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Exogenous nitric oxide inhibits Crimean Congo hemorrhagic fever virus

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Abstract

Crimean Congo hemorrhagic fever virus (CCHFV) is a geographically widespread pathogen that causes severe hemorrhagic fever with high mortality. Even though one of the main objectives focuses on the progress of antiviral agents, the research on CCHFV is strongly hampered due to its BSL-4 classification. Nitric oxide (NO), a mediator with broad biological effects, has been shown to possess inhibitory properties against various pathogens. The molecule constitutes a component of the innate immunity and serves to assist in the early immunological events where it contributes to clearance of microorganisms. In this study, we investigated the inhibitory properties of exogenous NO on CCHFV. We found that NO had a significant antiviral activity against CCHFV replication. By using the NO-donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) we were able to show up to 99% reduction in virion progeny yield. In contrast, 3-morpholinosydnonimine hydrochloride (SIN-1), a peroxynitrite donor, had no significant antiviral activity against CCHFV. Furthermore the expression of viral proteins; the nucleocapsid protein and the glycoprotein, were clearly reduced with increasing concentrations of SNAP. We have also shown that the amount of total vRNA in SNAP-treated cells was reduced by about 50% compared to the controls.

Keywords: CCHF; *Bunyaviridae*; Nitric Oxide; *S*-Nitroso-*N*-acetylpenicillamine (SNAP)

1. Introduction

The tick-borne Crimean Congo hemorrhagic fever virus (CCHFV) was first discovered in Crimea in 1944 (Chumakov, 1963) and 10 years later it was isolated in Congo, Africa (Casals, 1969). The two viruses were shown to be identical and the virus was subsequently named according to the two locations. CCHFV is a nairovirus in the family of *Bunyaviridae*, which also constitutes the genera of *Orthobunyavirus*, *Hantavirus*, *Phlebovirus* and *Tospovirus*. A common feature for this family is the genome, consisting of three segments of single stranded RNA, referred to as the S-, M- and L-segments. The S-segment encodes the viral nucelocapsid protein (NP), M the glycoproteins (G_N and G_C), and L the RNA dependent RNA polymerase (Bishop, 1996; Schmaljohn and Hopper, 2001). CCHFV is widely distributed over the world, with endemic areas in Africa (Simpson et al., 1967; Woodall et al., 1967), Eastern Europe, Middle East and Asia (Chinikar et al., 2004; Sanchez et al., 2002), but poses an impending threat also in non-endemic areas. The routes for transmission are through tick bites of the genus *Hyalomma* (family *Ixodidae*) (Shepherd et al., 1991), contact with infected blood, or nosocomial infections (Burney et al., 1980; Suleiman et al., 1980). The serious disease caused by CCHFV in man reaches mortality-levels from 15 to 60% (Swanepoel, 1994), while infection in animals and birds is frequently asymptomatic (Shepherd et al., 1987a,b). To current date there is no treatment, nor vaccine, for CCHFV.

Nitric oxide (NO) is a highly reactive, diffusible free radical generated by nitric oxide synthase (NOS). NO is synthesized in vivo from L-arginine by the calcium-dependent neuronal- and endothelial NOS, or the inducible calcium-independent NOS (iNOS) to citrulline and NO (Marletta et al., 1988; Nathan, 1992; Nathan and Xie, 1994). The molecule has numerous physiological functions in a variety of cells and tissues and may act as a vasodilator, neurotransmitter, immune regulator, antimicrobial and antiviral agent (Bogdan, 2001; Brunet, 2001; Cherayil and Antos, 2001; Reiss and Komatsu, 1998; Sanders, 1999). The microbiostatic and microbiocidal activities provide for protection against a wide range of pathogens such as parasites (Mauel

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et al., 1991), fungi (Nakamura et al., 1994) and mycobacteria (Doi et al., 1993).

During the past decade, NO has been subjected to antiviral research concerning several DNA and RNA viruses (Bi and Reiss, 1995; Croen, 1993; Gao et al., 1999; Karupiah and Harris, 1995; Lin et al., 1997; Rimmelzwaan et al., 1999; Zaragoza et al., 1997). However, nothing has so far been reported on the effects of SNAP or other NO donors on any know member of the family *Bunyaviridae*.

As an antiviral agent, foremost by the engagement of iNOS, NO is proposed to contribute during the initial stages of infection, before acquired immunity becomes fully functional. Although the course of action and assigned antiviral properties are not fully understood, NO remains a versatile player in the immune system.

In this study, we investigated the antiviral properties of exogenous NO against CCHFV. Our results revealed potential sites in the replication cycle of CCHFV, which were affected by NO. We observed a significant decrease in progeny virion yield and noted a specific inhibition of the CCHFV NP and G_N proteins while host cell proteins remained unaffected. We therefore attribute a potential role of innate immunity in CCHFV infection.

2. Materials and methods

2.1. Cell and virus

The CCHFV strain IbAr10200, originally isolated in Nigeria (Sokoto) in 1970, was used in all experiments (Andersson et al., 2004b; Flick et al., 2003). Propagation, as well as titration, was performed on Vero E6 cells (monkey kidney, ATCC CRL-1586). The virus stock titer was determined by focus forming units (FFU) and was assayed by an indirect immunofluoroscence assay (IFA) against CCHFV NP as previously described (Andersson et al., 2004b). The virus stock concentration was 2×10^5 FFU/ml and the infectious dose 0.03 multiplicity of infection (MOI). Vero E6 cells were maintained at 37 °C in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with fetal calf serum (FCS) 2 or 10%, 200 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco products, Invitrogen Corporation, Carlsbad, CA). All infections with virulent CCHFV were carried out in the Biosafety Level 4 (BSL4) laboratory at the Swedish Institute for Infectious Disease Control.

2.2. Antibodies and chemicals

Polyclonal antibodies against CCHFV NP were produced as described elsewhere (Andersson et al., 2004b). Polyclonal antibodies against the C-terminal peptide sequence EIHGDNYGGPGD of CCHFV glycoprotein (GP) G_N and to the peptide sequence CEEDEILNRSPRNRKPRRE of cellular calnexin were produced and the specificity confirmed by Agrisera, Vännäs, Sweden. Horseradish peroxidase (HRP)-conjugated (Bio-Rad Laboratories Inc., Hercules, CA), and fluorescein isothiocyanate (FITC)-conjugated antibodies (DAKO, Glostrup, Denmark), were purchased and used according to the instructions provided by the manufacturer. For all IFA, the antibodies were diluted in 0.1% Triton-X100 in PBS, supplemented with 0.2% BSA.

SNAP, a nitric oxide donor and its non-donor control counterpart, NAP, 3-morpholinosydnonimine hydrochloride (SIN-1), a peroxynitrite donor, were obtained from Sigma (Sigma–Aldrich, Saint Louis, MI). [^{35}S] Protein label mix was purchased from Perkin-Elmer (Perkin-Elmer Life Sciences Inc., Boston, MA), diluted to 1 μCi/μl in DMEM without methionine and cysteine and stored at −80 °C. For pulse labelling, 100 μCi was used in each well of a 12 well plate.

2.3. Chemical treatment, infection and harvesting

Vero E6 cells were grown confluent before treated with SNAP, NAP or methanol at various concentrations and time points. Infection was done by incubating virus (0.03 MOI) and cells in serum-free medium for 1 h in a humidified chamber (37 °C) followed by removal of excess virus. Infection was maintained for another 24 h under similar conditions. The various times used for chemical treatment were 6 or 3 h before infection and 1 h post infection. In the latter case, the chemicals were added solely after infection while pre-treatment was also succeeded by chemical addition after infection. Culture medium without additives was used as control. Chemicals were freshly prepared in DMEM with 2% FCS before added to the cells. Twenty-four hours post infection, the cells were harvested with glass beads, or cell layers homogenized in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 20 mMNaF, 100 mM Na_3VO_4, 1% Triton X-100 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and leupeptin) for protein analysis.

2.4. Immunoblot analysis

Vero E6 monolayers were seeded and grown in 12- or 24-well plates, CCHFV-infected (0.03 MOI) and treated with chemicals as described above. At the end of infection (24 h post infection), cells were collected and homogenized in lysis buffer. Lysates were mixed 1:1 in sample buffer (10 mM Tris–HCl, pH 7.5, 0.5% SDS, 10% glycerol, 2% β-mercaptoethanol, bromophenol blue) resolved in 10% Tris–glycine polyacrylamide gel and transferred onto a nitrocellulose membrane (Mini-PROTEAN 3, Bio-Rad Laboratories Inc., Hercules, CA). Blocking was performed over night at 4 °C in 5% non-fat dry-milk in 0.1% Tween-20 in PBS. The membranes were subsequently incubated with CCHFV NP-, CCHFV G_N-, or calnexin-specific primary antibodies for 1 h at room temperature, followed by HRP-conjugated secondary antibodies. Proteins were detected with ECL Plus Western Blotting Detection kit (Amersham Biosciences, Uppsala, Sweden).

2.5. CCHFV viral RNA

TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) was used for total RNA isolation, according to the manufacturer's instructions. RNA was extracted from cells harvested 24 h post infection (0.03 MOI) and subjected to reverse transcription fol-