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An Antiviral Mechanism of Nitric Oxide: Inhibition of a Viral Protease

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Summary

Although nitric oxide (NO) kills or inhibits the replication of a variety of intracellular pathogens, the antimicrobial mechanisms of NO are unknown. Here, we identify a viral protease as a target of NO. The life cycle of many viruses depends upon viral proteases that cleave viral polyproteins into individual polypeptides. NO inactivates the Coxsackievirus protease 3C, an enzyme necessary for the replication of Coxsackievirus. NO S-nitrosylates the cysteine residue in the active site of protease 3C, inhibiting protease activity and interrupting the viral life cycle. Substituting a serine residue for the active site cysteine renders protease 3C resistant to NO inhibition. Since cysteine proteases are critical for virulence or replication of many viruses, bacteria, and parasites, S-nitrosylation of pathogen cysteine proteases may be a general mechanism of antimicrobial host defenses.

Introduction

Nitric oxide (NO) is an antiviral effector of the immune system (Mannick, 1995; Fang, 1997; MacMicking et al., 1997; Reiss and Komatsu, 1998). Viral infection can induce expression of the inducible nitric oxide synthase (iNOS) in cells and in animals. NO inhibits the replication of a variety of viruses (Croen, 1993; Karupiah et al., 1993; Bi and Reiss, 1995; Bukrinsky et al., 1995; Hooper et al., 1995; Akaike et al., 1996; Komatsu et al., 1996; Tucker et al., 1996). We and others have previously shown that NO inhibits the replication of Coxsackievirus and other Picornaviruses (Komatsu et al., 1996; Lowenstein et al., 1996; Mikami et al., 1996; Flynn et al., 1997; Haddad et al., 1997; Zaragoza et al., 1997, 1998). Coxsackievirus B3 (CVB3) infection induces iNOS expression in macrophages in mice, and CVB3 replicates more rapidly to higher titers and causes more tissue damage in mice lacking iNOS, compared to infections of wild-type mice. Exogenous NO inhibits replication of Coxsackievirus and poliovirus in vitro, reducing viral RNA and protein synthesis. Thus, iNOS and NO are important components of the immune response to CVB3 infection. However, the viral targets of NO are unknown.

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Results

To explore the mechanism by which NO inhibits CVB3 replication, we first determined that NO inhibits CVB3 replication by blocking a step early in the viral life cycle. We infected HeLa cells with CVB3 and then at various times treated the infected cells with NO donors or control compounds; after 10 hr, we measured the amount of CVB3 produced during one viral life cycle. The NO donor S-nitroso-acetyl-penicillamine (SNAP) reduces the amount of CVB3 produced by infected HeLa cells, and the maximum effect of SNAP occurs when the NO donor is added 1–2 hr after infection (Figure 1). In contrast, the control compound acetyl-penicillamine (AP) has no effect. When SNAP is added to HeLa cells, most of the NO is released within 2 hr (data not shown). Since the life cycle of CVB3 lasts approximately 8 hr, these data suggest that NO inhibits an early step in the life cycle of CVB3.

Coxsackievirus, a member of the *Picornaviridae*, is a nonenveloped virus with a single-stranded RNA genome, whose life cycle includes virion attachment to the host cell, penetration, translation of the viral RNA into a polyprotein, autocleavage of the polyprotein by viral proteases into viral polypeptides, replication of the viral genome, assembly of the virion, and exit from the cell. Since protease activity is a critical step early in the life cycle of Coxsackievirus, we hypothesized that NO inhibits CVB3 proteases.

Exogenous NO Inhibits Protease Processing of an Endogenous Viral Polyprotein

In order to examine the effect of NO upon viral protease processing of viral proteins, we added the NO donor SNAP or its control AP to infected HeLa cells; cell lysates were harvested at various times after infection, fractionated by SDS-PAGE, and analyzed by immunoblotting for viral polypeptides. NO inhibits proteolysis of the viral protein precursor 2ABC, delaying and reducing the amount of the cleavage products 2BC and 2C detected by immunoblotting (Figure 2A). Since $3C^{pro}$ is responsible for cleavage of 2ABC, one interpretation of these data is that exogenous NO inhibits the proteolysis of the viral polypeptide 2BC.

Endogenous NO Inhibits Protease Processing of an Endogenous Viral Polyprotein

To answer the question of whether or not endogenous NO produced by iNOS would also interfere with viral proteolytic processing, we added macrophages that had been induced to express iNOS to infected cells. First, HeLa cells were infected with CVB3, and then cell culture inserts were added that contained RAW 264.7

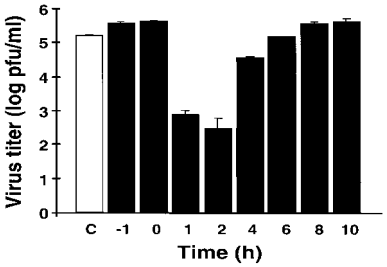

Figure 1. The NO Donor SNAP Inhibits CVB3 Replication

HeLa cells were infected with CVB3 at a multiplicity of infection of 10. SNAP (200 μM) was added at various times after infection as indicated. The amount of CVB3 after 10 hr of infection was measured by the plaque assay. To one set of infected cells (C), 200 μM AP was added 1 hr after infection. To another set of cells (-1), SNAP was added 1 hr before infection. n = 3 ± SD.

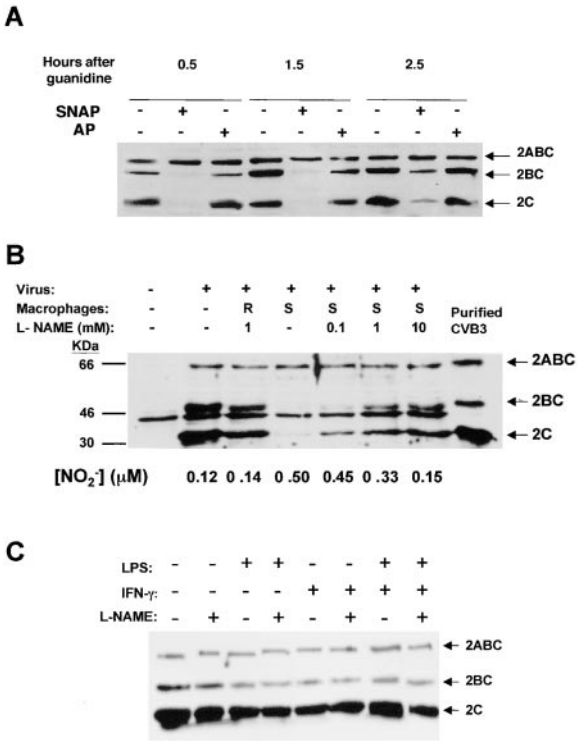

Figure 2. Figure 2. NO Inhibits Proteolysis of CVB3 Polypeptide 2BC in Infected Cells

(A) Exogenous NO donor SNAP inhibits proteolysis of CVB3 polypeptide. HeLa cells were infected with CVB3 at a multiplicity of infection of 10. After 1 hr, 200 μM of SNAP (+) or AP (-) was added, and after 3.5 hr guanidine was added to inhibit new viral RNA synthesis. HeLa cells were harvested at various times indicated after guanidine treatment. Cell lysates were fractionated by SDS-PAGE and immunoblotted with an antibody to CVB3 polypeptide 2C. These experiments were repeated twice with similar results.

(B) Endogenous NO from activated macrophages inhibits proteolysis of CVB3 polypeptide. HeLa cells were infected with CVB3 at a multiplicity of infection of 10. After 1 hr, plastic inserts containing resting (R) or LPS/IFN-stimulated (S) macrophages were added to the wells containing infected HeLa cells. Some cultures were also treated with the NOS inhibitor NAME (0–10 mM). Cells were harvested 5 hr after infection and cell lysates analyzed for CVB3 polypeptide 2C as above. This experiment was repeated five times with similar results.

(C) LPS or IFN do not directly affect CVB3 replication. HeLa cells were infected with CVB3 at a multiplicity of infection of 10. After 1 hr, LPS or IFN or media alone was added to the wells containing infected HeLa cells. Some cultures were also treated with the NOS inhibitor NAME (0–10 mM). Cells were harvested 5 hr after infection and cell lysates analyzed for CVB3 polypeptide 2C as above.

macrophages, activated or not with lipopolysaccharide and interferon-γ. The macrophages were separated from the infected HeLa cells by a 0.4 μM pore size membrane, which would allow the passage of NO from macrophages to infected cells. The NOS inhibitor nitro-arginine-methyl-ester (NAME) was added to some co-cultures of infected HeLa cells and activated macrophages, in order to confirm that any antiviral effect of activated macrophages was a result of NO, and not some other mechanism. Cells were harvested 5 hr after infection, and the cleavage of the viral polyprotein 2ABC was measured by immunoblotting as above.

Activated macrophages block proteolytic processing of the CVB3 polyprotein 2ABC, resulting in a decrease in the amount of 2BC and 2C (Figure 2B). Infected HeLa cells alone (lane 2) contain three prominent polypeptides recognized by the antibody to 2C (as well as another band also found in noninfected cells [lane 1]). These three polypeptides are 2ABC and its two proteolytic products 2BC and 2C. Resting macrophages (lane 3) do not affect the intensity of the three viral polypeptides. However, macrophages activated by lipopolysaccharide (LPS) and interferon-γ (IFNγ) (lane 4) release a soluble mediator that diffuses across the 4 μM pores and inhibits the processing of 2ABC into 2BC and 2C. This soluble mediator may be NO, since increasing concentrations of the NOS inhibitor NAME are associated with decreasing amounts of NO and also with increasing proteolytic processing of the viral polypeptide (lanes 5–7). (LPS or IFN alone have no effect upon viral replication in HeLa cells [Figure 2C].) Thus, NO synthesized by activated macrophages can diffuse into infected cells and inhibit the viral proteases that process the viral polyproteins.

Since exogenous and endogenous NO inhibit proteolytic processing of Coxsackievirus polyproteins, we therefore focused our attention on the effect of NO upon Coxsackievirus proteases.

NO Inhibits CVB3 Protease Activity

Picornaviruses encode two cysteine proteases, $2A^{pro}$ and $3C^{pro}$ (Kräusslich and Wimmer, 1988; Hellen et al., 1989; Palmberg, 1990; Dougherty and Semler, 1993). The protease $2A^{pro}$ cleaves the viral polyprotein at the boundary between the structural proteins and the nonstructural proteins, VP1 and 2A, producing two polyproteins. The protease $3C^{pro}$ is thought to cleave the resulting polyproteins into ten viral polypeptides, including the cleavage of 2BC into 2B and 2C (Ivanoff et al., 1986; Hammerle et al., 1991; Miyashita et al., 1993). The protease $3C^{pro}$ is a critical component of the Picornavirus life cycle: mutation of any of the three amino acids in the active site of $3C^{pro}$ inactivates the protease and abolishes viral replication (Ivanoff et al., 1986; Hammerle et al., 1991; Miyashita et al., 1993). One of the residues of the catalytic site of $3C^{pro}$ is cysteine at amino acid position 147, and since NO can nitrosylate cysteine residues

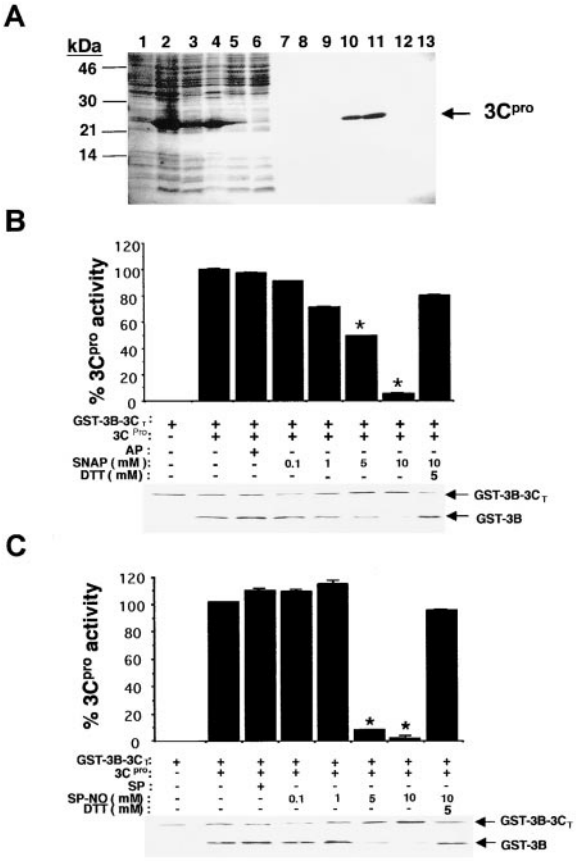

Figure 3. NO Inhibits Cleavage of a Viral Target by Purified 3C^{pro}

(A) 3C^{pro} was expressed in bacteria and purified. Bacterial lysates were subjected to SDS-PAGE: lane 1, noninduced bacteria; lane 2, bacteria induced with IPTG; lane 3, high-speed supernatant of induced bacteria; lane 4, flow-through from a Ni^{+} column; lanes 5–9, sequential column washes with 10 mM imidazole; and lanes 10–13, fractions from a Ni^{+} column eluted with 500 mM imidazole.

(B) The purified fusion protein GST-3B-3C$_T$, which contains an authentic 3C^{pro} cleavage site, was incubated with buffer, purified 3C^{pro}, or 3C^{pro} with the control compound AP, or the NO donor SNAP, or SNAP and DTT. The reaction mixture was analyzed by immunoblot with an antibody to GST and quantitated by densitometry. $n = 3 \pm$ SD, and * $p < 0.05$.

(C) The cleavage experiment was then repeated using spermine NONOate (SP-NO) as an NO donor or its control compound spermine (SP). $n = 3 \pm$ SD, and * $p < 0.05$.

(Stamler et al., 1992a, 1992b; Stamler, 1994), we hypothesized that NO inhibits the CVB3 3C^{pro}.

We therefore prepared pure 3C^{pro} and various protease substrates. We subcloned the cDNA encoding 3C^{pro} into a bacterial expression vector, expressed a fusion protein consisting of 3C^{pro} tagged with His_6 in bacteria, and then purified 3C^{pro} (Figure 3A) (Ghosh and Lowenstein, 1996). To prepare an authentic protease substrate, we subcloned, expressed, and purified a fusion polypeptide, consisting of glutathione-S-transferase, the entire CVB3 3B polypeptide, and the amino acid residues 1–140 of 3C; we refer to this fusion protein as GST-3B-3C$_T$. This fusion protein contains an authentic 3C^{pro} cleavage site between 3B and 3C, consisting of the 3C^{pro} recognition motif AXXQG (where Q is the P1 residue) (Kräusslich and Wimmer, 1988; Hellen et al., 1989; Lawson and Semler, 1990; Palmberg, 1990). (The fragment of 3C included in the fusion protein consists of the first 140 amino acids of 3C. It lacks the Cys^{147} of the active site and is thus incapable of protease activity.)

To test the activity of recombinant protease, purified 3C^{pro} was incubated with the protease substrate GST-3B-3C$_T$ and the reaction mixture analyzed by immunoblotting with an antibody to GST. Purified 3C^{pro} is capable of cleaving the polyprotein GST-3B-3C$_T$, as detected by immunoblotting (Figure 3B, immunoblot lanes 1 and 2).

In order to measure the susceptibility of 3C^{pro} to NO, we added a variety of NO donors or their controls to 3C^{pro} and a protease substrate. The NO donor SNAP inhibits the proteolytic activity of 3C^{pro} in a dose-dependent manner, while its control acetyl-penicillamine (AP), which has no nitroso group, has no effect (Figure 3B). DTT, an agent capable of reducing the nitrosylation of cysteine residues, reverses the NO inhibition of 3C^{pro}. Another NO donor, spermine NONOate, also inhibits 3C^{pro} cleavage of GST-3B-3CT. DTT also reverses this effect (Figure 3C).

To confirm these results, we used luciferase as another protease substrate (Holzman and Baldwin, 1980; Gorbalenya and Svitkin, 1983; Thompson et al., 1997). Firefly luciferase serves as a 3C^{pro} substrate because it contains the 3C^{pro} motif AXXQG. Cleavage of luciferase was measured by monitoring luciferase activity. 3C^{pro} cleaves luciferase in a dose- and time-dependent manner (Figures 4A and 4B). The NO donors SNAP or spermine NONOate but not their corresponding control drugs inhibit 3C^{pro} activity, and these inhibitions are reversed by DTT (Figure 4C). It was previously shown that the nonphysiological reagents iodoacetamide and N-ethylmaleimide can inhibit 3C^{pro} (Gorbalenya and Svitkin, 1983; Orr et al., 1989). Thus, NO inhibits the activity of 3C^{pro}.

NO Nitrosylates the CVB3 Protease Active Site Cysteine

3C^{pro} has only one cysteine, namely Cys^{147} in the active site. To determine whether or not NO nitrosylates this cysteine, we first measured the effect of NO donors upon its thiol group with the Ellman assay (Ellman, 1959; Zhang et al., 1996). Purified 3C^{pro} was incubated with NO donors for 1 hr and then dialyzed. 5,5′- Dithiobis (2-nitrobenzoic acid) was added, the increase of absorbance at 412 nm was measured, and the concentration of thiols was calculated from the molar extinction coefficient of the nitrothiobenzoate ion. NO donors but not their corresponding control compounds eliminate the sulfhydryl group from purified 3C^{pro} over time (Figure 5A).

We next measured the effect of NO donors upon the formation of a nitroso-thiol group by the Saville assay (Saville, 1958; Zhang et al., 1996). Purified 3C^{pro} was incubated with various NO donors or their controls, and excess NO donors and nitrite were removed from the sample with Sephadex G-25 column chromatography. 3C^{pro} was then incubated with $HgCl_2$ and the amount of nitrite was measured in the Griess reaction (Stamler et al., 1992a). For every 1 mole of 3C^{pro} added to the reaction, approximately 1 mole of nitrite is detected (Figure 5B). These data suggest that NO nitrosylates 3C^{pro} on a

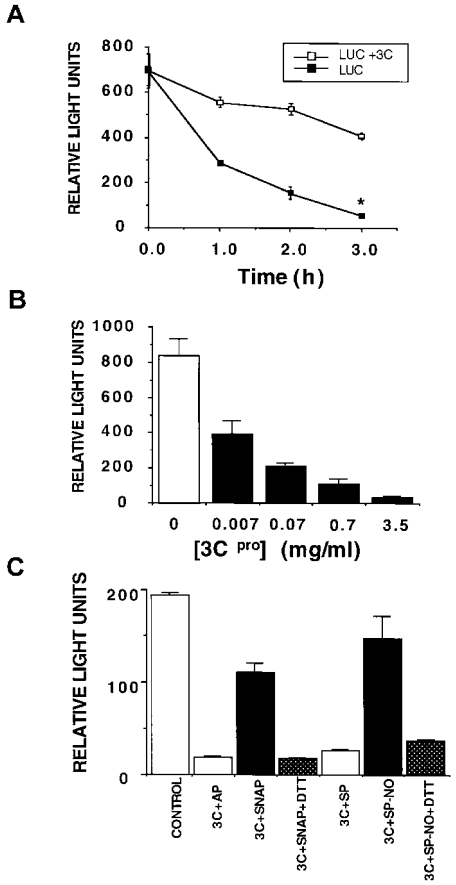

Figure 4. NO Inhibits $3C^{pro}$ Cleavage of Luciferase

(A) Purified $3C^{pro}$ was added to luciferase, which contains a $3C^{pro}$ consensus cleavage motif, and the activity of luciferase remaining at various times was assayed using a luminometer. n = 3 ± SD.
(B) Increasing amounts of $3C^{pro}$ were added to luciferase for 3 hr, and the amount of luciferase assayed as above. n = 3 ± SD.
(C) Luciferase was incubated alone, or in the presence of $3C^{pro}$ with the following additions: the control compound AP, the NO donor SNAP, SNAP and DTT, the control compound spermine (SP), the NO donor spermine NONOate (SP-NO), or spermine NONOate and DTT. n = 3 ± SD.

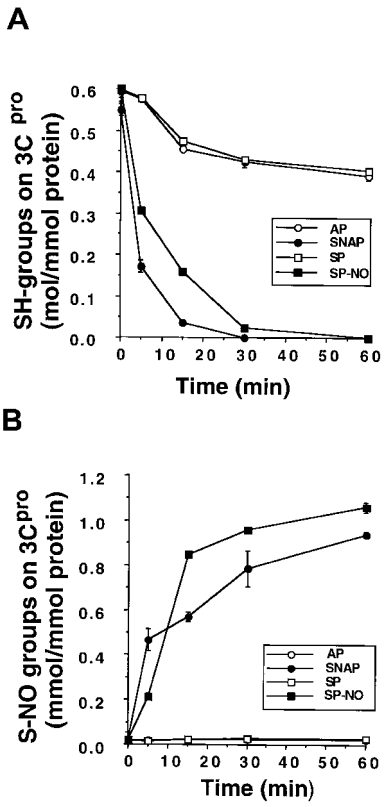

Figure 5. NO Eliminates a Sulfhydryl Group from $3C^{pro}$ and Nitrosylates $3C^{pro}$

Purified $3C^{pro}$ was incubated with AP, SNAP, spermine, or spermine NONOate. (A) Analysis of the $3C^{pro}$ sulfhydryl group. Treated $3C^{pro}$ was incubated with an excess of 5,5 dithiobis(2-nitrobenzoate) (DNTB) and its absorption measured at 412 nm. n = 4 ± SD. (B) Nitrosylation of the $3C^{pro}$ active site Cys^{147}. $3C^{pro}$ was exposed to NO donors or their controls, then incubated with $HgCl_2$, and the amount of nitrite released was measured by the Griess reaction. n = 4 ± SD.

cysteine residue. Since the active site Cys^{147} is the only cysteine residue in $3C^{pro}$, the data imply that NO nitrosylates the active site cysteine residue.

Mutating the CVB3 Protease Active Site Cysteine to Serine Renders the Protease Resistant to NO

To confirm that nitrosylation of the active site Cys^{147} is responsible for the inhibition of $3C^{pro}$, we created a mutant protease by PCR-directed mutagenesis, substituting a serine for the cysteine at position 147. The resultant mutant protease, $^{C147S}3C^{pro}$, still has catalytic activity, although at a level greatly reduced from wild-type protease, as others have shown (Ivanoff et al., 1986; Hammerle et al., 1991; Miyashita et al., 1993). The effect of NO upon protease activity was determined by adding NO donors and luciferase to either wild-type or mutant protease. NO reduces wild-type $3C^{pro}$ activity, and DTT restores wild-type protease activity (Figure 6). However, NO has no effect upon mutant $^{C147S}3C^{pro}$ activity, nor does DTT change mutant protease activity. Thus, the Cys^{147} of the catalytic triad is critical in determining the susceptibility of $3C^{pro}$ to NO.

Discussion

The major finding of this study is that NO inhibits Coxsackievirus replication at least in part by nitrosylating the cysteine residue in the active site of a viral protease, thereby drastically reducing the activity of this enzyme. This protease is critical to the life cycle of Picornaviruses. Nitrosylation of cysteine residues is one mechanism by which NO can directly modify a polypeptide, and NO in theory can reduce the activity of an enzyme if its catalytic site includes a cysteine residue (Stamler et al., 1992b, 1997; Stamler, 1994). For example, NO inhibits activity of the cysteine protease caspase-3 (Kim et al., 1997; Li et al., 1997; Mannick et al., 1997; Mohr et al., 1997). Although proteins capable of being nitrosylated at a cysteine residue contain a consensus nitrosylation motif, the $3C^{pro}$ lacks this sequence (Stamler et al., 1997). Coxsackievirus may have a selective advantage if

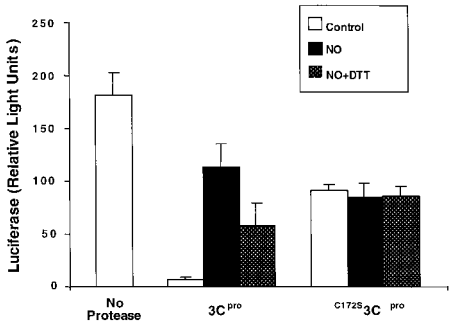

Figure 6. Mutation of the $3C^{pro}$ Active Site Cys^{147} Renders $3C^{pro}$ Resistant to NO

A mutant $3C^{pro}$ with serine substituted for cysteine at amino acid residue 147 was purified from bacteria. Wild-type $3C^{pro}$ (1 μg/ml) and mutated $3C^{pro}$ (5 μg/ml) were incubated for 3 hr with luciferase and AP, SNAP, or SNAP and DTT, and the activity of luciferase assayed in a luminometer. n = 3 ± SD, repeated twice with similar results.

its viral protease lacks a nitrosylation consensus sequence, decreasing cysteine reactivity to NO. The other protease of Coxsackievirus, 2Apro, is also a cysteine protease, and may be a target of NO as well.

Previously, we showed that NO inhibits Coxsackievirus replication in vitro and that NO reduces both viral RNA synthesis and viral protein synthesis (Zaragoza et al., 1997). NO inactivation of $3C^{pro}$ could explain this reduction in both viral RNA and viral protein levels, given the characteristics of the Coxsackievirus life cycle. After Coxsackievirus enters the cell, viral RNA is translated into a large polyprotein. The $3C^{pro}$ excises itself from the polyprotein and cleaves the polyprotein into its components, including $3D^{pol}$. This RNA-dependent RNA polymerase $3D^{pol}$ then replicates the viral genome. Thus, $3C^{pro}$ processing acts upstream of viral RNA synthesis and is necessary to generate $3D^{pol}$. Therefore, nitrosylation and inactivation of $3C^{pro}$ would be expected to block not only viral protein processing but also viral RNA synthesis.

Although our data suggest that NO inhibits activity of Coxsackievirus protease $3C^{pro}$, there are other potential viral targets of NO as well. For example, NO could affect the RNA polymerase activity of $3D^{pol}$. Although $3D^{pol}$ lacks the typical molecular targets of NO, cysteine residues or iron in its active site, NO could affect its activity by other mechanisms. It is also possible that NO could inactivate host proteins necessary for viral replication. The lack of effect of NO upon viral replication when NO is added to cells prior to infection argues against this possibility (Figure 1). Nonetheless, NO can affect a variety of host proteins; however, the net effect of NO upon host processes either harmful or beneficial to viral replication is unknown.

The genomes of many viruses encode a polyprotein that after translation is cleaved into smaller polypeptides by a viral protease. These proteases fall into several categories based on their active site residues, including cysteine proteases, serine proteases, and aspartic proteases (Kräusslich and Wimmer, 1988; Hellen et al., 1989; Babe and Craik, 1997). NO can inhibit the replication of other viruses that encode cysteine proteases, such as members of the Picornavirus family and the Coronavirus family (Mannick, 1995; Reiss and Komatsu, 1998). In contrast, NO does not inhibit the replication of some viruses that encode serine proteases, such as alphaviruses. However, NO also inhibits the replication of some viruses that do not encode cysteine proteases, implying that there exist other viral targets of NO. For example, the EBV transcription factor Zta and ribonucleotide reductase may be targets of NO (Mannick et al., 1994; Melkova and Esteban, 1995). Nonetheless, to our knowledge there are no viruses that have cysteine proteases that are resistant to NO.

Cysteine proteases are also critical to the replication and virulence of a variety of other micro-organisms. Inhibitors of cysteine proteases block the replication of *Plasmodium falciparum*, *Plasmodium berghei*, *Leishmania major*, *Schistosoma mansoni*, and *Trypanosoma cruzi*. (Bailly et al., 1992; Eakin et al., 1992; Harth et al., 1993; Kumar et al., 1994; Li et al., 1994; McGrath et al., 1995; Wasilewski et al., 1996; Selzer et al., 1997). Furthermore, inhibitors of cysteine proteases also reduce the virulence of *Streptococcus pyogenes*, *Porphyromonas gingivalis*, *Schistosoma mansoni*, *Naegleria fowler*, and *Plasmodium flaciparum*. (Kapur et al., 1993a, 1993b; Aldape et al., 1994; Bedi and Williams, 1994; Gluzman et al., 1994; Musser et al., 1996; Wasilewski et al., 1996; Kuramitsu et al., 1997; Lukomski et al., 1997; Gubba et al., 1998). (For example, the malaria parasite uses its cysteine protease falcipain to degrade hemoglobin for use as a source of amino acids, and cysteine protease inhibitors block the ability of the parasite to infect erythrocytes and to replicate [Gluzman et al., 1994; Dominguez et al., 1997].) It is striking that NO inhibits the replication of all of these parasites (Mannick, 1995; Fang, 1997; MacMicking et al., 1997; Reiss and Komatsu, 1998). NO production may thus be a general mechanism by which the host defends itself against infection by viruses and other pathogens whose life cycle depends upon cysteine proteases.

Experimental Procedures

Cells and Viruses

Coxsackievirus B3 (CVB3) (Nancy strain, generous gift of C.G. Gauntt) was grown in HeLa cells and measured by the plaque assay (Gauntt et al., 1979; Zaragoza et al., 1997).

To measure the effect of the NO donor SNAP upon proteolytic processing of CVB3 polypeptides in cells, CVB3 at an MOI of 10 was added to HeLa cell cultures and 200 μM SNAP was added after 1 hr, and cells were harvested at various times afterward and cell lysates fractionated by SDS-PAGE and analyzed by immunoblotting. Guanidine HCl 3 mM, a specific inhibitor of CVB3 RNA replication, was added to all infected cells 3 hr after infection in order to inhibit new viral RNA synthesis.

To measure the effect of endogenously synthesized NO upon proteolytic processing of CVB3 polypeptides in cells, CVB3 at an MOI of 10 was added to HeLa cell cultures in 6-well plates, and then macrophages (that previously had been plated onto cell culture inserts and then stimulated or not for 16 hr with LPS 10 ng/ml plus interferon-μ 50 U/ml) were added to the 6-well plates. In some wells containing infected HeLa cells and activated macrophages, nitro-arginine-methyl-ester (NAME) was added. Cells were harvested 5 hr after infection and cell lysates fractionated by SDS-PAGE and analyzed by immunoblotting.

Immunoblot Analysis

Immunoblotting was performed as described previously, using an antibody to CVB3 2C (generous gift of Christine Hohenadl) or antibody to GST (Pharmacia) (Zaragoza et al., 1997).

Northern Analysis

Total RNA was isolated from infected HeLa cells as described previously and probed with a radiolabeled CVB3 DNA probe (generous gift of R. Kandolf) (Klump et al., 1990; Zaragoza et al., 1997). Blots were rehybridized with a β-actin cDNA as an internal control.

Expression and Purification of Viral Polypeptides

The cDNA encoding CVB3 $3C^{pro}$ was cloned between the NdeI and BamHI sites of the expression vector pSG04 so that a $(His)_6$ amino terminal tag is fused to $3C^{pro}$ (Ghosh and Lowenstein, 1996). The $3C^{pro}$ mutant $^{C147S}3C^{pro}$ was constructed using PCR-directed mutagenesis, using as a mutagenic primer GAATGCTTATGTACAACTTCCCCACA AGAGCAGGCCAGTCTGGTGGA. The plasmid encoding the $3C^{pro}$ target polypeptide GST-3B-$3C_T$ was prepared by inserting the DNA encoding the 6 carboxy terminal amino acids of CVB3 3B and amino acid residues 1–140 of CVB3 3C into plasmid pGEX 4T2 (Pharmacia). The sequence of all constructions was confirmed by sequencing. The $3C^{pro}$ and $^{C147S}3C^{pro}$ were expressed in bacteria as described previously and purified by metal chelate chromatography (Ghosh and Lowenstein, 1996). The GST-3B/$3C_T$ fusion protein was expressed in bacteria and purified by glutathione-agarose column chromatography according to the manufacturer's instructions (Pharmacia). Proteins were dialyzed and stored at −70°C.

Measurement of $3C^{pro}$ Activity

The activity of the $3C^{pro}$ was assayed using two different substrates.

Viral Polypeptide Substrate

The GST-3B/$3C_T$ fragment (1 mg/ml) was incubated with $3C^{pro}$ (0.7 mg/ml) in a final volume of 500 μl for 16 hr at 30°C in protease buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, and 1 mM EDTA). The protease reaction mixture was subjected to 15% SDS-PAGE and immunoblotted using an antibody to GST.

Luciferase Substrate

A $1:10^8$ dilution of a 1 mg/ml solution of firefly luciferase (Sigma, St. Louis, MI) was incubated with $3C^{pro}$ in a final volume of 200 μl at 30°C in protease buffer. At various times, 20 μl aliquots were harvested, and the activity of the luciferase was measured using luciferin in a luminometer (Turner model 20e).

Determination of Thiol Content of $3C^{pro}$

Purified $3C^{pro}$ (0.5 mg) was incubated in protease buffer with various NO donors (5 mM SNAP or spermine NONOate) or their corresponding control drugs (5 mM aminopenicillamine or spermine) and then dialyzed in a buffer containing 0.1 M sodium phosphate, 6 M guanidine HCl, and 1 mM EDTA. 5,5′-dithiobis(2-nitrobenzoate) was added, and the absorbance at 412 nm was measured over time. The concentration of thiols was determined from the molar extinction coefficient.

Determination of S-Nitrosylation of $3C^{pro}$

Purified $3C^{pro}$ was incubated with NO donors for 1 hr and then dialyzed. 5,5′-Dithiobis (2-nitrobenzoic acid) was added to remove nitrite from the sample and then a solution of 3% sulfanilamide and 0.25% $HgCl_2$ was added, followed by 0.1% ethylenediamine. The mixture was incubated at 22°C for 10 min and the absorbance measured at 540 nm.

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