Nitric Oxide Inhibition of Coxsackievirus Replication In Vitro

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Abstract

Nitric oxide is a radical molecule with antibacterial, -parasitic, and -viral properties. We investigated the mechanism of NO inhibition of Coxsackievirus B3 (CVB3) replication in vitro by determining the effect of NO upon a single replicative cycle of CVB3 grown in HeLa cells. Transfection of inducible NO synthase cDNA into HeLa cells reduces the number of viral particles produced during a single cycle of growth. Similarly, a noncytotoxic concentration of the NO donor *S*-nitroso-amino-penicillamine reduces the number of viral particles in a dose-dependent manner. To explore the mechanisms by which NO exerts its antiviral effect, we assayed the attachment, replication, and translation steps of the CVB3 life cycle. NO does not affect the attachment of CVB3 to HeLa cells. However, NO inhibits CVB3 RNA synthesis, as shown by a [^{3}H]uridine incorporation assay, reverse transcription–PCR, and Northern analysis. In addition, NO inhibits CVB3 protein synthesis, as shown by [^{35}S]methionine protein labeling and Western blot analysis of infected cells. Thus, NO inhibits CVB3 replication in part by inhibiting viral RNA synthesis by an unknown mechanism. (*J. Clin. Invest.* 1997. 100:1760–1767.) Key words: enterovirus • myocarditis • autoimmune • nitric oxide synthase • nuclear factor kappa B

Introduction

Nitric oxide is a radical messenger molecule produced by the enzyme NO synthase (NOS)[1] (reviewed in references 1–7). Three NOS isoforms have been characterized: the constitutively expressed neuronal NOS, endothelial NOS (8–11), and also the inducible isoform iNOS (also called NOS2) (12–14). NO is a signaling and effector molecule with diverse roles. NO can defend the host against diverse pathogens, including bacteria, fungi, parasites, and viruses (2, 15).

Infection by RNA or DNA viruses can induce iNOS expression, and NO synthesized by iNOS can inhibit viral replication (reviewed in reference 16). Viral infections induce iNOS in cells (17–22), animals (17, 23–29), and humans (19, 30). Produced endogenously by iNOS or generated from exogenously added donors, NO inhibits viral replication in a variety of cells (25, 31–33). However, the effect of NO upon virus replication in vivo is more complex: depending on the system studied, administration of NOS inhibitors can increase or decrease viral replication (17, 24–28).

NO has been reported to interfere with specific stages in the life cycle of viruses. For example, NO inhibits vaccinia virus DNA synthesis, late protein translation, and virion assembly (18, 34, 35). One specific viral target of NO has been identified: NO can inhibit the function and expression of the Epstein-Barr virus immediate early transactivator *Zta* (20). Since NO can inhibit a variety of viruses, it is possible that NO also acts to inhibit cellular processes necessary for all viruses to replicate.

Coxsackievirus B3 (CVB3) is a human enterovirus in the *Picornaviridae* family (36). Its genome is a single-stranded RNA molecule of positive polarity that encodes a 2,185 amino acid polyprotein (37, 38). The CVB3 life cycle includes viral attachment to the host cell, penetration, replication of the RNA genome via a RNA intermediate, translation of the viral proteins, virion assembly, and exit from the host cell (39). Coxsackie B viruses cause acute and chronic infections in mice and humans, including myocarditis (40–52). Recently, we and others have demonstrated that CVB3 infection induces iNOS, and that NO has an antiviral effect in a murine myocarditis model (53, 54). However, the mechanism of NO inhibition is unknown. In this study, we explored the effect of NO upon different stages in the CVB3 replicative cycle in vitro.

Methods

Materials. *S*-nitroso-amino-penicillamine (SNAP) was made according to standard protocols (55). The iNOS cDNA was isolated by the authors (12). The CVB3 cDNA was a generous gift of R. Kandolf (Eberhard-Karls-Universität, Tubingen, Germany) (56). The rabbit polyclonal anti–murine-iNOS antibody was prepared by the authors (53). The anti–CVB3 2C antibody was a generous gift of Dr. C. Hohenadl, Max Planck-Institut fur Biochemie (Martinsried, Germany) (57).

Cell and viral culture. CVB3 (Nancy strain; generous gift of Charles J. Gauntt, University of Texas Health Science Center at San Antonio, San Antonio, TX) was grown and titered using HeLa cells. HeLa cells were cultured in growth media (MEM; GIBCO BRL, Gaithersburg, MD) supplemented with 1% L-glutamine (100 mmol), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. After infection, the cells were grown in infecting media: MEM with 1% FCS. Viral stocks were prepared by infecting an 80–90% confluent monolayer culture of HeLa cells at an MOI of 10. 2 d after incubation at 37°C, the cells were frozen and thawed three times, the suspension was centrifuged, and the supernatant was harvested and stored at −80°C. The amount of virus in the supernatant was measured by the plaque assay method (58). CVB3 stock titer was 2×10^9 plaque-forming units (pfu)/ml.

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1. *Abbreviations used in this paper:* AP, amino-penicillamine; CVB3, Coxsackievirus B3; iNOS, inducible NOS; NOS, nitric oxide synthase; RT, reverse transcription; SNAP, *S*-nitroso-AP.

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A stock of radioactively labeled virus was prepared by infecting a confluent monolayer of HeLa cells with CVB3 at an MOI of 10, in methionine-free medium containing 100 μCi/ml of *trans*^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine; > 1,000 Ci/mmol; ICN Biomedicals Inc., Costa Mesa, CA). After 24 h of incubation at 37°C and three freeze/thaw cycles, the cellular debris was spun down and the viral supernatant was centrifuged for 90 min at 45,000 rpm. The pellet was resuspended in PBS and the viral amount was measured. The labeled viral stock used in this work was 3.5×10^8 pfu/ml and 0.11 cpm/pfu.

To measure the amount of virus during infection of HeLa cells, we performed a plaque assay (Charles J. Gauntt). Serial dilutions of virus were added to six-well plates of 90% confluent HeLa cells in a volume of 200 μl for 1 h at 37°C with gentle rocking of the plates every 15 min. Equal volumes of 2% agar (DIFCO Laboratories Inc., Detroit, MI) and 2× infecting media at 42°C were mixed, and then 2 ml of the mixture were added to each well. Plates were incubated for 2 d, the wells were fixed with Carnoy's solution (acetic acid 25%, ethanol 75%), the agar plugs removed, the cells stained with Coomassie reagent, and the plaques counted.

CVB3 single-step growth curve. A 90% confluent monolayer of HeLa cells was infected with CVB3 at an MOI of 1. 1 h after incubation at 37°C, the infecting media was removed and the cells were washed three times with PBS and refed with fresh infecting media. At regular time points, 200 μl of culture supernatant was collected and titered by the plaque assay method.

Transient transfections of HeLa cells. HeLa cells that were 60% confluent in six-well plates were transiently transfected by a liposome-mediated method (Lipofectin reagent; GIBCO BRL) with the plasmid pCIS-iNOS (the murine iNOS cDNA placed downstream of a cytomegalovirus promoter) or with the plasmid pCIS-antisense-iNOS (a similar construct carrying an antisense orientation of the iNOS cDNA) (12). Between 0 and 10 μg plasmid and 10 μl lipofectin were incubated together for 15 min, added to 1 ml of OptiMem (GIBCO BRL), and then added to HeLa cells; the HeLa cells were then incubated for 16 h at 37°C. After 16 h of incubation, the transfection solution was removed and the cells were fed with media with 1% FCS. At regular time points, cells and culture supernatants were collected for analyses as described below.

Immunoblot analysis. Immunoblots were performed as previously described (53). In brief, cells were lysed in lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.17 mg/ml PMSF, and 2 μg/ml of the protein inhibitors leupeptin, pepstatin, antipain, and antitrypsin). The homogenate was briefly centrifuged and supernatants were collected. Proteins were quantified by Coomassie assay and the samples were stored at −20°C. 200 μg cell homogenate was fractionated by SDS-PAGE, and then transferred to a nylon membrane that was blocked and washed. iNOS protein expression was detected by hybridizing the membrane with anti–iNOS antibody generated in our laboratory (53), at a dilution of 1:2,000. CVB3 protein synthesis was monitored with a specific anti–CVB3 2C antibody (57), used at a dilution of 1:1,000. Membranes were washed, hybridized with the appropriate secondary antibody conjugated to horseradish peroxidase (Amersham Corp., Arlington Heights, IL), and developed with a chemiluminescent system (Enhanced Luminol Reagent; Dupont, Wilmington, DE) according to the manufacturer's instructions.

Nitrite assay. Nitrite concentration in culture supernatants was determined by a modification of the Griess assay (31). In brief, 50 μl of sample or sodium nitrite standards were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylene diamine, and 2.5% H_3PO_4) for 10 min at room temperature. The absorbance at 540 nm of each sample was measured in a microplate reader.

Cell viability. To determine the maximum dose of the NO donor SNAP with minimal cytotoxicity, HeLa cells were incubated with different concentrations of SNAP (0, 50, 100, 250, and 500 μM) for 24 h. The cells were trypsinized and counted in a 0.4% trypan blue solution on a glass hemocytometer plate.

Antiviral activity of SNAP. The antiviral effect of the NO donor SNAP was measured during a single growth cycle of CVB3. To determine the optimal timing of exposure to SNAP, 250 μM of SNAP or its drug control, amino-penicillamine (AP), were added 1 h before (−1), during (0), or 1 h after the infection (+1) of HeLa cells (MOI = 1). The amount of virus produced was measured 10 h after infection by plaque assay of supernatant cultures.

Uridine incorporation in total RNA. HeLa cell monolayers were infected with CVB3 at an MOI of 1. At the same time, 5 μg/ml of actinomycin D was added. Every hour after infection, 10 μCi of [^{3}H]uridine (30 Ci/mmol; Amersham Corp.) per milliliter was added to each plate. At regular time points, the medium was removed and the cells were treated with 5% trichloroacetic acid and washed with 100% ethanol. Cell monolayers were dried and dissolved in 1 N NaOH/1% SDS solution. The isotope incorporation of each sample was measured in a liquid scintillation counter.

CVB3 attachment to HeLa cells. HeLa monolayers growing in six-well plates were infected with [^{35}S]methionine- and [^{35}S]cysteine-labeled CVB3 particles at an MOI of 1 (0.11 cpm/pfu) and rocked at 4°C. The media was removed at 5, 15, 30, 45, and 60 min, and the cells were washed twice with PBS and harvested. The samples and a labeled virus control were suspended in EcoLite (ICN Biomedicals Inc.) and radioactivity measured in a liquid scintillation counter. In addition, a competition test was assayed separately by co-infecting HeLa cells with labeled CVB3 at an MOI of 1 and unlabeled virus at an MOI of 50. The effect of NO donors upon viral attachment to cells was assayed by the addition of 250 μM SNAP 1 h before (−1), during (0), or 1 h after (+1) the infection.

RNA isolation and Northern blot analysis. Northern analysis was performed as described previously (12). Total RNA was isolated from HeLa cells with the guanidinium thiocyanate-phenol-chloroform method (59), and 10 μg/lane was electrophoresed through a denaturing 1% agarose/0.66 M formaldehyde gels, transferred to GeneScreen Plus membranes (Dupont), and ultraviolet cross-linked. The filters were probed with a 1.5-kb PstI/XhoI cDNA fragment corresponding to the entire CVB3 genome (generous gift of R. Kandolf; reference 56). The cDNA probe was labeled with [^{32}P]dCTP using a randomly primed labeling kit (Amersham Corp.). The filters were hybridized for 16 h at 42°C in a buffer containing 5× SSPE, 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm (Sigma Chemical Co.). Membranes were washed at 55°C with 0.1× SSC/0.1% SDS, and exposed to a Dupont film using an intensifying screen.

Reverse transcription–PCR amplification of CVB3 RNA. For reverse transcription (RT)–PCR, total RNA was harvested from infected cells as above and used as a template to amplify a 200-bp cDNA fragment in a reverse transcription assay, followed by a polymerase chain reaction (GeneAmp; Perkin Elmer Corp., Norwalk, CT) according to the manufacturer's instructions (60). For the amplification of the corresponding cDNA fragment, the following primers based on the CVB3 genomic sequence (37, 38) were selected: sense primer: 5′-ACTCTGCAGCGGAACCGACTA-3′ (positions 526–546 in the CVB3 cDNA sequence), and antisense primer: 5′-GCTGTATTCAACTTAACAATG-3′ (positions 738–758 in the CVB3 cDNA sequence) (56). The amplification was performed using 25 cycles consisting of denaturation for 1 min at 94°C, primer annealing for 1 min at 60°C, and primer elongation for 1 min at 72°C. The PCR products were electrophoresed in 1% agarose and visualized with ethidium bromide staining.

Analysis of protein synthesis in infected cells. HeLa cell monolayers were infected with CVB3 at an MOI of 10. After incubation for 45 min at 4°C, cells were incubated at 37°C. At regular time points, the plates were washed twice with PBS and pulse labeled for 1 h in methionine/cysteine-medium–free media that contained 25 μCi/ml of *trans*^{35}S-label (> 1,000 Ci/mmol). The cells were lysed in lysis buffer as described above. Labeled proteins were electrophoresed in 12.5% SDS/PAGE. The gels were fixed, fluorographed with the Amplify reagent (Amersham Corp.), dried under vacuum, and exposed at −80°C to film for autoradiography.

Results

Antiviral effect of NO in a single CVB3 growth cycle. To explore the antiviral effect of NO, we first transfected the murine iNOS cDNA into HeLa cells and measured NO production over time. Cells transfected with an iNOS cDNA express iNOS protein as detected by immunoblot (Fig. 1 *A*), and produce NO as measured in the Griess reaction (Fig. 1 *B*). Maximum expression and activity is obtained by the transfection of 5 μg iNOS cDNA. The iNOS is active for at least 24 h after transfection. In contrast, control HeLa cells (transfected with antisense-iNOS cDNA, or with lipofectin, or with nothing) do not express iNOS and lack NOS activity.

Having determined the optimal conditions for transfection and expression of iNOS, we then infected CVB3 into HeLa cells 18 h after transfection with 5 μg iNOS cDNA. iNOS cDNA transfection inhibits CVB3 replication by 99% in infected HeLa cells (MOI = 1) as compared with viral growth in control transfected cells (Fig. 2). (The concentration of nitrite in supernatant of iNOS cDNA-transfected cells infected with CVB3 is 7.3 μM at 10 h after infection.)

To further explore the antiviral mechanism of NO, we used the NO donor *S*-nitroso-amino-penicillamine or its control drug amino-penicillamine. We first characterized the cytotoxicity of SNAP and its release of NO into HeLa cell cultures. Incubation of HeLa cells for 24 h with different concentrations of SNAP demonstrates that 250 μM is the maximal concentration of SNAP without any cytotoxic effect, as detected by trypan blue exclusion (Fig. 3 *A*). The time course of NO release by SNAP, measured by the Griess reaction, shows that 2 h after 250-μM SNAP administration, all the NO was released into the media (Fig. 3 *B*).

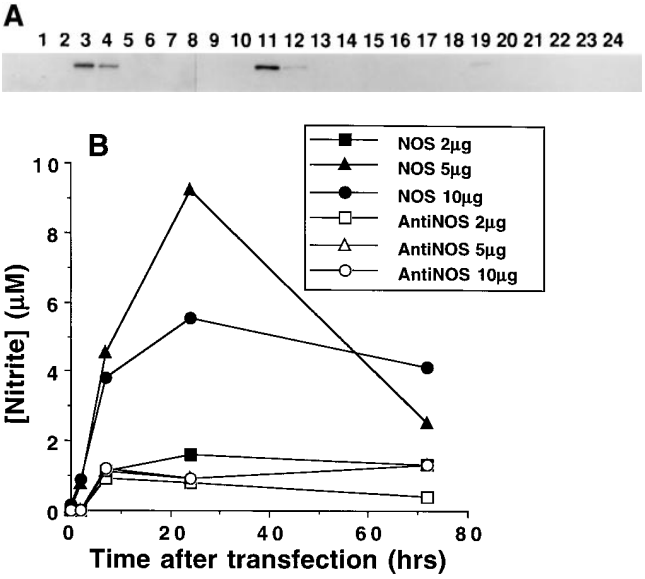

Figure 1. Murine iNOS cDNA is expressed and active in transiently transfected HeLa cells. HeLa cells were transiently transfected with either a plasmid expressing iNOS in the sense or antisense orientation, and proteins and culture supernatants were harvested from cells after transfection. (*A*) Maximal iNOS protein levels are detected by immunoblotting 24 h after transfection with 5 μg iNOS plasmid. Cells were transfected as follows: with 1, 2, 5, and 10 μg of pCIS-iNOS, and harvested after 7 h (lanes *1–4*, respectively); with similar amounts of pCIS-antisense-iNOS and harvested after 7 h (lanes *5–8*); with pCIS-iNOS and harvested after 24 h (lanes *9–12*); with pCIS-antisense-iNOS and harvested after 24 h (lanes *13–16*); with pCIS-iNOS and harvested after 48 h (lanes *17–20*); with pCIS-antisense-iNOS and harvested after 48 h (lanes *21–24*) (Data shown from single experiment repeated three times.) (*B*) Murine iNOS is active in transiently transfected HeLa cells. HeLa cells were transfected with pCIS-iNOS and pCIS-antisense-iNOS, and aliquots of supernatant were harvested and analyzed by the Griess assay ($n = 3 \pm$SD, although error bars are smaller than symbols).

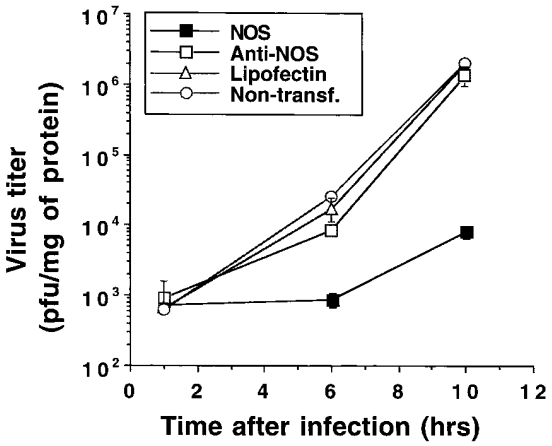

Figure 2. Murine iNOS inhibits CVB3 replication. HeLa cells were transfected with 5 μg of pCIS-iNOS, or pCIS-antisense-iNOS; 18 h after transfection, cells were infected with CVB3 at an MOI of 1. Aliquots of supernatant were collected at regular time points and the viral content was measured by the plaque assay ($n = 3 \pm$SD).

We used the conditions established above to determine the effect of NO upon HeLa cells. To determine the dose response to SNAP, we infected CVB3 at an MOI of 1 into HeLa cell monolayers, and then added different concentrations of SNAP. Increasing concentrations of SNAP inhibits viral replication of CVB3 during a single replicative cycle in a dose-dependent manner (Fig. 4 *A*). However, 500 μM of SNAP probably reduces viral growth by its cytotoxic effects upon host cells (Fig. 3 *A*). Based on these results, we chose to use 250 μM SNAP for further experiments.

To determine the optimal time to add SNAP, we infected HeLa cells (MOI = 1), adding 250 μM of SNAP 1 h before (−1), during (0), or 1 h after (+1) infection. SNAP always reduces the viral titer when compared with AP (Fig. 4 *B*). However, the maximal effect of SNAP occurs when it is added 1 h after infection (97% inhibition vs. control) (Fig. 4 *B*). The effect of SNAP is diminished if added more than 1 h after infection (data not shown). Since SNAP has a small effect upon CVB3 growth when added to cells before infection, it is possible that SNAP affects cellular targets necessary for viral replication.

SNAP inhibits CVB3 replication as shown by a CVB3 single-step growth curve (Fig. 4 *C*). SNAP was given to HeLa cells 1 h after infection, and viral amount was measured in supernatants every hour for 10 h. NO inhibits viral growth during the first 8 h of infection compared with the control. NO not only reduces the amount of virus produced, but also delays the logarithmic growth phase as well. We next investigated the effect of NO on individual steps of the viral life cycle.

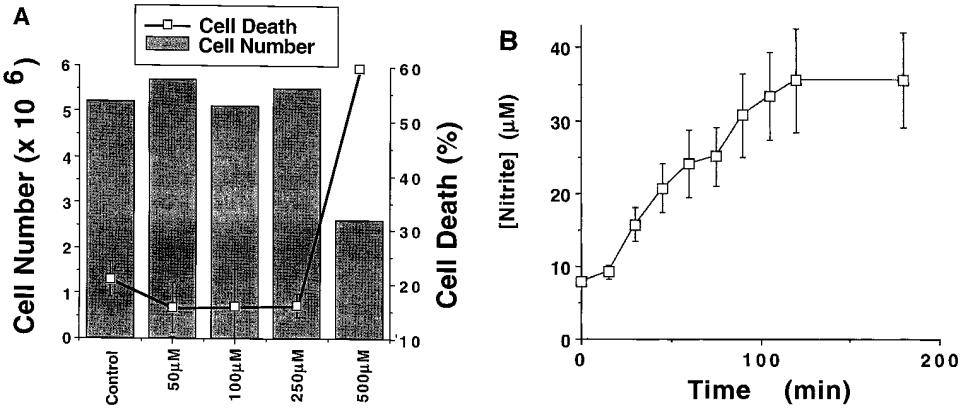

Figure 3. Effect of SNAP on HeLa cell viability. (*A*) HeLa cells were incubated with different concentrations of SNAP for 24 h. The cells were trypsinized and counted in a 0.4% trypan blue solution ($n = 3$; mean±SD). (*B*) NO release by SNAP in HeLa cells. The Griess assay was used to measure [nitrite] in HeLa culture supernatant over time after the addition of 250 μM SNAP ($n = 3$±SD).

SNAP does not affect the viral attachment of the CVB3. To test the effect of NO upon virus attachment to host cells, we incubated HeLa cells with radiolabeled CVB3 particles (MOI = 1, 0.1 cpm/pfu) for 1 h at 4°C, a temperature at which the virus can attach to the cell but not penetrate. We added 250 μM of SNAP or AP 1 h before, during, or 1 h after infection. Cells were then washed, and the radioactive viral particles associated with cells were counted. To check for the binding specificity, a competition assay with nonradioactive virus was used (MOI = 50). Neither SNAP nor AP has an effect upon viral attachment (Fig. 5). Therefore, we studied subsequent steps of the virus life cycle during exposure to NO.

SNAP inhibits CVB3 RNA synthesis. To examine the effect of NO on CVB3 RNA synthesis, we measured [^{3}H]uridine incorporation in infected HeLa cells treated with actinomycin D. When 250 μM SNAP is added 1 h after the infection, synthesis of RNA is completely blocked, and this effect is maintained for 5 h (Fig. 6). Infected HeLa cells treated with SNAP synthesize less RNA than control cells (infected but treated with AP or nothing). SNAP and AP do not affect the amount of total RNA synthesized by noninfected cells (Fig. 6). Thus, NO inhibits the production of viral RNA within infected cells (in which cellular mRNA synthesis has been blocked by both actinomycin D and viral proteases).

To determine more precisely the effect of NO upon viral RNA synthesis, we next performed Northern analysis and RT-PCR. The CVB3 cDNA was used to prepare a probe for a Northern blot of total RNA from infected HeLa cells treated with 250 μM SNAP or AP. CVB3 RNA is detected 4 h after infection in AP-treated cells, and increases 6 h after infection (Fig. 7 *A*). In contrast, CVB3 RNA is absent 4 h after infection of cells treated with SNAP, and present at low levels 6 h after infection.

RT-PCR confirms these results. CVB3 primers were used to prime and amplify total RNA from infected HeLa cells treated or not with SNAP. Viral RNA synthesis is reduced in cells treated with the NO donor SNAP (Fig. 7 *B*).

SNAP inhibits CVB3 protein synthesis. To determine if NO also affects CVB3 protein levels, we analyzed the kinetics of protein synthesis in infected HeLa cells. [^{35}S]Methionine and [^{35}S]cysteine were added to the media of infected HeLa cells at various times after infection (see Methods). Normally, CVB3 infection inhibits cellular protein synthesis between 2 and 5 h after infection, and CVB3 proteins are first produced between

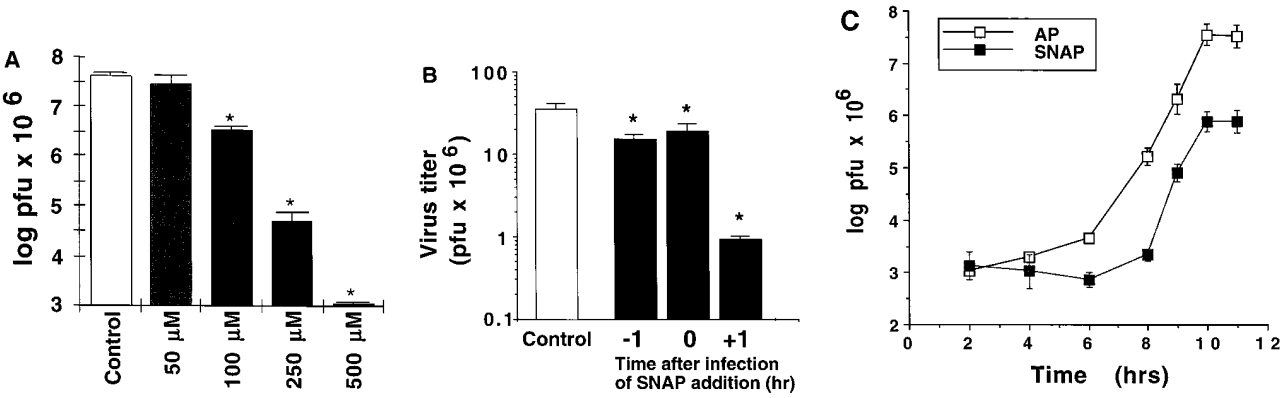

Figure 4. Time- and dose-dependent SNAP inhibition of CVB3 replication. (*A*) HeLa cells were infected with CVB3 at an MOI of 1, and different concentrations of SNAP were added 1 h after infection. Viral content in supernatants was measured by the plaque assay after 10 h of infection ($n = 3$±SD). (*B*) HeLa cells were infected as above, and 250 μM SNAP was added at different times after infection. Viral content in supernatants was measured by the plaque assay after 10 h of infection ($n = 3$±SD). (*C*) 250 μM SNAP was added 1 h after infection as above. Viral content in supernatants was measured by the plaque assay at various times after infection (*$P < 0.05$; $n = 3$±SD).

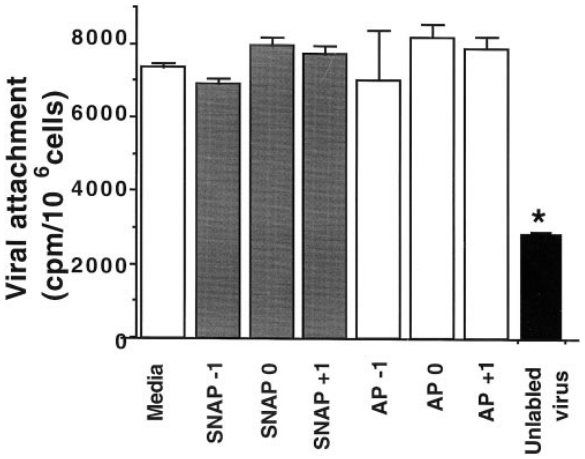

Figure 5. SNAP has no effect on CVB3 attachment to the surface of HeLa cells. HeLa cells were incubated with radiolabeled CVB3 particles at an MOI of 1 for 1 h at 4°C. 250 μM SNAP was added 1 h before (−1), at the same time (0), or 1 h after (+1) the infection. Cells were then washed and counted. In addition, to assay for specificity, HeLa cells were incubated with radiolabeled CVB3 at an MOI of 1 and unlabeled virus at an MOI of 50; ($*P < 0.05$ for cold competition vs. all other samples; $n = 3 \pm$SD).

2 and 5 h after infection (Fig. 8 *A*), as has been described for other picornaviruses such as poliovirus (61). Viral protein synthesis decreases dramatically by 7 h and stops after 10 h. However, SNAP reduces CVB3 protein synthesis 4 and 6 h after infection, compared with untreated cells or cells treated with AP.

Since NO has been reported to inhibit cellular protein synthesis in other systems, we examined the effect of SNAP upon protein synthesis in noninfected HeLa cells. Autoradiography

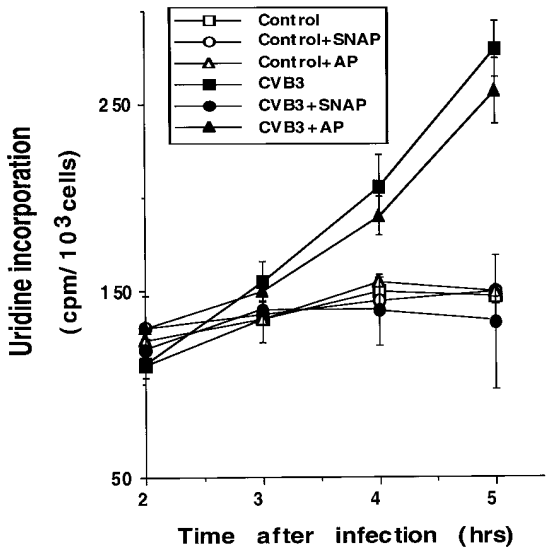

Figure 6. SNAP inhibits total RNA synthesis in HeLa cells infected with CVB3. HeLa cells were treated with actinomycin D, and simultaneously infected (*solid symbols*) or not (*open symbols*) with CVB3 at an MOI of 1. 250 μM SNAP or AP was added 1 h after infection. Every hour after infection, 10 μCi of [^{3}H]uridine was added. At regular time points, total RNA was harvested and counted in a liquid scintillation counter ($n = 5 \pm$SD).

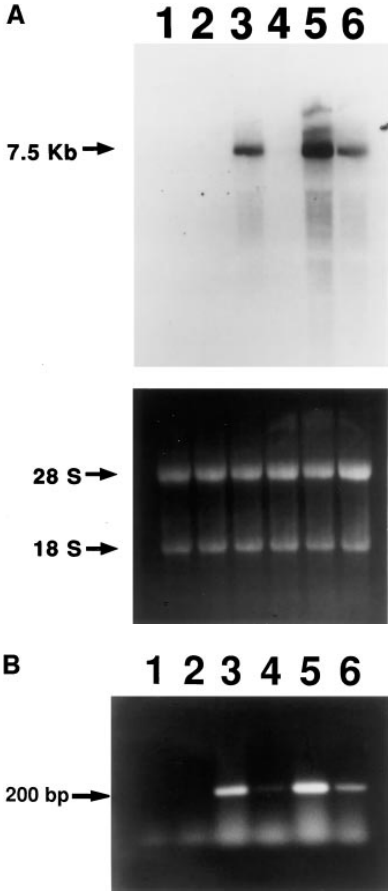

Figure 7. SNAP inhibits CVB3 RNA replication in infected HeLa cells. HeLa cells were infected with CVB3 at an MOI of 1, and 250 μM SNAP or AP was added 1 h after infection. At various times, total RNA was isolated, and assayed by Northern analysis (*A, top*) with ethidium bromide staining of total RNA (*A*, *below*) or (*B*) RT-PCR. Lanes *1*, *3*, and *5* contain RNA from cells treated with AP at 1 h, and then harvested 2, 4, and 6 h after infection, respectively. Lanes *2*, *4*, and *6* contain RNA from cells treated with SNAP at 1 h and harvested 2, 4, and 6 h after infection, respectively. (Data presented are single representative experiments from $n = 3$.)

of [^{35}S]methionine- and [^{35}S]cysteine-labeled proteins from mock-infected HeLa cells shows that SNAP has no effect upon host protein synthesis for 0–6 h, during the period when most viral RNA and viral protein synthesis occurs (Fig. 8 *B*). Although SNAP slightly reduces host protein levels after 7 h, and markedly reduces them after 10 h (Fig. 8 *B*), most viral protein synthesis is completed by 7 h (Fig. 8 *A*). Thus, SNAP has some effect upon host protein synthesis, and this effect could explain part of the antiviral effect of NO.

The metabolic labeling data show that NO reduces levels of protein. These proteins are viral proteins, since Picornaviridae infection inhibits cellular protein synthesis. Immunoblotting with a CVB3-specific antibody demonstrates that NO inhibits specific viral protein synthesis as well (Fig. 8 *C*). We used a rabbit polyclonal anti–CVB3 2C protein antibody to probe total cell lysates from HeLa cells infected with CVB3 at 5, 6, and 10 h after infection to focus on events during viral protein synthesis and after viral replication is completed. SNAP reduces CVB3 2C protein levels (and 2C precursor levels) 5, 6, and 10 h after infection.

Although our data show that NO reduces viral protein synthesis, we have not yet determined whether this reduction is due to a decrease in transcription of viral RNA or to a direct inhibition of translation of viral protein.

Discussion

Viral infection can induce iNOS, which synthesizes NO, an antiviral effector molecule. We previously demonstrated in a mu-

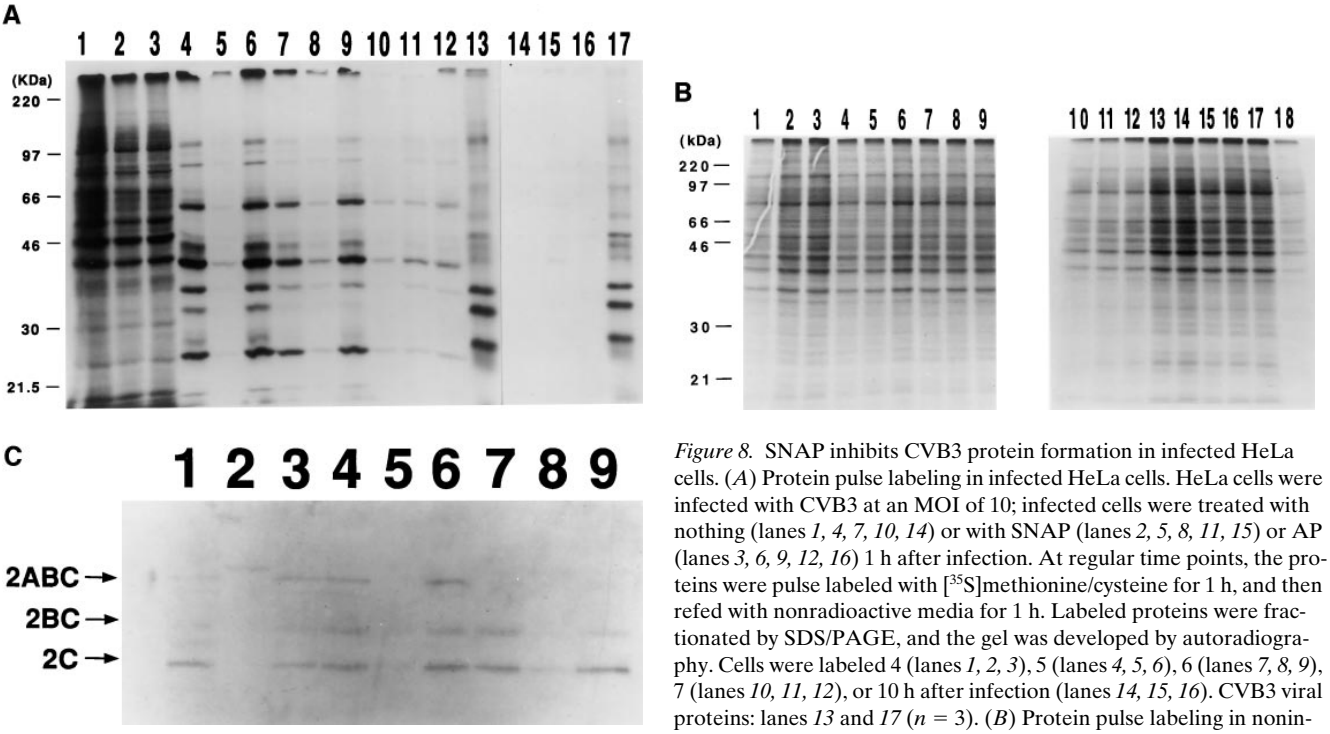

Figure 8. SNAP inhibits CVB3 protein formation in infected HeLa cells. (*A*) Protein pulse labeling in infected HeLa cells. HeLa cells were infected with CVB3 at an MOI of 10; infected cells were treated with nothing (lanes *1, 4, 7, 10, 14*) or with SNAP (lanes *2, 5, 8, 11, 15*) or AP (lanes *3, 6, 9, 12, 16*) 1 h after infection. At regular time points, the proteins were pulse labeled with [^{35}S]methionine/cysteine for 1 h, and then refed with nonradioactive media for 1 h. Labeled proteins were fractionated by SDS/PAGE, and the gel was developed by autoradiography. Cells were labeled 4 (lanes *1, 2, 3*), 5 (lanes *4, 5, 6*), 6 (lanes *7, 8, 9*), 7 (lanes *10, 11, 12*), or 10 h after infection (lanes *14, 15, 16*). CVB3 viral proteins: lanes *13* and *17* ($n = 3$). (*B*) Protein pulse labeling in noninfected HeLa cells. HeLa cells were treated with nothing (lanes *1, 4, 7, 10, 13, 16*), AP (lanes *2, 5, 8, 11, 14, 17*) or SNAP (lanes *3, 6, 9, 12, 15, 18*) 1 h after infection. At regular time points, the proteins were pulse labeled with [^{35}S]methionine/cysteine for 1 h, and then refed with nonradioactive media for 1 h. Labeled proteins were fractionated by SDS/PAGE, and the gel was developed by autoradiography. Cells were labeled at 0 (lanes *1, 2, 3*), 4 (lanes *4, 5, 6*), 5 (lanes *7, 8, 9*), 6 (lanes *10, 11, 12*), 7 (lanes *13, 14, 15*), or 10 h after mock infection (lanes *16, 17, 18*) ($n = 1$). (*C*) Western blot analysis of CVB3-2C protein. HeLa cells were infected with CVB3 at an MOI of 1, and nothing (lanes *1, 4, 7*), SNAP (lanes *2, 5, 8*), or AP (lanes *3, 6, 9*) was added 1 h after infection. Cells were lysed 5 (lanes *1, 2, 3*), 6 (lanes *4, 5, 6*), or 10 h after infection (lanes *7, 8, 9*). Proteins were electrophoresed, transferred, and hybridized with a specific anti–CVB3 2C antibody. (Data presented are single experiments from duplicate immunoblots.)

rine myocarditis model that CVB3 infection induces iNOS, and that NO has an antiviral effect (53). In this paper, we report the first direct evidence of an inhibitory effect by NO upon the CVB3 life cycle at the step of RNA synthesis.

NO inhibits CVB3 replication 100-fold, whether the source of NO is SNAP or iNOS expressed from transfected iNOS cDNA (Figs. 2 and 4 *C*). Although this reduction in viral replication is seen with NO itself (Fig. 2), it is possible that the antiviral effects of SNAP are due to some other NO-related species or SNAP itself. While the iNOS enzyme produces authentic nitric oxide, SNAP is a nitrosothiol that can produce nitric oxide or nitrosonium, and SNAP may also have direct effects mediated by a SNAP receptor (62–65). For the purposes of this discussion, we will refer to SNAP and its NO-related products as NO. After inhibition, CVB3 replication increases somewhat within 6 h after infection if exposed to SNAP, but CVB3 replication increases much less if exposed to NO synthesized from transfected iNOS; presumably because SNAP releases NO within 2 h (Fig. 3 *B*), whereas iNOS synthesizes NO continually (Fig. 1 *B*). Since CVB3 replication recovers slightly after transient exposure to NO, perhaps NO reversibly inhibits viral growth.

Two lines of evidence indicate that NO does not affect viral attachment to the host cell. First, SNAP maximally protects HeLa cells from CVB3 infection if added 1 h after infection (Fig. 4 *B*). Viral attachment occurs in the first hour of the viral life cycle (data not shown). Second, binding assays confirm that SNAP does not affect viral attachment to the host cell (Fig. 5). However, NO appears to act upon other viruses such as Vesicular Stomatitis virus early in their life cycle (66), so perhaps NO can affect the interaction between virus and receptor in other systems.

Our data demonstrate that SNAP reduces CVB3 RNA synthesis. This effect is not only shown directly by reduction of CVB3 RNA in Northern analysis, but also indirectly by the timing of the maximum effect of SNAP early in the CVB3 life cycle when RNA synthesis occurs (Figs. 4 *B*, 6, and 7). RNA synthesis is a critical step in Coxsackievirus replication, since the viral genome is a single strand of RNA with positive polarity that is copied via a negative-strand RNA intermediate. Any of the CVB3 proteins necessary for this RNA-dependent RNA polymerization are potential targets of NO. NO could act upon CVB3 replication by: (*a*) direct interaction with the CVB3 RNA-dependent RNA polymerase 3D^{pol} (67); (*b*) inhibition of the CVB3 proteases 2A^{pro} and 3C^{pro}, which cleave the CVB3 polyprotein, releasing the RNA polymerase 3D^{pol} (68–70); (*c*) inhibition of initiator activity of the viral protein VPg required by 3D^{pol} for polymerization (71–73); or (*d*) inhibition of the CVB3 nonstructural proteins 2B or 2C.

SNAP also inhibits CVB3 protein synthesis (Fig. 8). This effect might be due to NO inhibition of viral RNA synthesis. However, NO might act upon certain CVB3 targets necessary

for viral protein synthesis and processing. One potential viral target of NO is the CVB3 protease $2A^{pro}$, which shuts off cellular mRNA translation by cleaving the host p220 initiation factor (also known as eIF4G) (74–76). However, our data suggest but do not prove that NO does not directly inhibit CVB3 protein synthesis. NO has the biggest impact on viral replication when it is added relatively early in the viral life cycle, when CVB3 RNA synthesis, not protein synthesis, occurs (Fig. 4 *B*).

NO might also inhibit viral replication by acting upon host cell processes: our data show that adding SNAP 1 h before infection has a small effect upon viral production, and our data also show that SNAP inhibits host cell protein levels after 7–10 h. NO could inhibit cellular enzymes necessary for viral RNA synthesis (77) such as eIF-4G (78). NO might also inhibit the cellular apparatus for protein synthesis; NO has been shown to reduce over-all protein synthesis in hepatocytes (55). NO could reduce host protein synthesis, either by directly acting upon host translational enzymes or by reducing the levels of high energy phosphate compounds. For example, NO inhibits enzymes implicated in diverse metabolic processes, like glyceraldehyde-3-phosphate dehydrogenase (79), *cis*-aconitase (80), and NADPH-ubiquinone reductase (81), reducing the production of ATP.

In conclusion, we have demonstrated that NO inhibits CVB3 replication in HeLa cells by inhibiting synthesis of the viral RNA genome. However, the precise identity of the host and viral targets of NO are unknown.

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