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REPLICATION OF RESPIRATORY SYNCYTIAL VIRUS IS INHIBITED IN TARGET CELLS GENERATING NITRIC OXIDE *IN SITU*

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1. ABSTRACT

Nitric oxide (NO) is generated by recruited inflammatory cells and by pulmonary epithelial cells in response to respiratory virus infection, although the relative antiviral efficacy of NO from each of these sources had not been clarified. To compare the direct, antiviral potency of NO from an exogenous source with that generated by target epithelial cells *in situ*, we transduced HEp-2 epithelial cells with the retroviral construct, pMFGS-NOS and cloned transductant lines that generated NO constitutively. We found that NO-producing HEp-2 cells could be infected with RSV, but the titer correlated inversely with NO production, an effect that was reversed by the NOS inhibitor, N^G-methyl-L-arginine (N^GMMA). Our results demonstrate that NO has significant direct antiviral activity against RSV, and interestingly, that the inhibitory effect is more potent in the presence of continuous, endogenous NO production than in response to NO from an exogenous source.

2. INTRODUCTION

Nitric oxide (NO) is a free radical that mediates a broad range of physiologic processes and may act as a vasodilator, neurotransmitter, immune regulator, antimicrobial and antiviral agent (1-10). NO is generated catalytically by one of three isoforms of NO synthase (NOS) from its substrate, L-arginine, and NO generated by iNOS, the inducible form of NOS, is a significant host defense response to respiratory virus pathogens (3, 11-21). Respiratory syncytial virus infection is associated with the generation of NO in human tissues (17, 21), as well as with numerous other complex innate host responses (2, 11, 17, 22-27); as such, it is difficult to separate the specific biologic effects of NO on viral pathogens from the effects mediated by other pro-inflammatory stimuli. Of the potential sources of NO, we were particularly interested in the role and relative antiviral efficacy of NO produced *in situ* by pulmonary epithelial cells. To this end, we generated a series of HEp-2 epithelial cell clones that express iNOS constitutively and generate varying amounts of NO. Using these clones, we evaluated the antiviral activity of NO generated *in situ* and compared these results to those obtained from parent cells cultured with an exogenous source of NO, specifically with the chemical NO donor, S-nitroso-N-aceytylpenicillamine (SNAP).

3. MATERIALS AND METHODS

3.1. Cell Culture

HEp-2 cells (American Type Culture Collection, Manassas, VA), and all iNOS-expressing HEp-2 transductants were grown in RPMI-1640 (GIBCO, Grand Island, NY), supplemented with 10% FBS (Hyclone, Logan UT), 2mM glutamine (GIBCO) and penicillin/streptomycin (GIBCO) (referred to as cRPMI). Where indicated, the NOS inhibitor, N^G-methyl-L-arginine (N^GMMA; Sigma, St

Louis, MO) was added at a concentration of 100 µM. For some experiments, medium containing 0-400 µM of the NO donor, S-nitroso-N-acetylpenicillamine (SNAP) or its control compound N-acetylpenicillamine (NAP) (Sigma) was included.

3.2. Preparation of RSV stock suspensions

RSV (ATCC VR-1401) was obtained from American Type Culture Collection (ATCC, Manassas, VA) and used to inoculate 180 cm^2 flasks containing semi-confluent monolayers of HEp-2 cells. When cytopathic effect reached ~80% (at 72 to 96 hrs), the culture supernatants were harvested and cellular debris removed by centrifugation at 500g. Aliquots of the virus suspensions were flash frozen and stored at –80°C. Infectivity of virus stocks, determined by plaque assay on HEp-2 cells, ranged from 10^5 to 1.5 $x10^6$ plaque forming units (pfu)/ml. For subsequent experiments, RSV was used to inoculate HEp-2 and HEp-2 transductant monolayers at a multiplicity of infection approximating 0.1.

3.3. Generation of HEp-2 clones that express iNOS and generate nitric oxide

The psi-crip-NOS34 cell line was grown in 75 cm^2 flasks containing IMDM supplemented with 10% FBS, 2 mM glutamine, and penicillin/streptomycin (referred to as cIMDM) as previously described (28, 29). In brief, this high titer producing packaging cell line was generated by transfection with the retroviral vector pMFGSNOS which contains the entire open reading frame of iNOS ligated to the Nco I cloning site of pMFGS (28). When psi-crip-NOS34 cells reached confluency, the medium was replaced with 5 ml of fresh c-IMDM and collected every 24 hours for three consecutive days. To optimize transduction efficiency, HEp-2 monolayers growing in 75 cm^2 flasks, were transduced with 5 ml freshly harvested retroviral supernatent in the presence of 6 µg/ml protamine (Sigma) for three consecutive days, replacing the transducing medium every 16 hours. Twenty-four hours after the third transduction, limiting dilutions were performed for single cell cloning. Clonal transductants were then expanded and further characterized. Expanded clones were screened for constituitive nitric oxide production by measuring nitrite accumulation in each of their culture media as described below. Several of the propagated clones (Clones A, B, C, D, E) were selected for additional experiments based on the varying amounts of NO generated. Sham transductants were generated in an identical fashion using conditioned supernatant harvested from the parent Ψ-crip cell line. The growth characteristics for several sham transductant clones (X, Y and Z) were similar (not shown). For all subsequent experiments, cells were seeded at 3 x 10^5cells/ml. Cell counts and viability were determined using a hemocytometer and trypan blue exclusion. Clone doubling time was determined by sequential cell counts performed between 24 and 72 hours after seeding. Aliquots of culture supernatants for each RSV-infected clone were retrieved at regular intervals, flash frozen in ethanol/dry ice and stored for viral titration at a later date.

3.4. Northern Analysis

Total RNA was extracted from clonal transductants using RNAzol (Tel-Test, Friendswood, TX). RNA samples of 20 µg each were loaded on formaldehyde-agarose gels, electrophoresed, transferred to a nylon membrane, and UV crosslinked. After prehybridization at 37°C, the membranes were hybridized at 37° C in standard hybridization solution with a ^{32}P-radiolabeled iNOS cDNA probe (28), then washed in 0.2X SSPE/0.1% SDS twice at 55°C and exposed to X-ray film. The same membrane was stripped with boiling water/1% SDS and hybridized with ^{32}P-radiolabeled β-actin as a control for RNA loading.

3.5. Nitrite Assay

The accumulation of nitrite, an oxidation product of NO, in the culture medium of the cells was measured colorimetrically after mixing 100 µL each of culture media and freshly prepared Griess reagent (0.5% naphthylethylenediamine in water and 1% sulfanilamide in 5% phosphoric acid mixed 1:1) (30). Absorbances at 550 nm were recorded on a microplate reader against standard solution of sodium nitrite prepared in media.

3.6. Plaque Assays

RSV plaque assays were performed on HEp-2 monolayers as previously described (31). After 6 days of incubation, plaque assay monolayers were fixed in 10% formaldehyde over 6 hours, stained with 0.03% methylene blue, and counted. All experiments were performed in triplicate.

3.7. Statistical Analysis

Data points represent the average +/- standard error of the mean (SEM) of samples from 3 or more trials. Unpaired t tests were used to compare continuous data as per the algorithms of the Microsoft Excel data analysis program.

4. RESULTS

4.1. Characterization and iNOS expression in clonal transductants

Northern analysis performed on total cellular RNA obtained from HEp-2 NOS transductant clones confirmed iNOS expression (Figure 1A). Clones A, B, C, D, and E expressed iNOS mRNA in varying amounts (Figure 1B). No mRNA encoding iNOS was detected in Clone X, the empty-vector transductant control, or in the parent cell line.

4.2. Growth of and nitrite accumulation from clonal transductants

Growth characteristics and nitrite accumulation for each of these clones both with and without the NOS inhibitor, N^GMMA, are shown in Table 1. Nitrite accumulation ranged from a low of 1.1 +/- 0.01 µM (Clone E) to a high of 23.4 +/- 2.8 µM (Clone A), values that correlated with mRNA expression. The two clones with the highest rates of NO production, A and B, demonstrated less vigorous growth, with doubling times of 24.1 +/- 1.6 hrs and 18.3 +/- 0.9 hrs, respectively vs. 14.3 +/- 0.8 hrs for the iNOS-minus Clone X ($p < 0.05$). Both Clones A and B demonstrated a more rapid doubling time when grown in the presence of 100 µM N^GMMA, with no significant differences from the doubling time observed for Clone X

Table 1. Characterization of iNOS clonal transductants

	No N^GMMA		+ N^GMMA	
iNOS transductant	Doubling time (hrs)	Nitrite (μM)	Doubling time (hrs)	Nitrite (μM)
Clone A	24.1 ± 1.6*	23.4 ± 2.8	16.1 ± 0.8	2.5 ± 0.2
Clone B	18.3 ± 0.9*	11.8 ± 3.1	15.8 ± 1.2	1.4 ± 0.03
Clone C	13.7 ± 1.2	4.9 ± 0.8	14 ± 1.2	0
Clone D	14.6 ± 2.0	2.5 ± 0.03	13.7 ± 1.0	0
Clone E	15.2 ± 1.4	1.1 ± 0.01	14.4 ± 0.2	0
Clone X	14.3 ± 0.6	0	13.8 ± 0.5	0
Parent cell line	14.1 ± 0.8	0	14.3 ± 1.1	0

Clones A-E (iNOS transductants) and clone X (vector-alone transductant control) were derived and generated from single cell clones as described in the Methods. Data on growth rates (doubling time) and nitrite accumulation in the culture supernatants at 60 hrs post-inoculation both in the absence and presence of the iNOS inhibitor, 100 μM N^GMMA are results of triplicate experiments +/- standard error (SE); *$p < 0.05$ vs. Clone X (vector-transductant control).

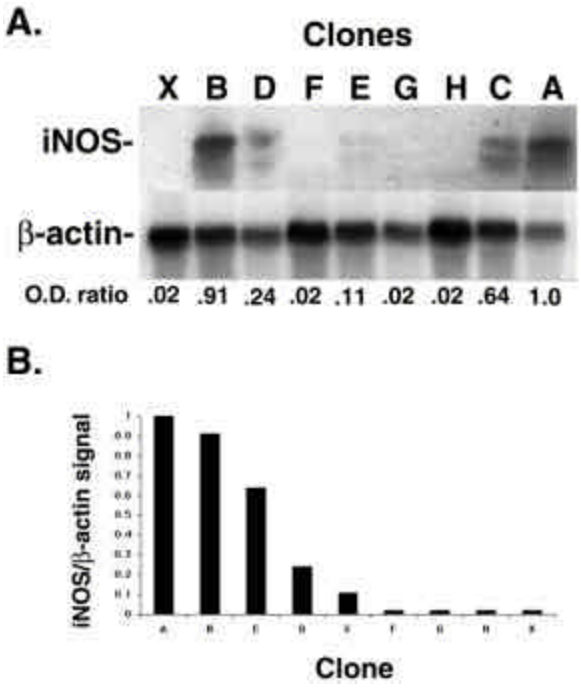

Figure 1. Shown is a Northern analysis of RNA extracted from HEp-NOS clones A-H, and from control clone X probed with radiolabelled iNOS cDNA. The β-actin signal is shown as a control for RNA loading. Figure 1B shows the relative signal intensities in rank order, as determined using imaging densitometry for iNOS to β-actin ratios where clone A is set at 1.0.

under the identical conditions, suggesting that growth inhibition was a direct consequence of the NO generated, as opposed to iNOS expression per se. However, as depressed growth would present a confounding variable for studies of virus replication, we have not utilized these high-producing clones for studies of virus replication. In contrast, no growth inhibition was detected among Clones C, D, and E when compared to Clone X, nor was there any significant change in their growth characteristics in the presence of N^GMMA. Clones C, D, and E produced significant quantities of NO, with accumulations of 4.9 +/- 0.08, 2.5 +/- 0.03, and 1.1 +/- 0.01 μM, respectively, detected at t = 60 hrs post-plating. These clones were selected for virus replication studies.

4.3. Quantitation of nitrite accumulation from exogenous NO donor

Cells of the HEp-2 parent line were treated with increasing concentrations (0 – 400 μM) of the chemical NO donor, N-acetyl-penicillamine (SNAP) or the control, N-acetyl-penicillamine (NAP) and nitrite accumulation was measured at t = 60 hrs as shown (Table 2). As anticipated, nitrite accumulation increased parallel with increasing SNAP concentration, with 5.8 +/- 1.2 μM nitrite detected in response to 50 μM SNAP matching that produced endogenously by the HEp-2 iNOS transductant, Clone C (4.9 +/- 0.8 μM at t = 60 hrs). Accumulations reaching 56.7 +/- 2.1 μM were obtained in response to 400 μM SNAP; no nitrite was detected in response to NAP administration, nor was growth inhibition observed under any of the conditions presented (not shown).

4.4. Virus replication in response to NO from endogenous vs. exogenous source

With these data, we compared the effects of endogenous vs. exogenous NO on virus replication (Table 3). Beginning with endogenous NO, we observe a 7-10 fold reduction in virus titer in the supernatants of RSV-infected Clone C (generating ~5 μM nitrite / 60 hrs) as compared to either the parent cell line or the empty-vector transductant Clone X. In contrast, virus replication proceeds unhindered in parent cells treated with 50 μM SNAP, which likewise results in the generation of ~5 μM NO in 60 hrs. Virus replication is inhibited to some extent as well in Clones D and E, while a 5-fold decrease in virus replication was observed in response to exogenous NO only when > 50 μM nitrite concentrations (400 μM SNAP) were achieved. No replication inhibition was observed in response to NAP, and inhibition of virus replication in the HEp-2 NOS transductant Clones C, D, and E was circumvented by the addition of 100 μM N^GMMA.

5. DISCUSSION

In this work, we demonstrate that heterologous expression of iNOS and generation of nitric oxide by target HEp-2 epithelial cells result in a reversible, dose-dependent inhibition of RSV replication. Furthermore, we

Table 2. Accumulation of nitrite (µM) at t = 60 hrs in culture supernatants by SNAP and control compound, NAP

µM	+ SNAP	+NAP
0	0	0
50	5.8 +/- 1.2	0
100	12.7 +/- 1.5	0
200	22.2 +/- 1.7	0
400	56.7 +/- 2.1	0

Increasing concentrations of SNAP were administered to and used to generate NO in supernatants of cells of the HEp-2 parent cell line. Experimental details as per Methods and legend to Table 1.

Table 3. Virus replication in response to NO from endogenous and exogenous sources

Cell type	NO Source	No N^GMMA		
		48 hrs (x 10^{-2})	60 hrs (x 10^{-2})	Fold reduction
Clone X	-	1480 +/- 250	2210 +/- 260	-
Clone C	Endogenous	118 +/- 14**	340 +/- 70**	6.5
Clone D	Endogenous	840 +/- 100**	1260 +/- 190**	1.8
Clone E	Endogenous	1010 +/- 170	1780 +/- 100**	1.2
Parent	-	1270 +/- 200	2240 +/- 380	-
Parent	50 µM SNAP	1180 +/- 210	2180 +/- 440	-
Parent	100 µM SNAP	1240 +/- 120	2000 +/- 500	-
Parent	200 µM SNAP	980 +/- 94	1680 +/- 320	-
Parent	400 µM SNAP	260 +/- 120*	440 +/- 80*	5.1
		+ 100 µM N^GMMA		
Clone X	-	1290 +/- 310	1980 +/- 340	-
Clone C	Endogenous	1460 +/- 380	2010 +/- 150	-
Clone D	Endogenous	1090 +/- 300	1960 +/- 240	-
Clone E	Endogenous	1400 +/- 80(no inhibitor)	2340 +/- 480	-
Parent	-	1210 +/- 140	2300 +/- 400	-
Parent	50 µM NAP	1090 +/- 340	2510 +/- 360	-
Parent	100 µM NAP	1290 +/- 240	2360 +/- 280	-
Parent	200 µM NAP	1110 +/- 90	2110 +/- 320	-
Parent	400 µM NAP	1240 +/- 120	2200 +/- 200	-

Data presented as plaque forming units (pfu) per ml culture supernatant sampled at 48 and 60 hours post-inoculation +/- SE; *p < 0.05 compared to 0 µM SNAP; **p < 0.05 compared to vector-alone transductant, Clone X. Fold reduction calculated for statistically significant differences at t = 60 hrs.

demonstrated that NO derived from endogenous iNOS expression was much more efficient at inhibiting RSV replication than was the addition of the exogenous NO, in this case via the chemical donor, SNAP.

To date, efforts to document a direct association between nitric oxide and viral infections *in vitro* have used two basic experimental strategies. Exogenous NO donors such as SNAP, sodium nitroprusside (SNP), or 3-morpholino-sydononimine (SIN-1), have been used as a source of NO, while analogs of the substrate L-arginine, such as L-N^GMMA, or L-NAME have been used to inhibit endogenous enzymatic activity (1, 10, 13-15, 18, 19). While these approaches have led to some interesting observations regarding the broad antiviral spectrum of NO (1), they fail to demonstrate the potential effects of intracellular, continuous generation of NO *in situ*. Many cell types, including respiratory epithelium can be stimulated to express endogenous iNOS at high levels, but this requires the presence of proinflammatory cytokines, which by themselves offer potential (and potentially confounding, from an experimental point of view) antiviral and immunomodulatory effects. Low levels of iNOS gene expression and NO production have already been demonstrated by RSV-infected A459 cells, an effect that was greatly enhanced by the presence of exogenous cytokines (17). Under these conditions, it is impossible to separate potential direct antiviral effects of NO from the direct and indirect effects of the proinflammmatory cytokines. In this work, we addressed this problem directly by generating epithelial cells that constituitively express iNOS and generate NO. Interestingly, under conditions of moderate to minimal NO generation (clones C, D, and E), intracellular production of NO was more efficient at inhibiting RSV replication than were significantly higher concentrations of NO provided by chemical donors added exogenously. As such, it seems unlikely that treatment of infants with severe RSV infection with chemical NO donors or inhaled NO would offer substantial antiviral activity. One study has already failed to demonstrate a bronchodilating effect of inhaled NO in the treatment of mechanically-ventilated, RSV-infected infants (28). Therapeutic immunomodulatory strategies that increase endogenous NO production may provide more substantial benefits.

The most simple and straightforward explanation for the differences in exogenous vs. endogenous production is the barrier function of the cell membrane. NO and its derivative, peroxynitrite, are highly reactive molecules, and it is likely that most of the exogenous-source mediator reacts with lipids in the cell membrane before reaching the intracellular compartment. As such, although diffusible, the extracellular concentration (measured as accumulated nitrite) is likely to be significantly higher than the intracellular. In the same vein, and for the same reason, the

intracellular concentration of endogenous-source NO is probably significantly higher than what can be detected as accumulated NO in the culture supernatant. Our data fit easily into this simple model, although we cannot rule out more subtle distribution, sequestering or even biochemical effects at this time.

Several different mechanisms for the observed antiviral activity of NO can be proposed. NO has been shown to inhibit picornavirus replication by blocking RNA and protein synthesis (18), vesicular stomatitis virus via nitrosylation of viral structural proteins, and vaccinia virus through inhibition of DNA replication (1). NO may also inhibit one or more of the cysteine-containing proteases that are critical for viral protein production (19) or activate antiviral pathways such as the double stranded RNA-dependent protein kinase (32) or ribonuclease L (33). One or more of these, or other as yet unknown mechanisms of antiviral action may be initiated more efficiently when NO is generated endogenously. Taken together, our data support the hypothesis that induction of iNOS during acute viral infections is an important component of the innate antiviral immune response and might be a reasonable focus of a broad-reaching immunomodulatory strategy.

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