Nitric oxide and peroxynitrite have different antiviral effects against hantavirus replication and free mature virions

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Reactive nitrogen intermediates (RNI), like nitric oxide (NO) and peroxynitrite, have antiviral effects against certain viruses. Hantaviruses, like other members of the Bunyaviridae family, have previously not been shown to be sensitive to RNI. In this study, we compared the effects of NO and peroxynitrite on hantavirus replication and free mature virions *in vitro*, and of inducible nitric oxide synthase (iNOS) in hantavirus-infected suckling mice. The NO-generating compound S-nitroso-N-acetylpenicillamine (SNAP), as well as cytokine-induced NO, strongly inhibited hantavirus replication in Vero E6 cells, while pretreatment of free virions with SNAP only had a limited effect on their viability. In contrast, 3-morpholinosydnonimine hydrochloride (SIN-1), a peroxynitrite donor, inhibited virus replication only to a very low extent *in vitro*, but pretreatment of virus with SIN-1 led to a considerably lowered viability of the virions. Infections of various human cell types *per se* did not induce NO production. The viral titers in iNOS$^{-/-}$ mice were higher compared to the controls, suggesting that NO inhibits hantavirus replication *in vivo*. In conclusion, we show that NO has strong antiviral effects on hantavirus replication, and peryoxynitrite on mature free virions, suggesting that different RNI can have different effects on various parts of the replication cycle for the same virus.

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Abbreviations: **D-NMMA:** N^G-monomethyl-D-arginine · **DOBV:** Dobrava hantavirus · **FFU:** focus forming unit · **HCPS:** hantavirus cardiopulmonary syndrome · **HFRS:** hemorrhagic fever with renal syndrome · **HTNV:** Hantaan hantavirus · **L-NMMA:** N^G-monomethyl-L-arginine · **N:** nucleocapsid · **NAP:** N-acetylpenicillamine · **O$_2^-$:** superoxide · **PUUV:** Puumala hantavirus · **RNI:** reactive nitrogen intermediates · **SAAV:** Saaremaa hantavirus · **SIN-1:** 3-morpholinosydnonimine hydrochloride · **SNAP:** S-nitroso-N-acetylpenicillamine

Introduction

Hantaviruses cause two severe and often fatal human zoonotic diseases, hemorrhagic fever with renal syndrome (HFRS) in the old world and hantavirus cardiopulmonary syndrome (HCPS) in the new world. Hantaviruses, belonging to the Bunyaviridae family, have a negative sense tripartite RNA genome encoding four structural proteins: the nucleocapsid (N) protein, two glycoproteins, and an RNA-dependent RNA polymerase [1]. The natural hosts are rodents, and the virus is transmitted to humans *via* inhaled contaminated rodent excreta. In contrast to human infections, the natural rodent hosts do not show any symptoms after infection [2]. The pathogenesis in man is only poorly

understood, but immune-mediated mechanisms have been suggested [3, 4].

Nitric oxide (NO), a gaseous free radical, is an important molecule playing a key role in a wide range of biological processes, such as vasomotor tone regulation, neurotransmission, and immune responses. NO inhibits the replication of certain DNA and RNA viruses, for instance poliovirus, Japanese encephalitis virus, mouse hepatitis virus, vesicular stomatitis virus, herpes simplex virus type 1, vaccinia virus, Epstein-Barr virus, influenza virus and SARS coronavirus [5–7]. However, the possible antiviral effect of NO and peroxynitrite on hantaviruses, or other viruses within the Bunyaviridae family, has previously not been reported.

During inflammation, NO and superoxide (O_2^-) together form peroxynitrite ($ONOO^-$), and other reactive nitrogen intermediates (RNI) [8]. Recently, it was shown that peroxynitrite has antiviral capacities both against Coxsackievirus replication and free virions, suggesting that also other viruses might be sensitive to peroxynitrite [9].

Although NO and peroxynitrite can inhibit viral replication, and thereby contribute to the clearance of virus from the circulation, highly elevated levels of RNI during disease can be deleterious [5, 10], due to oxidation and nitration of cellular lipids, DNA and proteins [8]. Elevated levels of nitrate/nitrite, stable end-products of NO, have been found in HIV-infected individuals, and NO has been suggested to play a role in the pathogenesis of AIDS [11]. Influenza virus pneumonia [12] and neurotropic virus infections are other diseases where NO is believed to contribute to the pathogenesis [10], and we have detected elevated levels of NO production in suckling mice that succumbed to hantavirus infection [13]. On the other hand, inducible nitric oxide synthase (iNOS) deficiency had no impact on the pathology in vaccinia virus and corona virus infections of mice [14], showing that NO-induced pathology is not a general feature during virus infections.

Stable end-products of RNI have been found at elevated levels in both HFRS and HCPS patients [15–17], as well as in monkeys infected with Puumala hantavirus (PUUV) [18]. In contrast, infection of the natural host *Peromyscus maniculatus* with Sin Nombre hantavirus (SNV) does not induce NO production [15]. The elevated levels of RNI detected in HFRS/HCPS patients have been suggested to play a part in the pathogenesis [15].

A variety of cell types and tissues generate NO through the conversion of L-arginine into L-citrulline through three distinct isoforms of the enzyme nitric oxide synthase (NOS) [19]. Two forms of NOS, neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3) are constitutively expressed, whereas iNOS (or NOS2) is strongly induced by cytokines and other immunoregulatory stimuli [20].

In the present study, we investigated the effect of NO and peroxynitrite on hantavirus replication in Vero E6 cells and on free mature virions by using S-nitroso-N-acetylpenicillamine (SNAP; an NO-donor), 3-morpholinosydnonimine hydrochloride (SIN-1; a peroxynitrite donor), and by stimulating endogenous NO production by iNOS with cytokines. Furthermore, the effect on NO production by hantavirus infection of several different types of cells *in vitro* was measured to test if hantavirus infection *per se* could induce NO production, and iNOS$^{-/-}$ suckling mice were infected to test if iNOS is a part of the antiviral response against hantaviruses, and/or the pathogenesis, in suckling mice.

Results

HTNV replication does not induce NO production *in vitro*

In a first set of experiments, we tested if Hantaan hantavirus (HTNV) infection of different cell lines induced NO production. Cells were infected with 1 multiplicity of infection (MOI) of HTNV and then incubated without change of media.

Elevated levels of nitrite were detected neither in the supernatants from the human lung epithelial cell lines HL and A549, the monkey kidney epithelial cell lines Vero and Vero E6 8 days post infection, from the human hepatoma cell line HuH-7 5 days post infection, nor from the human cervix epithelial cell line HeLa, a primary culture of human umbilical vein endothelial cells (HUVEC) or the human monocytic cell line MonoMac 4 days post infection.

Exogenous NO inhibition of hantavirus replication

The viability of HTNV-infected cells treated with 100 μM SNAP or 100 μM of the control N-acetylpenicillamine (NAP), or medium alone, starting 12 h before infection and then replenished every 12 h, was examined. No toxicity of SNAP could be measured using an MTT test 54 h post infection (data not shown), showing that 100 μM SNAP is not toxic for Vero E6 cells. The nitrite concentration in medium measured 12 h after SNAP treatment was approximately half of the concentration of SNAP added to the medium, clearly showing that NO was released by SNAP under these conditions (Fig. 1). As expected, no nitrate was detected in medium from cells incubated with NAP for 12 h (Fig. 1).

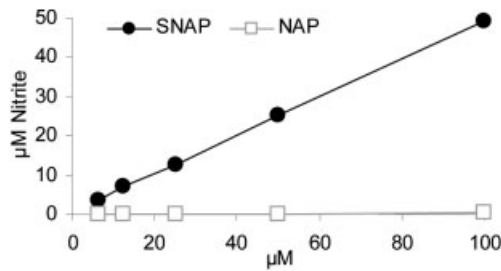Whether NO had an effect on HTNV replication was then tested. HTNV-infected cells were incubated with

Figure 1. Levels of nitrite in media from Vero E6 cells after 12 h of incubation with various concentrations of SNAP and NAP.

6.25–100 µM SNAP or NAP, starting 1 h after infection. The media were subsequently changed every 12 h, and supernatants were drawn at 30 h post infection and titrated. No viable viruses were detected in supernatants from cells treated with 100 µM SNAP (Fig. 2A). From cells treated with 50 µM SNAP, 85% less viable virus was obtained, whereas lower concentrations of SNAP had no detectable effect on the virus replication, as compared to NAP-treated or medium controls (Fig. 2A, and data not shown).

To test if the replication of other hantaviruses was also sensitive to SNAP, cells were infected with PUUV, Dobrava hantavirus (DOBV) and Saaremaa hantavirus (SAAV), followed by treatment with 100 µM SNAP, 100 µM NAP, or medium alone from 1 h post infection. A complete inhibition of released virus was observed for PUUV at 54 h post infection, and for DOBV at 30 h post infection, whereas an approximately 90% inhibition was detected for SAAV at 30 h post infection (Fig. 2B).

Since it has been shown that replication of vesicular stomatitis virus is more efficiently inhibited when the cells are pretreated with exogenous NO donors [21], we compared treatment of Vero E6 cells with 100 µM SNAP starting 12 h before, 1 h after, or 12 h after HTNV infection. The media were changed every 12 h, and supernatants were sampled 30 h post infection. No difference was observed between adding SNAP 12 h before infection or 1 h post infection (Fig. 2C), showing that pretreatment is not needed for NO-induced inhibition of replication. However, a stronger inhibition was observed when SNAP was added 12 h before or 1 h post infection, as compared to 12 h post infection
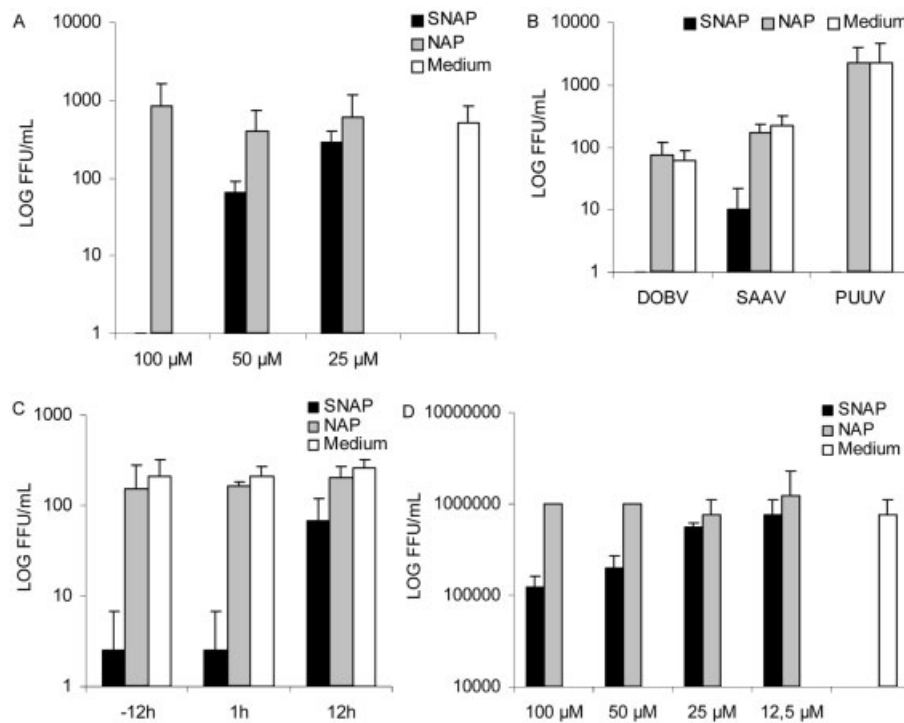

Figure 2. Effect of exogenously administered NO on hantavirus replication in Vero E6 cells. (A) Titers of HTNV in supernatants sampled 30 h post infection of Vero E6 cells incubated with medium or various concentrations of SNAP or NAP, from 1 h post infection. The data represent means ± SD of three individual experiments. (B) Titers of DOBV, PUUV and SAAV in supernatants sampled 30 h (DOBV and SAAV) and 54 h (PUUV) post infection of Vero E6 cells incubated with medium alone or with 100 µM SNAP or NAP, from 1 h post infection. The data represent means ± SD of three individual experiments. (C) Titers of HTNV in supernatants sampled 30 h post infection. Vero E6 cells were incubated with 100 µM SNAP, with treatment starting 12 h before, 1 h after, or 12 h post infection. The data represent means ± SD of three individual experiments. (D) Vero E6 cells infected with HTNV were incubated for 7 days followed by incubation with various concentrations of SNAP or NAP for 30 h. The data represent means ± SD of one representative experiment.

(Fig. 2C). The same pattern was observed for DOBV and SAAV (data not shown).

To investigate if NO could inhibit hantavirus replication in an already established infection, we infected cells with HTNV and incubated them for 1 wk (at this time point all cells were infected; data not shown). Cells were then treated with 12.5–100 µM SNAP. At 30 h after the initial treatment with 50 and 100 µM SNAP, the titers of released virus were approximately 80 and 87.5% lower, respectively, as compared to cells treated with NAP or medium alone (Fig. 2D). Lower concentrations of SNAP had no effect on the virus titers (Fig. 2D, and data not shown).

Endogenously produced NO inhibition of HTNV replication

We have previously shown that IL-1β together with IFN-γ up-regulates iNOS expression in Vero E6 cells [7]. Here, we further examined the effect of cytokine-induced NO on hantavirus replication *in vitro*. Treatment of cells with IL-1β (10 ng/mL) alone had no effect on HTNV replication (data not shown), whereas IFN-γ (400 U/mL), as reported earlier [22], inhibited the viral replication (data not shown). No increased level of nitrite was detected in cells treated with IL-1β or IFN-γ alone (data not shown), suggesting that the expression of iNOS in Vero E6 cells requires both IL-1β and IFN-γ. Approximately 20 µM of nitrite was detected in the medium 48 h after stimulation with IL-1β and IFN-γ.

To test the effect of cytokine-induced NO on HTNV replication, cells were infected with HTNV. After 1 h, cells were stimulated with IL-1β and/or IFN-γ in the presence of 1 mM of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) or the control N^G-monomethyl-D-arginine (D-NMMA). L-NMMA and D-NMMA were subsequently added to the media also 24 h post infection. Supernatants were collected 48 h post infec-
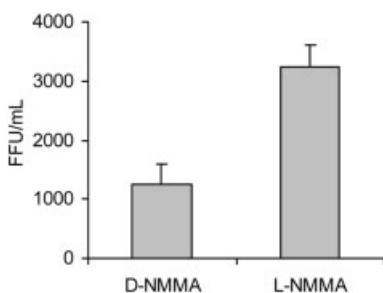
tion for virus titration. The viral titers in supernatants from cells stimulated by IL-1β combined with L-NMMA or D-NMMA showed no clear differences (approximately 6% more viruses in L-NMMA + IL-1β-treated cells compared to D-NMMA + IL-1β-treated cells). Similarly, L-NMMA or D-NMMA had no clear effect on the viral titers from cells stimulated by IFN-γ (approximately 5% more viruses in D-NMMA + IFN-γ-treated cells compared to L-NMMA + IFN-γ-treated cells), showing that the inhibition of virus replication by IFN-γ alone is NO independent. In contrast, approximately 260% higher HTNV titers were observed in supernatants from cells treated with the NOS inhibitor L-NMMA, as compared to supernatants from cells treated with the control D-NMMA, in cells incubated in the presence of IL-1β together with IFN-γ (Fig. 3). Thus, cytokine-induced NO can inhibit HTNV replication in Vero E6 cells.

Lower level of N protein and viral RNA in NO-treated cells

We further tested if NO affected the levels of N protein expressed after infection of Vero E6 cells. Cells were treated with 100 µM SNAP, 100 µM NAP, or medium alone, with start at 1 h post infection, and then media were changed every 12 h. Treatment with 100 µM SNAP had no effect on the total cellular protein, or β-actin, levels in Vero E6 cells ([7], and data not shown). In samples drawn 30 h post infection, HTNV N protein was detected in NAP-treated and untreated cells, but not in SNAP-treated cells (Fig. 4A).
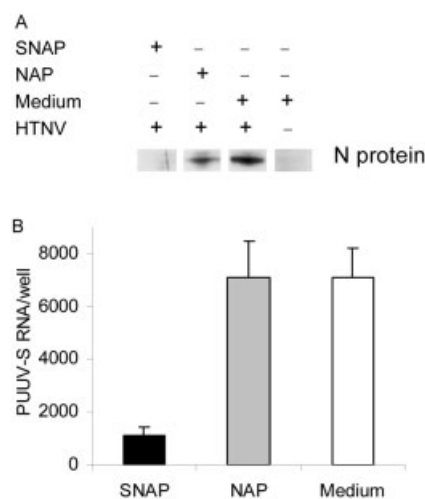
Figure 4. NO inhibits production of N protein and viral RNA. (A) Western blot: Cells were infected with HTNV and subsequently treated with SNAP, NAP or medium alone for 30 h. N protein = HTNV nucleocapsid protein. (B) Real-time PCR: Cells were infected with PUUV and then treated as described for Western blot. RNA from PUUV stock with known titer (FFU on Vero E6 cells) was used as a standard.

Figure 3. Endogenously produced NO inhibits hantavirus replication. Cells were infected with HTNV and then stimulated with IL-1β and IFN-γ in the presence of 1 mM L-NMMA or 1 mM D-NMMA. At 48 h post infection, virus titers in the supernatants were determined. The data represent means ± SD of one representative experiment.

We then tested if NO also had an effect on viral RNA by performing real-time PCR on the PUUV S-segment. Cells were infected with PUUV and treated with 100 μM SNAP, 100 μM NAP or normal medium, as described above. The levels of viral RNA in the cells 30 h post infection were analyzed. Approximately 85% less viral RNA was detected in SNAP-treated cells as compared to the NAP- or medium-treated cells (Fig. 4B).

Peroxynitrite inhibition of hantavirus replication

As peroxynitrite was recently shown to have antiviral capacities [9], we then investigated if hantavirus replication was sensitive to treatment with peroxynitrite. In medium with 100 μM SIN-1, 66 μM nitrite was detected 12 h after incubation, showing that most of the added SIN-1 had decayed (data not shown). To Vero E6 cells, 100 μM SIN-1 was added after infection with HTNV, and fresh medium containing SIN-1 was added at 12 h and 24 h after infection. At 30 h post infection, the supernatant was collected and titrated. Approximately 40% less viable virus were observed in cells incubated with 100 μM SIN-1, as compared to controls (Fig. 5).

NO and peroxynitrite inactivation of free HTNV

To test if NO or peroxynitrite had a direct inhibitory effect on free mature virions, HTNV was incubated with 1 μM to 1 mM SNAP, 1 μM to 1 mM SIN-1, 1 mM NAP, or with normal medium for 4 days at +4°C before titration. The levels of nitrite detected in the medium at this time point corresponded to approximately half of the added concentration of SNAP and SIN-1 (data not shown). No nitrite was detected after addition of 1 mM NAP to the media (data not shown). The virus was 1000-fold diluted before titration to rule out the effect of
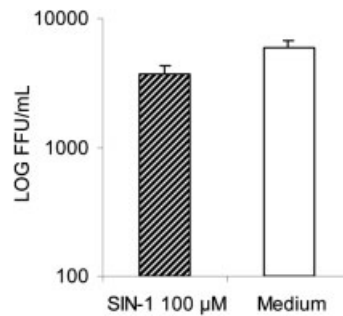

Figure 5. Effect of exogenously administered peroxynitrite on hantavirus replication in Vero E6 cells. Titers of HTNV in supernatants sampled 30 h post infection of Vero E6 cells treated with 100 μM SIN-1 or medium, starting at 1 h post infection. The data represent means ± SD of three individual experiments.

potential inhibition of viral replication by residual SNAP or SIN-1.

While 10 μM SIN-1 inactivated approximately 75% of the virus, 100 μM SIN-1 inactivated more than 90% of the virus, and 1 mM SIN-1 almost all (Fig. 6). Approximately 25% reduction in viability was observed for 1 mM and 100 μM SNAP, as compared to the NAP or medium controls (Fig. 6).

Elevated levels of hantavirus in brains of suckling iNOS$^{-/-}$ mice

To test if NO has a role in the antiviral defense *in vivo*, we infected suckling C57BL/6 mice (iNOS$^{-/-}$ and iNOS$^{+/+}$ controls) with 5000 focus forming units (FFU) DOBV, previously shown to induce NO production in and to be lethal for suckling mice [13]. All mice in the two groups (iNOS$^{-/-}$, $n = 6$; controls, $n = 6$) showed ruffled fur, paralysis of the limbs, and progressively diminishing mobility, at day 15 after infection, and were sacrificed at
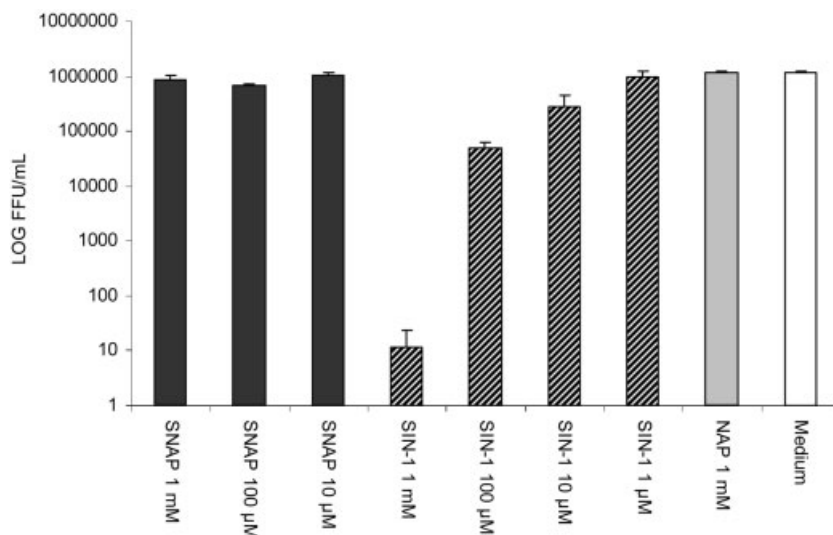

Figure 6. Effect of NO and peroxynitrite on free virions. HTNV was incubated with various concentrations of SNAP and SIN-1, and 1 mM NAP or in normal medium for 4 days at +4°C before titration on Vero E6 cells. The data represent means ± SD of one representative experiment.
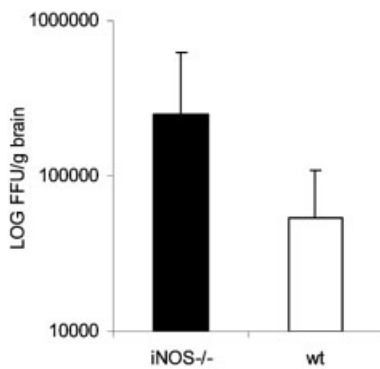
Figure 7. Increased levels of replicating hantavirus in iNOS knockout mice. Virus titers in brains of suckling iNOS$^{-/-}$ C57BL/6 mice ($n = 6$), and suckling normal (wt) C57BL/6 mice ($n = 6$) infected with 5000 FFU of DOBV and sacrificed at day 15 post infection. The data represent means ± SD.

this time point. None of the mice showed any signs of symptoms at day 14 after infection.

Higher levels of DOBV were observed in the iNOS$^{-/-}$ (249 593 ± 370 463 FFU/g brain) compared to the control (53 688 ± 55 172 FFU/g brain) mice (Fig. 7), suggesting that NO produced by iNOS has antiviral properties against hantaviruses *in vivo*.

Discussion

The major finding in this study is that different RNI can have different effects on various parts of the replication cycle for viruses; NO showed a strong antiviral effect on the hantavirus replication *in vitro* but only a minor effect on free viruses, while the opposite was observed for peroxynitrite. Furthermore, we showed for the first time that a member within the Bunyaviridae family is sensitive to NO and peroxynitrite.

It should be noted that although SNAP releases NO into the medium, some NO might escape into the atmosphere, and furthermore, a portion of the NO radicals produced most probably oxidizes into various reaction products of NO·, such as NONOates, S-nitrosothiols, nitrite, and nitrous acid that could account for parts of the activity. NO will also react with O_2^- in the medium and form peroxynitrite. And although most of the NO and O_2^- produced by SIN-1 will immediately form peroxynitrite, some NO will be produced that might not react with O_2^-. Superoxide can also form hydrogen peroxide, which in turn can form hypochlorus acid and other oxidants. These reactive nitrogen intermediates and reactive oxygen intermediates can penetrate cellular membranes and react with pathogen targets. Thus, the effect we have observed might be even more polarized, as some of the effect of SNAP observed on viable free virions might be explained by the formation of small amounts of peroxynitrite and other nitrogen intermediates, and the minor antiviral effect of SIN-1 observed on hantavirus replication *in vitro* might partly be explained by the production of NO and other intermediates.

The half-life of NO and peroxynitrite, endogenously produced or formed after decomposition of SNAP and SIN-1, is very short, and it is therefore difficult to adequately measure the amounts of NO or peroxynitrite at a given time point. The levels of nitrate/nitrite observed in patients indicate the accumulated levels of NO and/or peroxynitrite, but say little about the concentration of them at a certain time point. It is therefore not possible to state that the amount of NO and peroxynitrite formed *in vitro* by SNAP and SIN-1, respectively, and shown to be antiviral against hantaviruses, are physiologically relevant. However, the finding that iNOS$^{-/-}$ suckling mice had higher levels of replicating virus than controls is in line with the finding that cytokine-stimulated NO production inhibited hantavirus replication in Vero E6 cells. Furthermore, Davis and coworkers recently reported clearly elevated levels of nitrate/nitrite in HCPS patients [15], and Groeneveld and coworkers showed the same for HFRS patients [16]. Thus, our results suggest that the levels of NO and peroxynitrite formed in patients might reduce hantavirus replication and/or damage free virions.

Not all the mechanisms behind the antiviral effect of RNI are known. However, at least three different mechanisms are known for NO and one for peroxynitrite: (I) S-Nitrosylation of cysteine residues of viral proteins needed for replication; for instance, the inhibition of Coxsackievirus replication is related to S-nitrosylation of cysteine protease 3B [23]. (II) Enhanced mutation rate: NO has been shown to enhance the mutation rate of another RNA virus, the Sendai virus [24]. (III) S-Nitrosylation of host cellular proteins needed for virus replication: The antiviral effect of NO against some viruses depends on pretreatment of cells with NO before infection [21, 25]. Peroxynitrite has been reported to inhibit Coxsackievirus RNA entry into host cells [9].

The mechanisms behind the antiviral effect of NO and peroxynitrite on hantavirus replication and free virions, respectively, remain to be studied. The finding that pretreatment of cells with SNAP was not needed for inhibition of the virus replication suggests that S-nitrosylation of host cellular proteins is not instrumental for NO-mediated inhibition of hantavirus replication. Furthermore, it seems likely that NO and peroxynitrite have different targets, as they only show a minor overlap in their potential to interfere with replication and to inactivate free virions.

iNOS is the major source of NO during virus infection. There are two pathways for iNOS induction during infections: direct up-regulation by the virus or indirect up-regulation *via* cytokine-dependent mechanisms [10]. Direct up-regulation by virus has been shown for respiratory syncytial virus, human immunodeficiency virus, and hepatitis C virus infection [26–29]. The exact mechanism(s) leading to elevated levels of RNI during human hantavirus infection are currently not known, but the finding that infection of cells *in vitro* did not induce detectable levels of nitrite suggests that RNI are produced as a response to the elevated levels of cytokines, like TNF-α and IFN-γ, detected in patients [3].

RNI has been suggested to be involved in hantavirus pathogenesis [15]. Elevated levels of NO have been detected in man [15–17] and monkeys [18], in whom hantavirus infections induce clinical symptoms, but are normally cleared within weeks after infection. We previously showed that DOBV, but not SAAV, was lethal for suckling mice and that increased levels of NO production were detected in lethally infected mice [13]. Furthermore, we also observed replicating virus in SAAV-inoculated mice 34 days after infection, whereas mice that survived DOBV infection had no replicating viruses in the brain [13]. Together with the finding that iNOS$^{-/-}$ suckling mice showed higher titers of replicating virus in the brain compared to normal C57BL/6 mice and that both strains showed severe symptoms at the same day after infection, the results might indicate that NO, at least in mice, is more likely to be involved in viral clearance than in pathogenesis.

In conclusion, we report that NO and peroxynitrite, two RNI, both have antiviral effects on hantaviruses, and that this effect is caused by inhibition of viral replication by NO at an early step in infection, and by direct inactivation of free virions by peroxynitrite. Furthermore, our results strengthens the suggestion that peroxynitrite is an endogenous effector of the antiviral immune response [9].

Materials and methods

Viruses and cells

The viruses used were the Vero E6 cell line-adapted HTNV, strain 76-118 [30], DOBV, strain Slovenia [31], SAAV [32], and PUUV, strain Kazan E6 [33]. Propagation and titration of the viruses were performed on Vero E6 cells [VERO C1008; American Type Culture Collection (ATCC), Manassas, VA], as described [34].

The cells used were A549, HeLa, HL, Huh-7, HUVEC (Clonetics, BioWhittaker, Walkersville, WV), MonoMac (kindly provided by Åsa Björndal, Swedish Institute for Infectious Disease Control, Solna, Sweden), Vero and Vero E6. A549, HeLa, HL, HuH-7, Vero, and Vero E6 cells were grown in EMEM supplemented with 2% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1.5 g/L bicarbonate (Sigma, St. Louis, MO), HUVEC in EGM-2-MV medium supplemented with EGM-2-MV singlequots (Clonetics), and MonoMac in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin.

Mice

Suckling C57BL/6 and C57BL/6 iNOS$^{-/-}$ mice, were purchased from MTC, Breeding unit, Karolinska Institutet, Stockholm, Sweden. Suckling mice were inoculated intracerebrally with 20 µL DOBV. Infected mice were kept in biological safety isolators. After sacrifice, hearts and brains were removed aseptically; brains were minced in PBS and stored at –70°C until further use. Hearts were stored at –70°C with PBS for 24 h; after thawing, the supernatant was transferred to a new tube and used for the detection of hantavirus-specific antibodies [13]. The care of all animals used in the present study was in compliance with the relevant guidelines and requirements of the Swedish Institute for Infectious Disease Control, Stockholm, Sweden.

Cytokines and chemicals

Recombinant human IL-1β and IFN-γ were purchased from Peprotech (London, UK). SIN-1, SNAP and NAP, were obtained from Sigma. L-NMMA and D-NMMA were purchased from Calbiochem (La Jolla, CA).

Titration of hantavirus

Samples were diluted tenfold in HBSS supplemented with 2% HEPES, 2% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin, and incubated on confluent Vero E6 cells in 24-well plates. After 1 h of incubation, cells were overlaid with 0.5% agarose-medium and incubated for a further 6–9 days, depending on the virus, at 37°C, 5% CO_2. Foci of infected cells were stained with polyclonal rabbit anti-HTNV or rabbit anti-PUUV sera, followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA) and were visualized with 3,3′,5,5′-tetramethylbenzidin (Sigma) and counted [33].

Assays of NO and peroxynitrite antiviral activity

Confluent Vero E6 cells grown on 24-well plates were washed, and medium alone or medium containing SIN-1, SNAP or NAP was added. Cells were infected with 1000 FFU of hantavirus, corresponding to 0.005 MOI. Media, with or without chemicals, were changed every 12 h. Supernatants were collected 6 h after the last treatment and were subsequently titrated on Vero E6 cells as described above.

Essentially the same protocol was used for endogenously produced NO [7]: 10 ng/mL IL-1β and/or 400 U/mL IFN-γ was added to the media 1 h after virus infection. L-NMMA (1 mM), a general NOS inhibitor, or D-NMMA as control for L-NMMA, was added to the cells at 1 and 24 h after virus infection. Supernatants were collected for virus titration 48 h post infection.

MTT assay

An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay was used to measure mitochondrial function, which served as an index of viable cells, in the SNAP-treated, NAP-treated and untreated cells. The MTT cell proliferation assay was carried out according to the manufacturer's instructions (ATCC).

NO assays

NO rapidly reacts with oxygen to form nitrite and nitrate, its two stable end-products [35]. Production of NO *in vitro*, and release of NO from SNAP and of peroxynitrite from SIN-1, was measured indirectly in cell culture supernatants by determination of the level of nitrite using the Griess assay. Supernatant samples, and sodium nitrite as standard, were mixed with equal volumes of Griess reagents (1% sulfanilamide and 0.1% naphtylethylenediamide, in 5% phosphoric acid), and the optical density at 540 nm was measured by spectrophotometry. The nitrite standard was diluted in the same medium as used for the samples.

Western blot

Vero E6 cells were infected with HTNV and treated with SNAP, NAP or medium alone as described above. At the end of infection, cells were collected and homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin and leupeptin). Lysates were mixed 1 : 1 in sample buffer (10 mM Tris-HCl, pH 7.5, 0.5% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue) resolved in 10% Tris-glycine polyacrylamide gels and transferred to nitrocellulose membranes. Blocking was performed overnight at 4°C in 5% nonfat dry milk in 0.1% Tween-20 in PBS. The membranes were subsequently incubated with hantavirus N-specific mAb 1C12 [36] and β-actin-specific mAb 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).

Real-time PCR

RNA was extracted from PUUV-infected cells using TriPure (Roche Diagnostics, Lewes, UK), according to the manufacturer's instructions. First-strand cDNA synthesis (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was performed according to the manufacturer's instructions with primer pd(N)$_6$. TaqMan real-time PCR was performed with 300 nM of primer 983F 5′-GTGCACCAGATCGGTGTCC-3′, 900 nM of primer 1038R 5′-CAATTCAGCCATCCCAGCA-3′ and 200 nM of TaqMan MGB probe 1003T 5′-CCTACATGCATTTATG-3′ on a 7900HT sequence detection system (Applied Biosystems, Foster City, CA) with software SDS version 2.0 [37]. RNA extracted from stocks of PUUV Kazan-E6 with known concentrations of virus (measured as FFU on Vero E6 cells) was used as a standard.

References

1 **Plyusnin, A., Vapalahti, O. and Vaheri, A.,** Hantaviruses: Genome structure, expression and evolution. *J. Gen. Virol.* 1996. **77:** 2677–2687.

2 **Schmaljohn, C. and Hjelle, B.,** Hantaviruses: A global disease problem. *Emerg. Infect. Dis.* 1997. **3:** 95–104.

3 **Khaiboullina, S. F. and St Jeor, S. C.,** Hantavirus immunology. *Viral Immunol.* 2002. **15:** 609–625.

4 **Settergren, B.,** Clinical aspects of nephropathia epidemica (Puumala virus infection) in Europe: A review. *Scand. J. Infect. Dis.* 2000. **32:** 125–132.

5 **Reiss, C. S. and Komatsu, T.,** Does nitric oxide play a critical role in viral infections? *J. Virol.* 1998. **72:** 4547–4551.

6 **Rimmelzwaan, G. F., Baars, M. M. J. W., de Lijster, P., Fouchier, R. M. and Osterhaus, A. D. M. E.,** Inhibition of influenza virus replication by nitric oxide. *J. Virol.* 1999. **73:** 8880–8883.

7 **Åkerström, S., Mousavi-Jazi, M., Klingström, J., Leijon, M., Lundkvist, Å. and Mirazimi, A.,** Nitric oxide inhibits the replication cycle of severe acute respiratory syndrome coronavirus. *J. Virol.* 2005. **79:** 1966–1969.

8 **Nathan, C.,** Specificity of a third kind: Reactive oxygen and nitrogen intermediates in cell signaling. *J. Clin. Invest.* 2003. **111:** 769–778.

9 **Padalko, E., Ohnishi, T., Matsushita, K., Sun, H., Fox-Talbot, K., Bao, C. and Baldwin 3rd, W. M.,** Peroxynitrite inhibition of Coxsackievirus infection by prevention of viral RNA entry. *Proc. Natl. Acad. Sci. USA* 2004. **101:** 11731–11736.

10 **Akaike, T. and Maeda, H.,** Nitric oxide and virus infection. *Immunology* 2000. **101:** 300–308.

11 **Evans, T. G., Rasmussen, K., Wiebke, G. and Hibbs, J. B.,** Nitric oxide synthesis in patients with advanced HIV infection. *Clin. Exp. Immunol.* 1994. **97:** 83–86.

12 **Karupiah, G., Chen, J. H., Mahalingam, S., Nathan, C. F. and MacMicking, J. D.,** Rapid interferon gamma-dependent clearance of influenza A virus and protection from consolidating pneumonitis in nitric oxide synthase 2-deficient mice. *J. Exp. Med.* 1998. **188:** 1541–1546.

13 **Klingström, J., Hardestam, J. and Lundkvist, Å.,** Dobrava, but not Saaremaa, hantavirus is lethal and induces nitric oxide production in suckling mice. *Microbes Infect* 2006. **8:** 728–737.

14 **van den Broek, M., Bachmann, M. F., Höhler, G., Barner, M., Escher, R., Zinkernagel, R. and Kopf, M.,** IL-4 and IL-10 antagonize IL-12-mediated protection against acute vaccinia virus infection with a limited role of IFN-γ and nitric oxide synthetase 2. *J. Immunol.* 2000. **164:** 371–378.

15 **Davis, I. C., Zajac, A. J., Nolte, K. B., Botten, J., Hjelle, B. and Matalon, S.,** Elevated generation of reactive oxygen/nitrogen species in hantavirus cardiopulmonary syndrome. *J. Virol.* 2002. **76:** 8347–8359.

16 **Groeneveld, P., Colson, P., Kwappenberg, K. and Clement, J.,** Increased production of nitric oxide in patients infected with the European variant of Hantavirus. *Scand. J. Infect. Dis.* 1995. **27:** 453–456.

17 **Linderholm, M., Groeneveld, P. and Tärnvik, A.,** Increased production of nitric oxide in patients with hemorrhagic fever with renal syndrome – relation to arterial hypotension and tumor necrosis factor. *Infection* 1996. **24:** 337–340.

18 **Klingström, J., Plyusnin, A., Vaheri, A. and Lundkvist, Å.,** Wild-type Puumala hantavirus infection induces cytokines, C-reactive protein, creatinine, and nitric oxide in *Cynomolgus* macaques. *J. Virol.* 2002. **76:** 444–449.

19 **MacMicking, J., Xie, Q. and Nathan, C.,** Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 1997. **15:** 323–350.

20 **Bogdan, C., Röllinghoff, N. and Diefenbach, A.,** The role of nitric oxide in innate immunity. *Immunol. Rev.* 2000. **173:** 17–26.

21 **Bi, Z. and Reiss, C. A.,** Inhibition of vesicular stomatitis virus infection by nitric oxide. *J. Virol.* 1995. **69:** 2208–2213.

22 **Tamura, M., Asada, H., Kondo, K., Takahashi, M. and Yamanashi, K.,** Effects of human and murine interferons against hemorrhagic fever with renal syndrome (HFRS) virus (Hantaan virus). *Antivir. Res.* 1987. **8:** 171–178.

23 **Saura, M., Zaragoza, C., McMillan, A., Quick, R. A., Hohenadl, C., Lowenstein, J. M. and Lowenstein, C. J.,** An antiviral mechanism of nitric oxide: Inhibition of a viral protease. *Immunity* 1999. **10:** 21–28.

24 **Akaike, T., Fujii, S., Kato, A., Yoshitake, J., Miyamoto, Y., Sawa, T. and Okamoto, S. et al.,** Viral mutation accelerated by nitric oxide production during infection *in vivo*. *FASEB J.* 2000. **14:** 1447–1454.

25 **Colasanti, M., Persichini, T., Venturini, G. and Ascenzi, P.,** S-Nitrosylation of viral proteins: Molecular bases for antiviral effect of nitric oxide. *IUBMB Life* 1999. **48:** 25–31.

26 **Kao, Y. J., Piedra, P.-A., Larsen, G. L. and Colasurdo, G. N.,** Induction and regulation of nitric oxide synthase in airway epithelial cells by respiratory syncytial virus. *Am. J. Respir. Crit. Care Med.* 1999. **163:** 532–539.

27 **Tsutsumi, H., Takeuchi, R., Ohsaki, M., Seki, K. and Chiba, S.,** Respiratory syncytial virus infection of human respiratory epithelial cells enhances inducible nitric oxide synthase gene expression. *J. Leukoc. Biol.* 1999. **66:** 99–104.

28 **Blond, D., Raoul, H., Le Grand, R. and Dormont, D.,** Nitric oxide synthesis enhances human immunodeficiency virus replication in primary human macrophages. *J. Virol.* 2000. **74:** 8904–8912.

29 **Machida, K., Cheng, K. T.-H., Sung, V. M.-H., Lee, K. J., Levine, A. M. and Lai, M. M. C.,** Hepatitis C virus infection activates the immunological (type II) isoform of nitric oxide synthase and thereby enhances DNA damage and mutations of cellular genes. *J. Virol.* 2004. **78:** 8835–8843.

30 **Lee, H. W., Lee, P. W. and Johnson, K. M.,** Isolation of the etiological agent of Korean hemorrhagic fever. *J. Infect. Dis.* 1978. **137:** 298–308.

31 **Avsic-Zupanc, T., Xiao, S. Y., Stojanovic, R., Gligic, A., van der Groen, G. and LeDuc, J. W.,** Characterization of Dobrava virus: A hantavirus from Slovenia, Yugoslavia. *J. Med. Virol.* 1992. **38:** 132–137.

32 **Nemirov, K., Vapalahti, O., Lundkvist, Å., Vasilenko, V., Golovljova, I., Plyusnina, A. and Niemimaa, J. et al.,** Isolation and characterization of Dobrava hantavirus carried by the striped field mouse (*Apodemus agrarius*) in Estonia. *J. Gen. Virol.* 1999. **80:** 371–379.

33 **Lundkvist, Å., Cheng, Y., Brus Sjölander, K., Niklasson, B., Vaheri, A. and Plyusnin, A.,** Cell culture adaption of Puumala hantavirus changes the infectivity for its natural reservoir, *Clethrionomys glareolus*, and leads to accumulation of mutants with altered genomic RNA S segment. *J. Virol.* 1997. **71:** 9515–9523.

34 **Klingström, J., Maljkovic, I., Zuber, B., Rollman, E., Kjerrström, A. and Lundkvist, Å.,** Vaccination of C57/BL6 mice with Dobrava hantavirus nucleocapsid protein in Freund's adjuvant induced partial protection against challenge. *Vaccine* 2004. **22:** 4029–4034.

35 **Stamler, J. S., Singel, D. J. and Loscalzo, J.,** Biochemistry of nitric oxide and its redox-activated forms. *Science* 1992. **258:** 1896–1902.

36 **Lundkvist, Å., Fatouros, A. and Niklasson, B.,** Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies. *J. Gen. Virol.* 1991. **72:** 2097–2103.

37 **Klingström, J., Falk, K. I. and Lundkvist, Å.,** Delayed viremia and antibody responses in Puumala hantavirus challenged passively immunized *Cynomolgus* macaques. *Arch. Virol.* 2005. **150:** 79–92.