Inhibition of Vesicular Stomatitis Virus Infection by Nitric Oxide

ZHENGBIAO BI[1] AND CAROL S. REISS[1,2,3]*

Department of Biology,[1] Center for Neural Science,[2] and Kaplan Comprehensive Cancer Center,[3]
New York University, New York, New York 10003-5181

Inhibitory effects of nitric oxide (NO) on vesicular stomatitis virus (VSV) infection were investigated by using a VSV-susceptible mouse neuroblastoma cell line, NB41A3. Productive VSV infection of NB41A3 cells was significantly inhibited by an organic NO donor, S-nitro-N-acetylpenicillamine (SNAP), while the control compound N-acetylpenicillamine (NAP) had no effect. Survival rate of VSV-infected cells was greatly increased by the treatment with SNAP, while the NAP treatment did not have any effect. Adding SNAP 30 min prior to infection resulted in complete inhibition of viral production when a low multiplicity of infection (MOI) was used. Substantial inhibition of viral production was also obtained with treating cells 6 h earlier before infection with a higher MOI. Activating the neuronal NO synthase by treating cells with N-methyl-D-aspartate (NMDA) led to significant inhibition of viral production by cells infected at the three doses of virus tested (MOIs of 0.1, 1, and 5). The inhibitory effect of NMDA on viral infection was totally blocked by the NO synthase inhibitor N-methyl-L-arginine. However, adding hemoglobin, a strong NO-binding protein and thus an inactivator of NO activity, did not reverse the NMDA-induced inhibition of viral production, suggesting that NO might exert its antiviral effects inside the NO-producing cells. Collectively, these data support the anti-VSV effects of NO, which might be one of the important factors of natural immunity in controlling the initial stages of VSV infection in the central nervous system.

Both innate and acquired immunity are believed to be important in controlling viral infections. Innate immunity, which is rapidly induced or activated by viral infections, is expected to play a predominant role in controlling the initial events of viral infections. Acquired immunity, on the other hand, takes days, not hours, for its activation by viral infections and is likely to be more important at relatively later stages of infections and viral clearance. Although the interaction between the central nervous system (CNS) and peripheral immune systems might exist (6), the CNS is isolated from the peripheral blood by the blood-brain barrier, which when its integrity is not yet altered by viral infections, efficiently prevents perfusion of blood-borne factors (25), and the local innate immunity of the CNS may be especially critical in controlling the initial neurotropic viral infections.

Vesicular stomatitis virus (VSV), a member of the *Rhabdoviridae* family, is an enveloped, single-stranded, negative-sense RNA virus (46). Intranasal instillation of VSV into mice leads to initial infection of olfactory receptor neurons (38) and then the olfactory bulb, followed by an acute infection of the CNS (9, 15, 16). When mortality occurs, infected mice die between days 7 and 10 postinfection. All surviving mice completely clear the virus from the CNS by 12 days postinfection. No obvious damage to the CNS could be observed in these surviving mice. The host defense mechanisms which control the neurotropic viral infection in the CNS and mediate viral clearance are not fully understood.

As a neurotransmitter having many important physiological functions in the CNS (35, 36, 40), nitric oxide (NO) has also been demonstrated to have inhibitory effects on viral infections. Recent research showed that NO has an inhibitory effect on ectromelia, vaccinia, and herpes simplex type 1 viruses (21) and may mediate Lentinan-induced protection against influenza virus (17).

NO is synthesized from L-arginine by a group of enzymes called NO synthases (28, 35, 36). The olfactory bulb is one of the few areas in the adult CNS where neuronal NO synthase is readily expressed (2). The expression of constitutive neuronal NO synthase in olfactory receptor neurons declines after birth, but it is readily and rapidly induced after injury (39). Inducible NO synthase, which produces NO in large quantities, can also be induced in activated astrocytes and microglia cells in the CNS (5, 11). It is notable that the areas where NO synthases are highly expressed or rapidly induced are the places where VSV infection initially takes place. Considering the antiviral effects of NO, it is intriguing to hypothesize that NO might be especially crucial in controlling the initial infection before specific immune responses develop and infiltrate the infected CNS. Therefore, it is important to determine directly the effect(s) of NO on VSV infection, especially in neurons. A VSV-susceptible mouse neuroblastoma cell line, NB41A3, was used in our studies to investigate the effects of NO on VSV infection.

MATERIALS AND METHODS

Virus. VSV Indiana strain, San Juan serotype, was propagated in Chinese hamster ovary cells and purified over a sucrose gradient as previously described (14).

Mouse neuroblastoma cell line NB41A3. VSV-susceptible neuroblastoma NB41A3 cells were purchased from American Type Culture Collection and cultured in Ham's F-10 medium plus 15% horse serum and 2.5% noninactivated fetal bovine serum (Sigma). Cells were subcultured two to three times per week by adding fresh trypsin (0.25%) solution.

Reagents. S-Nitroso-N-acetylpenicillamine (SNAP), N-methyl-D-aspartic acid (NMDA), and hemoglobin were purchased from Calbiochem-Novabiochem, Inc. All other chemicals, including N-acetylpenicillamine (NAP) and N-methyl-L-arginine (L-NMA), were purchased from Sigma. Stock solutions were prepared as 5 mM SNAP, 5 mM NAP, 50 mM NMDA, 3 mM L-NMA, and 5 mM hemoglobin.

Plaque assay of infectious viral titer. Infectious virus was quantitated on NB41A3 monolayers. Monolayers of NB41A3 cells were prepared by inoculating

* Corresponding author. Mailing address: Biology Department, Main Building Room 1009, New York University, 100 Washington Square East, New York, NY 10003-5181. Phone: (212) 998-8269. Fax: (212) 995-4015. Electronic mail address: ReissC@ACFcluster.nyu.edu.
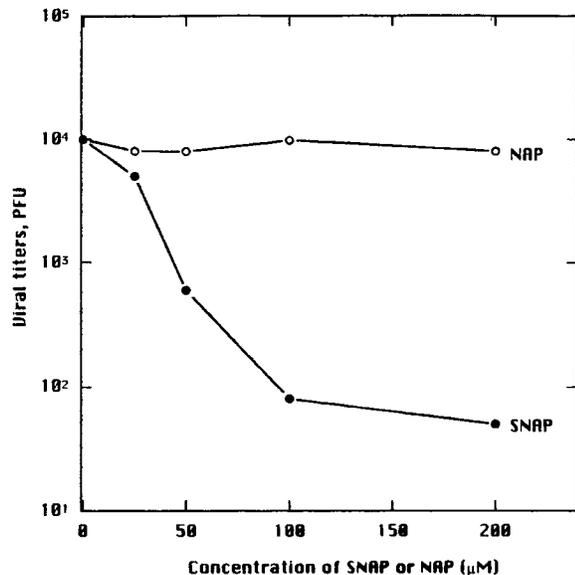
FIG. 1. Inhibition of VSV infection by SNAP. NB41A3 cells (5×10^4) were infected with VSV at an MOI of 0.1 and cotreated with SNAP (●) or NAP (○). Medium-treated infected cells are shown at 0 μM. SNAP and NAP were replenished once, 4 h later. Eight hours postinfection, infectious viral titers in the supernatants of treated infected cells were determined. Results shown are the averages of three replicates. The experimental results are similar to those of three repetitions of these conditions.
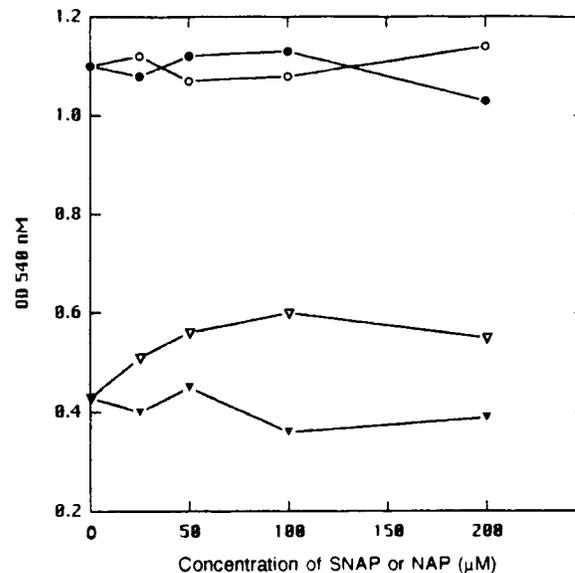

FIG. 2. Increased survival rate of infected cells by the treatment of SNAP. NB41A3 cells (5×10^4) were either uninfected or infected with VSV at an MOI of 5 and cotreated with SNAP or NAP. Cells treated with medium are shown at 0 μM. SNAP and NAP were added every 4 h. Sixteen hours postinfection, cellular viability was determined with the MTT assay. No cytotoxicity was observed when uninfected cells were treated with either SNAP or NAP. Symbols: open circles, uninfected, treated with NAP; closed circles, uninfected, treated with SNAP; open triangles, infected, treated with SNAP; closed triangles, infected, treated with NAP.

20×10^4 cells in 1 ml per well (24-well plate; Nunc) and incubated for 2 days at 37°C. The medium was removed, 0.1 ml of each dilution of samples (10-fold serial dilutions) was added to each well, and the wells were then incubated for 30 min at 37°C. The medium was removed, 1 ml of the mixture of equal volumes of 1.8% agar (kept at 45°C) and 2× F-10 medium (kept at 37°C) was added to each well, and the wells were then incubated at 37°C for 24 h. Plaques were fixed with 10% formaldehyde for 30 min and stained with 0.5% methylene blue.

Determination of NO concentration. The concentration of NO was determined by assaying its stable end product, NO_2^- (3). Briefly, equal volumes of samples and Greiss reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine, 5% H_3PO_4) (Sigma) were incubated at room temperature for 10 min. The reaction produces a pink color, which was quantified at 540 nm against standards in the same buffer on a automated microplate reader (model EL 309; Bio-Tek, Inc.).

MTT assay for cytotoxicity. The assay employs the pale yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT; Sigma), which is cleaved by active mitochondria to form a dark blue formazan product that can be completely solubilized in acidic isopropanol. MTT was dissolved in 0.1 M Tris-buffered saline to make a 5-mg/ml solution, which was filtered and removed any insoluble residues. Ten microliters of MTT solution was added to each well containing 100 μl of cells (96-well plate) and incubated 4 h in a 37°C, 5% CO_2 humidified incubator. One hundred microliters of 0.04 N HCl in isopropanol was added to each well and mixed vigorously to dissolve the dark blue crystals. The plate was then read at 540 nm with the automated plate reader.

RESULTS

Inhibition of VSV infection by the organic NO donor SNAP. To investigate the effects of NO on VSV infection of NB41A3 cells, NO was directly provided to infected cells at the time of infection by the addition of the organic NO donor SNAP. The same concentrations of the control compound NAP were added in the control group. Significant inhibition of infectious VSV production was obtained upon treatment with SNAP (Fig. 1). Eight hours postinfection, untreated infected cells produced about 10^4 PFU of VSV per ml. Similar viral yield was produced by NAP-treated infected cells. However, more than a 100-fold reduction of infectious viral titer was reproducibly observed upon treatment with 100 μM SNAP.

Since NO has been shown to be cytotoxic at high concentrations (4, 7, 31, 32, 35), the antiviral effect of SNAP might

result from toxicity to cells. The MTT assay, which is a measure of mitochondrial functions, an index of living cells, was used to assess cellular viability of SNAP- or NAP-treated uninfected and infected NB41A3 cells. Although there was a slight decrease (about 10%) of survival rate of uninfected NB41A3 cells treated with the highest dose of SNAP tested, 200 μM, compared with cells treated with medium or NAP, no cytotoxicity was induced by 100 μM or lower concentrations of SNAP (Fig. 2). In fact, infected cells were saved from dying by the SNAP treatment. The survival rate of VSV-infected NB41A3 cells was increased about 50% by 100 μM SNAP. Treatment with NAP had no effect on the survival rate of both uninfected and infected cells at any concentration. The antiviral effect of SNAP is not likely due to the result of cytotoxicity.

Kinetics of VSV production by the SNAP-treated infected cells. The inhibitory effect of SNAP on VSV infection of NB41A3 cells was further investigated by determining the kinetics of VSV production by the SNAP-treated infected cells. A significant inhibition of viral release was obtained with 100 μM SNAP for multiplicities of infection (MOIs) of both 0.1 and 1 (Fig. 3). The same concentration of NAP had no effect on the viral production. A lower level of inhibition was observed when NB41A3 cells were infected with VSV at an MOI of 5. It is possible that high viral load might kill infected cells rapidly and impair the cellular ability to convert SNAP to NO and thus is more resistant to the treatment with SNAP. This possibility, however, was ruled out, in that similar concentrations of NO_2^- were produced by both uninfected and infected cells (MOIs of 0.1, 1, and 5) treated with SNAP (data not shown). Thus, the cellular ability to produce NO from SNAP was not impaired even if in the presence of high viral multiplicity.

Treating infected cells with 100 μM SNAP 6 or 3 h prior to infection resulted in greater inhibition than adding SNAP at
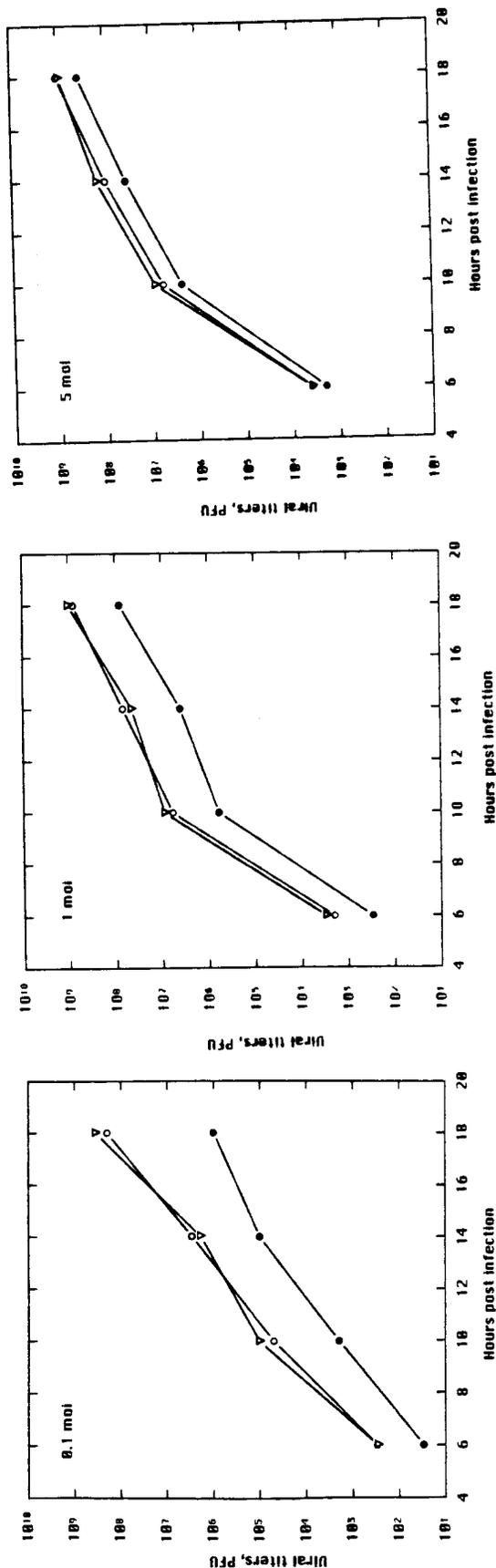
FIG. 3. Kinetics of VSV production by infected cells treated by SNAP. NB41A3 cells (5×10^4) were infected with VSV at an MOI of 0.1, 1, or 5 and cotreated with either medium ($\triangledown$), 100 μM SNAP ($\bullet$), or 100 μM NAP ($\bigcirc$). SNAP and NAP were replenished every 4 h. At 6, 10, 14, and 18 h postinfection, infectious viral titers in the supernatants of treated cells were determined. The averages of three replicates are shown.

the time of infection (Fig. 4). Viral production by cells infected with VSV at an MOI of 0.1 was completely inhibited by adding SNAP as late as 30 min before infection. When the stringent test of use of an MOI of 5 was performed, higher level of inhibition of viral production was also obtained when infected cells were pretreated with 100 μM SNAP 6 h before infection. Considering that the half-life of NO is only several seconds and NO is highly active and forms many products with other molecules inside living cells (13), this result suggests that NO inhibits VSV infection indirectly rather than inhibiting VSV infection directly by itself.

Inhibition of VSV infection by NMDA-induced activation of neuronal NO synthase. NMDA has been used as an activator of neuronal NO synthase (3, 7). Significant inhibition of VSV production by cells infected by all the three doses of virus tested (MOIs of 0.1, 1, and 5) was obtained by treating NB41A3 cells for 2 min with 500 μM NMDA (Fig. 5). Adding 300 μM L-NMA, a competitive inhibitor of NO synthase, completely reversed the inhibitory effect of NMDA on VSV infection, suggesting that the inhibitory effect of NMDA was mediated by activation of neuronal NO synthase. Treatment of NB41A3 cells with 500 μM NMDA for 2 min did not result cytotoxicity (data not shown). Adding 300 μM L-NMA alone to the infected cells before infection did not have any effect on VSV production. Unexpectedly, adding 500 μM hemoglobin, a strong NO-binding protein and thus an inactivator of NO activity (32), did not prevent NMDA-induced inhibition of VSV production. The same concentration of hemoglobin added alone to infected cells also had no effect on viral production. Since L-NMA can efficiently diffuse into living cells whereas hemoglobin cannot, it is reasonable to infer that NO exerts its inhibitory effect on VSV infection inside NO-generating cells rather than by an autocrine or paracrine means.

DISCUSSION

The effect of NO on VSV infection was investigated in this study. Productive VSV infection of NB41A3 cells was greatly inhibited by 100 μM SNAP (Fig. 1). The survival rate of VSV-infected cells was also significantly increased by the treatment with 100 μM SNAP (Fig. 2). Greater inhibition was obtained by pretreating cells with SNAP before infection (Fig. 4). Activating the neuronal NO synthase by treating cells with 500 μM NMDA led to significant inhibition of viral production by infected cells (Fig. 5). The data strongly support the anti-VSV effect of NO.

Although one of the smallest and simplest biosynthetic molecules, NO has now been recognized as a very important factor in mammalian cells (35, 36, 40). First identified as an endothelium-derived blood vessel-relaxing factor (10), NO has since been demonstrated to be involved in a wide variety of functions especially in the CNS, including serving as a neurotransmitter (42), influencing synaptic plasticity and expression of long-term potentiation (1a), facilitating NMDA receptor-mediated neurotransmitter release (33), mediating the formation of synaptic connections in developing and regenerating olfactory receptor neurons (39), and formation of smell (12).

The importance of NO in immune responses is beginning to be recognized. It has been demonstrated that NO is a key component in the host defense against tumor cells, protozoan parasites, fungi, bacteria, and viruses (21, 24, 41, 45). NO induces a marked increase in tumor necrosis factor alpha and interleukin-1β mRNAs through a transcription control (27). Moreover, NO rapidly activates lymphocytes by enhancing the NF-κB activity and *src* family protein tyrosine kinase p56[lck] (23).
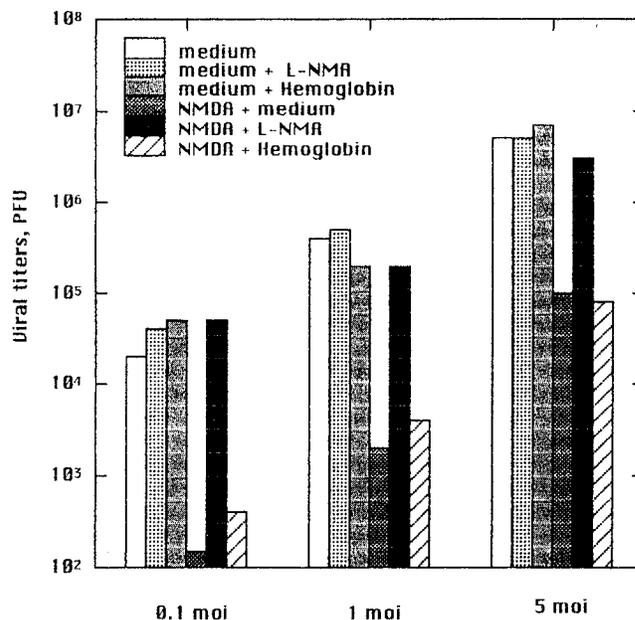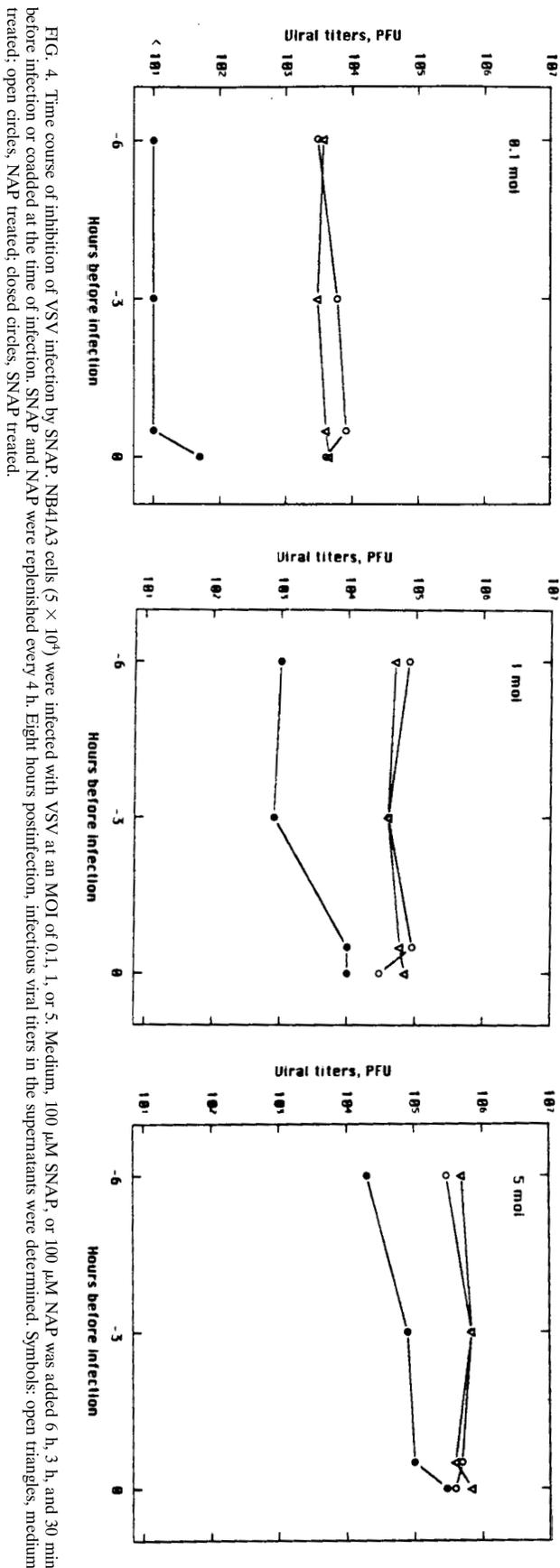
FIG. 4. Time course of inhibition of VSV infection by SNAP. NB41A3 cells (5×10^4) were infected with VSV at an MOI of 0.1, 1, or 5. Medium, 100 μM SNAP, or 100 μM NAP was added 6 h, 3 h, and 30 min before infection or coadded at the time of infection. SNAP and NAP were replenished every 4 h. Eight hours postinfection, infectious viral titers in the supernatants were determined. Symbols: open triangles, medium treated; open circles, NAP treated; closed circles, SNAP treated.

FIG. 5. Reversion of NMDA-induced inhibition of VSV infection by L-NMA. NB41A3 cells (5×10^4) were treated with either medium or 500 μM NMDA for 2 min. Cells were extensively washed and cultured for 30 min before being infected with VSV at an MOI of 0.1, 1, or 5. L-NMA (300 μM) or hemoglobin (500 μM) was added in either medium-treated or NMDA-treated infected cells. Eight hours postinfection, infectious viral titers in the supernatants were determined on NB41A3 monolayers.

Many cellular targets of NO have been identified in the living cells. Heme-, transition metal-, or thiol-containing proteins serve as the major targets of NO (35, 43, 44) which, by binding to these proteins, can inhibit their activities. These include such essential enzymes as complexes I and II of the mitochondrial respiratory chain, aconitase, and ribonucleotide reductase (13). In addition, NO activates cGMP synthetase (3) and poly(ADP-ribose) synthetase (47). The iron concentration can also be controlled by NO, which modulates both ferritin mRNA translation and transferrin receptor mRNA stability (8). Many other functions are mediated by more complex and more active forms of NO formed by reacting with O_2^- or H_2O_2 (43, 44). Studies reported in this article clearly showed that NO has an inhibitory effect on VSV infection, but the mechanisms involved are far from clear. Some of the functions of NO mentioned above or other, not yet identified ones may mediate its inhibition.

As it has been ruled out in other viral infections (21), interference of NO itself with viral infectivity seems less likely. The greater inhibition observed by treating cells with SNAP several hours before infection suggests that NO might work indirectly by inducing and/or activating other antiviral factors inside cells. Furthermore, contrary to the general belief that NO diffuses out of the generating cells and mediates its activities through acting on neighboring cells, the lack of reversion by hemoglobin of NMDA-induced inhibition of VSV infection suggests that NO mediates its antiviral effects inside cells. Antiviral actions of NO inside NO-generating neurons might be advantageous for the host, considering the fact that NO-generating neurons are selectively resistant to toxicity of NO, but the neighboring neurons are sensitive to the NO-induced neurotoxicity (7).

In addition to its potential protective role of controlling initial infections, NO might also be important in viral clearance

from the CNS. Viral clearance in the periphery is primarily mediated by virus-specific T cells. However, the mechanisms of viral clearance from the CNS, especially from the infected neurons, are not thoroughly understood but are likely to differ from those employed in the periphery, because T cells can recognize only viral antigens presented by self major histocompatibility complex (MHC) molecules and neurons lack the constitutive expression of MHC molecules (18). Furthermore, neurons do not have an efficient system of loading viral antigens to MHC class I molecules (19). One prominent advantage of NO as an antiviral agent is its action independent of immune recognition of the infected cells. As most neurons in the CNS are terminally differentiated and cannot regenerate after death, clearance of virus from neurons by a noncytopathic mechanism is also advantageous.

Research with interferon regulator factor 1 (IRF-1) knockout mice provides provocative results on potential effect(s) of NO on VSV infection. IRF-1, a transcriptional factor, is involved in the antiviral responses of interferons (IFNs) (22). While the expression of most of IFN-inducible genes (2',5'-oligoadenylate synthetase, double-stranded RNA-dependent protein kinases, 1-8, 9-27, and MHC class I H-$2K^b$ genes) is not significantly impaired by IRF-1 inactivation (22, 30), the expression of inducible NO synthase is severely inhibited in IRF-1 knockout mice (20, 29). VSV infection of IRF-1 knockout mice results in earlier death than in wild-type mice (30), suggesting an anti-VSV effect of NO in vivo. However, other unidentified impairments resulted from the deficiency of IRF-1 might complicate the interpretation. In a recent report, Kimura et al. (22) suggested the nonessential role of IRF-1 and inducible NO synthase in antiviral responses of IFNs to VSV. Although the expression of the inducible NO synthase is abrogated by inactivation of IRF-1, the expression of inducible NO synthase in the wild-type cells that they used in response to IFNs is very weak, if expressed at all. Their experimental condition (treating embryonic fibroblast cells of wild-type and IRF-1 knockout mice with IFNs 18 h before VSV infection) might not be physiological, since in vivo IFNs are produced only after viral infections. The finding that VSV infection was not altered in IFN-γ receptor knockout mice (34) conflicts with their assumption of the importance of IFN-γ in anti-VSV infection. Overexpression of the IRF-1 cDNA induces an antiviral state to VSV (22), further suggesting the importance of IRF-1 in anti-VSV responses.

Clearance of Sindbis virus from neurons by antibodies through a noncytopathic mechanism has been demonstrated (26), but anti-VSV antibodies are less likely to be important in the control of CNS infection by VSV because anti-VSV immunoglobulin G appears in the blood only after 8 days postinfection (30) and infiltration of B cells into the CNS was not detected until day 14 postinfection (1). The fact that mice devoid of functional type I IFN (type I IFN receptor knockout) are extremely sensitive to intravenous VSV infection but type II IFN receptor knockout mice are as resistant as wild-type mice to intravenous VSV infection suggests the protective role of type I IFN but not type II IFN in VSV infection of the periphery (34). However, the roles of IFNs in controlling VSV infection in the CNS are await investigation.

Although the results of this study clearly support the anti-VSV effect of NO, the mechanisms involved are not understood. Further studies in vitro may help to dissect the molecular mechanisms involved in the antiviral effects of NO.

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