity was determined in a similar way to the virus titration described in Section 2.3. After virus was added to the cells, 25 μl of osmolyte or salt at various concentrations were added to the infected cells.

To determine the effect of osmolyte concentration on antiviral activity, 25 μl of either TMAO or glycine with a final concentration ranging from 0.00 to 0.30 M was added to the infected cells. To determine the effect of the time between infection and osmolyte addition on antiviral activity, 0.20 M of either TMAO or glycine was added at various times post-infection. MTT reagent addition was performed after five days, as described in Section 2.3. Calculation of the log reduction is shown in Eq. (1).

$$\text{log reduction} = -\log\left(\frac{\text{virus titer with osmolyte}}{\text{virus titer of control}}\right) \quad (1)$$

2.5. Osmolyte toxicity

Cell viability was assessed with an MTT assay and was used to determine the toxicity of TMAO and glycine to PK-13 cells. Cells were seeded as described in Section 2.3 in 100 μl of media. Osmolytes diluted to a final concentration ranging from 0.00 to 0.60 M in NanoPure water were added to the cells after 24 h at a volume of 25 μl. MTT reagent addition was conducted after five days, as described in Section 2.3. Calculation of the % survival of cells is shown in Eq. (2).

$$\%\ \text{survival} = \left(\frac{\text{absorbance with osmolyte}}{\text{absorbance of control}}\right) \times 100 \quad (2)$$

2.6. Plaque reduction assay

Plaque assays were performed as described previously (Heldt et al., 2006). Briefly, PK-13 cells were seeded into 25 cm^2 tissue culture flasks with a final concentration of 4×10^5 cells per flask and incubated at 37 °C and 5% CO_2 until 70% confluent. Ten-fold serial dilutions of 10^8 MTT_{50}/ml were made in either PBS with 3% FBS or PBS with 3% FBS containing 0.20 M TMAO or 0.20 M glycine. Cells were infected with 200 μl of different sample dilutions. After 1 h of incubation, virus inoculum was removed and infected cells were overlaid with 1:1 of 2% agarose in Nanopure water and 2× supplemented media. Overlay media for osmolytes samples also contained 0.20 M glycine or 0.20 M TMAO. Flasks were stained with 2 ml of 4% neutral red at 3.3 g/L in the overlay media after 4 days of incubation. Plaques were counted 4–8 h after staining.

2.7. Yield reduction assay

Intracellular and extracellular viable virus particles were measured. PK-13 cells were seeded in 6-well plates at the same cell density as the MTT Assay described in Section 2.3. Cells were infected with 2×10^3 MTT_{50} of PPV. After 1 h, the virus inoculum was removed and media added. Osmolytes were added either at this time (Treatment 1) or 5 min prior to sample collection (Treatment 2). Virus supernatant was removed at various times to measure extracellular viable virus particles. To assay intracellular viable virus particles, cells were detached by the addition of trypsin/EDTA and equal volume of media was added to deactivate the trypsin. All samples were frozen at −20 °C for 24 h, thawed at room temperature, and centrifuged at 5000 rpm in a Sorvall ST16R Centrifuge (Thermo Scientific, Pittsburgh, PA) for 15 min at 4 °C. The supernatants were removed and titrated as described in Section 2.3.

2.8. Immunohistochemical detection of virus capsid protein production

Intracellular virus capsid protein production was assessed through immunohistochemistry of PPV-infected cells with and without osmolytes. Glass slides (25 × 75 mm) from VWR (Radnor,