Nitric Oxide Inhibits the Replication Cycle of Severe Acute Respiratory Syndrome Coronavirus

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Nitric oxide (NO) is an important signaling molecule between cells which has been shown to have an inhibitory effect on some virus infections. The purpose of this study was to examine whether NO inhibits the replication cycle of the severe acute respiratory syndrome coronavirus (SARS CoV) in vitro. We found that an organic NO donor, *S*-nitroso-*N*-acetylpenicillamine, significantly inhibited the replication cycle of SARS CoV in a concentration-dependent manner. We also show here that NO inhibits viral protein and RNA synthesis. Furthermore, we demonstrate that NO generated by inducible nitric oxide synthase, an enzyme that produces NO, inhibits the SARS CoV replication cycle.

Severe acute respiratory syndrome (SARS), which is associated with a novel coronavirus (CoV), was first identified during fall 2002 in Guangdong Province, China (24, 32, 34). The mortality rate of SARS appears to range from 6 to 55% (12, 20, 21). Coronaviruses are enveloped single-stranded positive-sense RNA viruses with genomes of about 27 to 30 kb (21). Coronaviruses belong to the family *Coronaviridae*, in which SARS CoV forms a distinct group within the genus *Coronavirus* (9, 29).

Nitric oxide (NO) is an important signaling molecule between cells and is involved in a wide range of processes (9, 27). An antimicrobial activity of NO has been described for several bacteria and protozoa and for some viruses (1, 18, 27). NO is produced by three enzymes that catalyze the oxidation of L-arginine to NO and L-citrulline (9). Two of the enzymes, neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS), are constitutively expressed and are calcium dependent (27). Inducible NOS (iNOS) is expressed only in activated cells and is calcium independent (11). The up-regulation of iNOS is common during an infection, and it is known that some viruses and bacteria are either inhibited or stimulated by increased levels of NO (1, 2, 11, 26, 30). It has also been demonstrated that iNOS is expressed after interferon stimulation in murine macrophages, mouse T cells, human hepatocytes, mononuclear cells, human airway epithelial cells, and alveolar macrophages (6, 13, 15, 25, 31, 33).

To investigate the role of NO in SARS CoV infection, we infected Vero E6 cells with SARS CoV at a multiplicity of infection (MOI) of 1. At 1 h postinfection (hpi), the cells were washed twice and then mock treated or treated with different concentrations of the organic NO donor *S*-nitroso-*N*-acetyl-

penicillamine (SNAP; Sigma, St. Louis, Mo.) or, as a negative control, *N*-acetylpenicillamine (NAP; Sigma), which lacks the NO-donating *S*-nitroso group (3, 7, 18, 19, 27). At 24 hpi, the amount of virus was deduced by the 50% tissue culture infective dose ($TCID_{50}$), which was calculated from the cytopathic effect induced in cell culture by different dilutions of the harvested virus. As shown in Fig. 1A, SNAP inhibited the replication cycle of SARS CoV in a dose-dependent manner. Treatment with 100 μM SNAP resulted in a 2-log reduction in the yield of progeny virus, and the inhibitory effect was even more pronounced with 400 μM SNAP. As shown in Fig. 1B, the observed inhibitory effect correlated with the release of NO_2 into the culture medium, as determined by the use of Griess reagent (14, 16, 28). To exclude the possibility that the detected antiviral effect of SNAP might have resulted from toxicity to the cells, we performed an MTT cell proliferation test (American Type Culture Collection). These results clearly excluded the possibility that the antiviral effect of SNAP was due to general cytotoxicity (Fig. 1C).

The inhibitory effect of NO on SARS CoV infection in Vero E6 cells was further demonstrated by an immunofluorescence assay and Western blotting as described previously (5), using rabbit polyclonal antibodies directed against SARS CoV NP (kindly provided by Luis Martínez-Sobrido and Adolfo García-Sastre). The antibodies were raised against purified recombinant NP of SARS CoV expressed in bacteria (L. Martínez-Sobrido, personal communication). Vero E6 cells were mock infected or infected with SARS CoV at an MOI of 1. At 1 hpi, the cells were mock treated or treated with SNAP or NAP (400 μM). At 12 or 24 hpi, the cells were fixed or lysed and then analyzed. The results clearly showed that treatment with 400 μM SNAP reduced the number of infected cells (Fig. 2A) and also demonstrated a significant reduction in NP expression (Fig. 2B).

To exclude the possibility that the reduction in NP expres-

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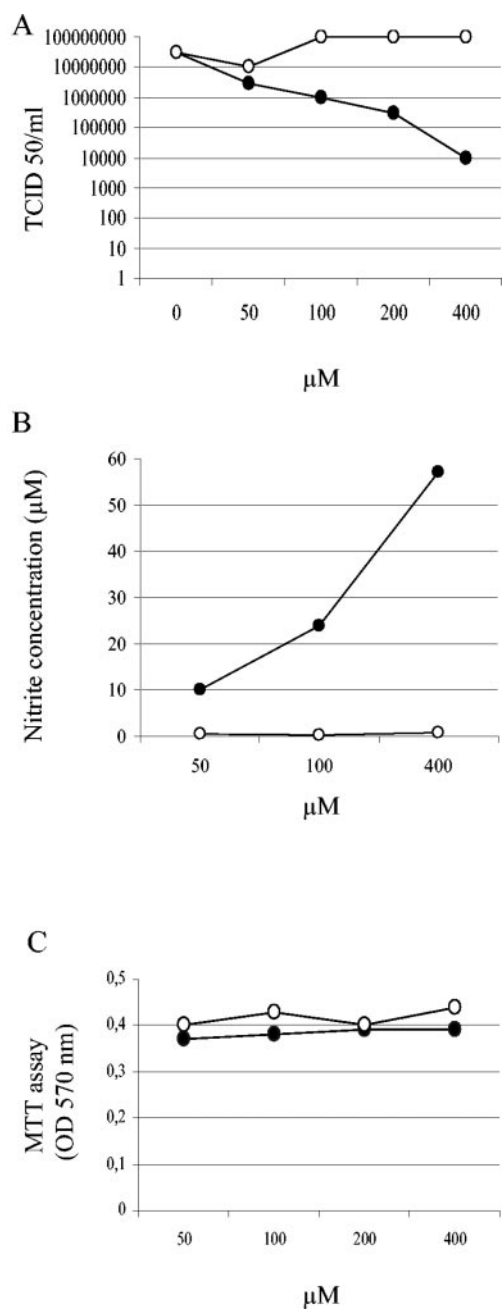
FIG. 1. NO has an antiviral effect on SARS CoV infection. Vero E6 cells were infected with SARS CoV at an MOI of 1.0. At 1 hpi, the cells were treated with different concentrations of SNAP (●) and NAP (○). (A) Viruses was harvested at 24 hpi and titers were determined. (B) Nitrite concentrations produced at 24 h posttreatment with different concentrations of SNAP and NAP. (C) Cell viability, as determined by MTT assays. The mean values from two experiments are indicated.

sion was due to a common inhibition of protein translation, we mock treated Vero E6 cells or treated them with SNAP or NAP (400 μM). At 24 h posttreatment, the cells were pulsed with 250 μCi of radiation/ml for 1 h. The cells were lysed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (22). The results clearly

showed that the total amount of protein translation was not decreased in SNAP-treated cells compared to that in control cells (Fig. 2C).

To investigate whether NO inhibits the viral RNA replication process of SARS CoV, we infected Vero E6 cells with SARS CoV at an MOI of 0.1. At 1 hpi, the cells were treated with 400 μM SNAP or NAP and then lysed by the use of Trizol (Gibco/Life Technologies/Invitrogen, Groningen, The Netherlands) at different times, and total RNAs were isolated as previously described (4). Viral RNAs were subsequently quantified by real-time PCR as described below. A ReSSQ SARS assay (LightUp Technologies AB, Stockholm, Sweden) was used for real-time PCR quantification with a Light-Cycler 1.0 instrument (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. One of the gene fragments targeted by the ReSSQ assay was designed to be almost identical to the fragment used in the SARS standard distributed by the Bernhard-Nocht Institute for Tropical Medicine (BNI) to enable the use of this standard for quantification; hence, all reported quantifications are based on the BNI standard. As shown in Fig. 3, we demonstrated that viral RNA production was significantly inhibited by 400 μM SNAP.

To investigate the effect of iNOS on the replication cycle of SARS CoV in cell culture, we mock treated Vero E6 cells or treated them with 10 ng of recombinant human interleukin-1β (IL-1β) (1 U/ml; Peprotech, London, England)/ml, together with 400 U of recombinant human gamma interferon (IFN-γ) (Peprotech)/ml, to induce iNOS (26).

After 48 h of stimulation, the cells were mock infected or infected with SARS CoV at an MOI of 0.1. At 24 hpi, the virus was harvested and the titer was determined as described previously.

We found that the induction of iNOS reduced the yield of progeny virus by about 82% (from 2.1×10^7 to 3.9×10^6 $TCID_{50}$) (Fig. 4A). This result confirmed a recent report which showed that IFN-γ has an antiviral effect on the replication cycle of SARS CoV in Vero cells (10).

The measurement of NO levels (Fig. 4B) demonstrated that the concentration of nitrite produced by the cytokine treatment reached approximately the same level as that seen with 50 μM SNAP.

Most interestingly, we observed the same level of inhibition of the virus replication cycle with 50 μM SNAP as that with the cytokine treatment. In order to confirm that the NO production was dependent on iNOS induction, we treated cells with a 1 mM concentration of the iNOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) (8, 26, 27, 30), together with IL-1β and IFN-γ. L-NMMA significantly inhibited the production of NO and thereby restored the replication cycle of SARS CoV. However, it should be mentioned that the virus titer was still 30% lower than that of the control. One possible explanation for this observation may be that L-NMMA could not completely inhibit iNOS and that some NO was therefore still produced, which may have inhibited the virus (Fig. 4).

Our results demonstrated that NO specifically inhibits the replication cycle of SARS CoV, most probably during the early steps of infection, suggesting that the production of NO by iNOS results in an antiviral effect. However, the production of NO should be adjusted to exert antiviral rather than damaging
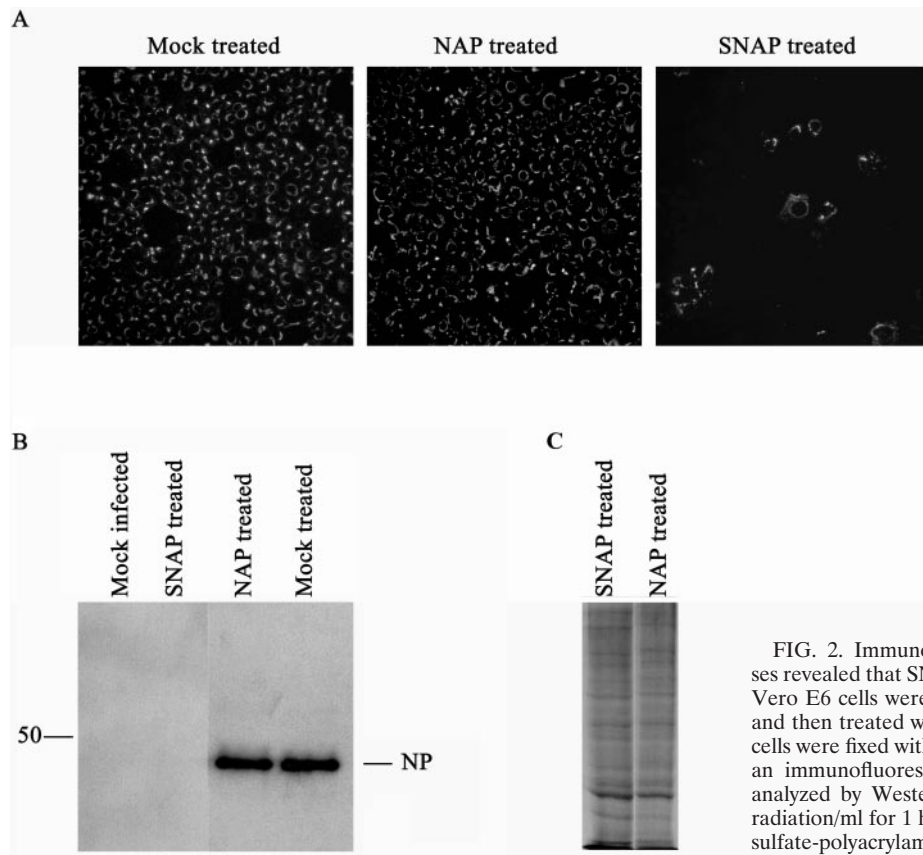
FIG. 2. Immunofluorescence assays and Western blot analyses revealed that SNAP inhibits the SARS CoV replication cycle. Vero E6 cells were infected with SARS CoV at an MOI of 1.0 and then treated with 400 μM SNAP or NAP at 1 hpi. Vero E6 cells were fixed with 80% acetone at 24 hpi and then analyzed by an immunofluorescence assay (A), lysed at 12 hpi and then analyzed by Western blotting (B), or pulsed with 250 μCi of radiation/ml for 1 h, lysed, and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (C).

effects. At present, there is no information concerning the levels of NO in SARS patients. Previous studies have shown that NO plays a role in the pathogenesis of influenza virus pneumonia in mice (2, 17). This pathological effect, however, has been suggested to be associated with the mouse model of pneumonia, since the peak of NO in infected humans was not associated with clinical symptoms (23). Thus, the role of NO during SARS infection in animal models and the level of NO in SARS patients constitute important areas for future studies.
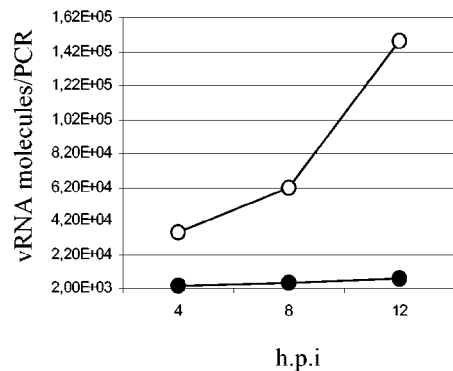

FIG. 3. SNAP blocks viral RNA replication of SARS CoV. Vero E6 cells were infected at an MOI of 0.01 and then treated with 400 μM SNAP (●) or NAP (○) at 1 hpi. Treated cells were lysed by the use of Trizol at different times, and viral RNAs were quantified as described in the text.
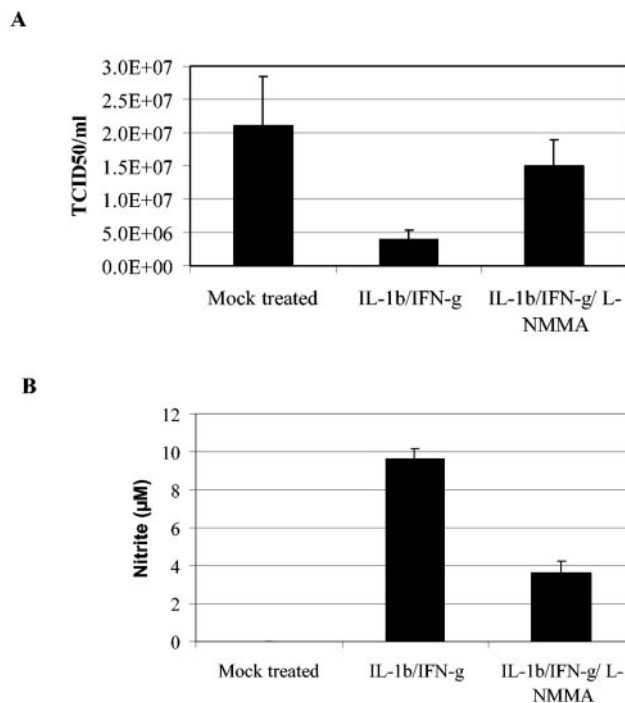
FIG. 4. iNOS has an inhibitory effect on the replication cycle of SARS CoV. Vero E6 cells were mock treated or treated with IL-1β and IFN-γ, with or without the iNOS inhibitor L-NMMA. At 48 h posttreatment, the cells were infected with SARS CoV at an MOI of 0.1. Progeny virus was harvested at 24 hpi, and the titer was deduced by calculating the $TCID_{50}$ (A). Nitrite concentrations were determined by the use of Griess reagent (B). The mean values from three separate experiments are given, and error bars indicate standard deviations.

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