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Targeting Cysteine Residues of Human Immunodeficiency Virus Type 1 Protease by Reactive Free Radical Species

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ABSTRACT

Nitric oxide (NO) is a naturally occurring free radical with many functions. The oxidized form of NO, the nitrosonium ion, reacts with the thiol group of cysteine residues resulting in their modification to *S*-nitrosothiols. The human immunodeficiency virus type 1 (HIV-1) protease (HIV-PR) has two cysteine residues that are conserved amongst different viral isolates found in patients with acquired immunodeficiency syndrome (AIDS). In an active dimer, these residues are located near the surface of the protease. We have found that treatment of HIV-PR with different NO congeners results in loss of its proteolytic activity and simultaneous formation of *S*-nitrosothiols. Sodium nitroprusside inhibited HIV-PR up to 70% and *S*-nitroso-*N*-acetylpenicillamine completely inhibited the protease within 5 min of treatment. The pattern of inhibition by NO donors is comparable to its inhibition by *N*-acetyl pepstatin. Using electrospray ionization-mass spectrometry, we identified the modification of HIV-PR by NO as that of *S*-nitrosation. Our findings point towards a possible role of NO in mediating resistance to HIV-1 infection. Antiox. Redox Signal. 1, 105–112.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS type 1 (HIV-1) is a positive-strand RNA virus encoding two large precursor polyproteins, Pr^{gag} and $Pr^{gag\text{-}pol}$. These polyproteins are processed post-translationally to their individual components. Pr^{gag} is cleaved to yield structural proteins and $Pr^{gag\text{-}pol}$ yields both structural proteins and replicative enzymes. The *pol* gene products include reverse transcriptase, RNase-H, integrase, and a protease. The processing of Pr^{gag} generates p17 (matrix protein), p24 (capsid protein), p2, p7 (nucleocapsid protein), p1, and p6. The Gag protein possesses different domains for membrane association and for protein–protein and protein–nucleic acid interactions (Garnier *et al.*, 1998). It also confers infectivity of the mature virus by assisting in its entry, disassembly, unloading of the nucleocapsular packet, and nuclear import of the preintegrative complex (Henderson *et al.*, 1992; Reicin *et al.*, 1996). Cleavage of Gag precursor has been shown to occur before the assembled

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virus buds from the surface of the infected cell and protease activity is required for the efficient budding process of the virus (Wang and Barklis, 1993; Kaplan *et al.*, 1994). The HIV-1 protease, therefore, is essential for the maturity and infectivity of the virus. The protease releases itself from the precursor polyprotein by autocatalysis and acts upon the precursor proteins to release the individual components (Wan *et al.*, 1996). The *gag* gene products p6 and p7 have reportedly been involved in the processing of protease. p6 inhibits autoprocessing while p7 facilitates both autoprocessing and dimerization of the protease (Partin *et al.*, 1991; Zybarth and Carter, 1995).

HIV-1 protease (HIV-PR) is the smallest of all retrovirus proteases, containing 99 amino acids (MW 10,778.8 Da), and is active as a homodimer. Its crystal structure has been solved to 2.7 angstrom resolution (Lapatto *et al.*, 1989; Navia *et al.*, 1989; Wlodawer *et al.*, 1989), revealing that this protein is related both structurally and functionally to the eukaryotic aspartic proteinase family of enzymes, which can be inhibited by aspartic protease inhibitors (*e.g.*, *N*-acetyl pepstatin). The active center, formed at the interface of the dimers, consists of an Asp-Thr-Gly motif contributed by each monomer. The dimer is stabilized by antiparallel interactions involving the active site triad. Dimerization of the protease is pH-dependent and is favored under acidic conditions. Two types of cleavage sites have been identified for the HIV-PR; it cleaves between an aromatic residue and proline residue and between two adjacent hydrophobic residues (Griffiths *et al.*, 1992; Dunn *et al.*, 1994). Two cysteine residues present in each monomer of HIV-PR are highly conserved among viral isolates from HIV-1-infected patients, suggesting a critical function. Both of these residues are located near the surface of the protease. While Cys67 is exposed to the surface of the molecule, Cys95 is buried in a hydrophobic environment. These cysteine residues are susceptible to oxidation by metal ions like copper and mercury resulting in inhibition of the enzyme (Karlstrom and Levine, 1991). These resides are also sensitive to different thiol-modifying reagents such as 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide, iodoacetamide, and glutathione, which affect enzyme activity to varying degrees (Karlstrom *et al.*, 1993; Davis *et al.*, 1996). Nonactive-site cysteine residues have been shown to serve regulatory roles in many proteins, *e.g.*, citrate synthetase, glutathione transferase P, and E2 protein of papilloma virus (Donald *et al.*, 1991; Tamai *et al.*, 1991; McBride *et al.*, 1992).

Nitric oxide (NO), a naturally occurring, fast acting free radical, is generated in different cell types under normal and stressed conditions. The oxidized form of NO, the nitrosonium cation (NO^+), is known to react predominately with cysteine residues of proteins forming nitrosothiol adducts (R-S-NO). The reactivity of NO^+ toward thiol groups of cysteine residues in proteins is at least 1000-fold greater than its reactivity toward the OH group of tyrosine and secondary amines of lysine and arginine. Nitrosothiol modification alters the activity and biological functions of many proteins including enzymes, signaling proteins, receptors, antioxidants, transcription factors, and ion channels (Stamler, 1994). Here we present evidence that *S*-nitrosation of cysteine residues of HIV-PR by NO causes a dramatic decrease in its proteolytic activity.

MATERIALS AND METHODS

Materials

Sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP), isopropyl β-D-thiogalactopyranoside, and protease inhibitors were purchased from Sigma (St. Louis, MO). SNP and SNAP solutions were prepared fresh each time immediately before use. SNAP was dissolved in dimethylsulfoxide (DMSO) and then diluted to achieve the desired concentration. *N*-acetyl pepstatin and Luria Broth were purchased from Boehringer Mannheim (Indianapolis, IN). Pepstatin was prepared in ethanol and stored at −20°C. All other chemicals were of analytical grade.

Expression and purification of HIV-1 protease

The synthetic cDNA of HIV-PR was expressed as a 156-amino-acid-long precursor protein from a modified pET3a expression vec-

tor, pET3AM (Cheng *et al.*, 1990) in BL21 (DE3)pLysS cells (Novagen, Inc., Madison, WI). The bacteria were grown to an OD_{600} value of 0.3 and induced with 0.4 m*M* IPTG for 3 hr at 37°C. Cells were then harvested, resuspended in lysis buffer, and stored at −20°C. The protease was purified from inclusion bodies by selective extraction and membrane fractionation using 50% acetic acid as described by Gustafson *et al.* with slight modifications (Gustafson *et al.*, 1995). The renatured protease was dialyzed against phosphate buffer (100 m*M* at pH 6.7) containing 10% glycerol and stored in the same buffer with 40% glycerol at −20°C for long-term use. Original pilot experiments used HIV-PR supplied by the NIH AIDS Research and Reference Program.

Protease assay

The HIV-1 protease assay was performed according to Matayoshi *et al.* (Matayoshi *et al.*, 1989) with slight modifications. The fluorogenic substrate (R-E-(EDANS)-S-Q-N-Y-P-I-V-Q-K-(DABCYL)-R was obtained from Molecular Probes, Inc. (Eugene, Oregon). The assays were performed at 37°C in 100 μl of buffer, 0.1 *M* Na-acetate, pH 4.5 containing 1.0 *M* NaCl, 10% DMSO, 1.0 m*M* EDTA, and 1 mg/ml BSA in standard 96-well FluorNunc plates. The concentrations of enzyme (0.2 μ*M*) and substrate (4.0 μ*M*) were kept constant in all our experiments. Samples were treated as indicated and the activity was measured for 5 min. The reaction mixtures were excited at 360 nm and fluorescence was measured at 535 nm in a fluorescence microplate reader (Spectrafluor, TECAN, Research Triangle Park, NC). The assay was linear over 5 min. In a typical experiment, about 10,000 fluorescence units were generated upon addition of substrate to a sample containing enzyme alone. The change in fluorescence was expressed either as percent activity or as relative fluorescence intensity. Each data point is an average of three experiments, each performed in triplicate.

Mass spectrometry

Electrospray ionization-mass spectrometry of HIV-PR was performed using a Quattro II Triple Quadrupole Mass Spectrometer (Micromass Ltd., Wythenshawe, UK). HIV-PR (5 μ*M*) was treated with 100 μ*M* of an NO-saturated solution in 5 m*M* ammonium acetate buffer at pH 4.9 for 5 min at room temperature. For *N*-ethylmaleimide (NEM) studies, 5 μ*M* HIV-1 protease (in ammonium acetate buffer, 5 m*M* at pH 4.9) was modified with 1 m*M* NEM for 5 minutes at 22°C. Combined modification of HIV-1 protease with NO and NEM was achieved by allowing the protease to react with 1 m*M* NEM and 100 μ*M* NO-gas solution for 10 min. The treated protease was then mixed with 50% methanol and infused into the electrospray source of the mass spectrometer. The molecular mass of the protease and its modified forms were obtained from positive-ion electrospray spectra using a maximum entropy deconvolution routine. Conditions for the identification of NO-modified species on proteins using electrospray–ionization mass spectrometry has previously been described (Mirza *et al.*, 1995).

RESULTS

Expression of HIV-PR in bacteria and its purification from inclusion bodies

HIV-PR was expressed in BL21(DE3)pLysS cells and purified from the inclusion bodies by selective extraction and membrane fractionation using 50% acetic acid. The protein was refolded and its activity was examined. Figure 1 shows different fractions of HIV-PR during its purification from bacterial extract that include crude bacterial extract (lane 1), inclusion bodies (lane 2), high-speed supernatant (lane 3), and the purified protein (lane 4). The processed form of recombinant protease (shown by an arrow) was obtained at greater than 90% purity as determined by SDS-PAGE analysis and Coomassie Blue staining (lane 4). The unprocessed form of HIV-PR (156 amino acids) was co-purified with the processed form (99 amino acids) as shown by the arrowhead. The ratio of processed and unprocessed form was estimated to be 90:10. The yield of the recombinant protease from 1 liter of bacterial culture was approximately 3.0 mg.

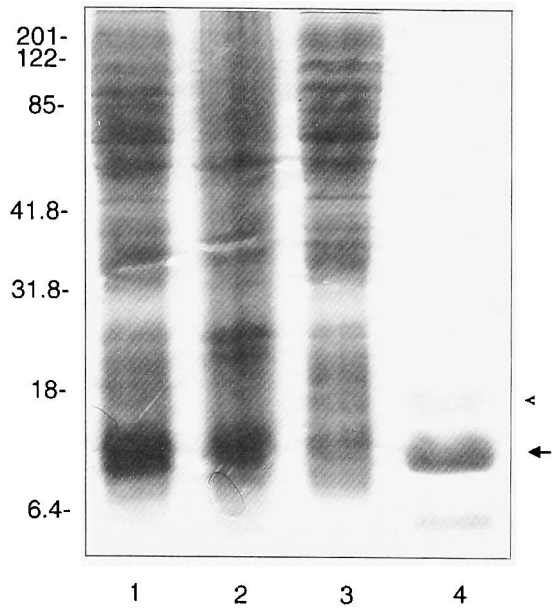

FIG. 1. Purification of HIV-PR from bacterial extract. HIV-PR was expressed in *E. coli* and purified from inclusion bodies as described in Materials and Methods. Different fractions were analyzed on a 15% polyacrylamide gel, stained with Coomassie Blue and destained. *Lane 1*, Crude extract (15 μg); *lane 2*, high-speed supernatant (15 μg); *lane 3*, isolated inclusion bodies (10 μg); *lane 4*, purified HIV-PR (4 μg). The processed and unprocessed forms are shown by an arrow and arrowhead, respectively. The molecular mass standards (kDa) are indicated on the left margin.

Activity assays of HIV-PR in the presence of protease inhibitors

In an attempt to determine if the purified recombinant HIV-PR preparation contained nonspecific proteases that might affect its activity, enzyme assays were performed in the presence of leupeptin, aprotinin, soybean trypsin inhibitor (1.0 μg/ml each), and phenylmethylsulfonyl fluoride (PMSF, 0.5 m*M*). As shown in Fig. 2A, the activity of HIV-PR was unaffected by these serine- and cysteine-specific protease inhibitors. These data suggest that there was no contribution of nonspecific proteases toward the activity of HIV-PR in our assays.

N-Acetyl pepstatin is known to inhibit aspartic proteases (Fitzgerald *et al.*, 1990). To examine its effectiveness in inhibiting HIV-PR, we performed protease assays in the presence of *N*-acetyl pepstatin. As can be seen in Fig. 2B increasing the concentration of pepstatin resulted in a gradual decrease in HIV-PR activity. In fact, at 500 n*M* pepstatin, only 2.5% of the original activity was detected. These data, coupled with our purification experiments, strongly suggest that our assay is specifically measuring HIV-PR activity.

Inhibition of HIV-PR by SNP and SNAP

To determine the effect of NO on the activity of HIV-PR, the enzyme was preincubated in the presence and absence of various concen-

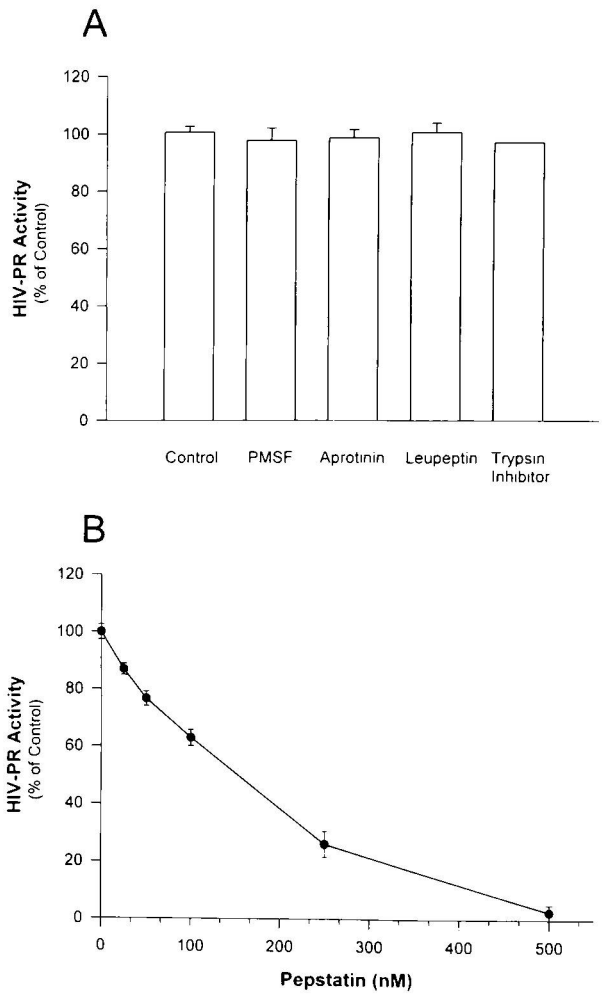

FIG. 2. A. HIV-PR preparation is free of nonspecific protease contamination. Activities of HIV-PR were assayed in the presence or absence of PMSF (0.5 m*M*), aprotinin (1 μg/ml), leupeptin (1 μg/ml), and soybean trypsin inhibitor (1 μg/ml) for 5 min at 37°C. The observed fluorescence was converted to enzyme activity and compared with the control. Each bar is an average of three experiments and the standard errors are shown. **B.** Pepstatin inhibits HIV-PR. The HIV-PR was assayed in the presence of the indicated concentrations of *N*-acetyl pepstatin for 5 min at room temperature. The fluorescence measured was converted to enzyme activity and compared with control. Each data point is an average of three experiments and the standard errors are shown.

trations of SNP or SNAP for 5 min at room temperature. As seen in Fig. 3A, preincubation of HIV-PR at low concentrations of SNP resulted in a nominal decrease in its activity, whereas 62.4 ± 0.94% inhibition was achieved at 5 m*M* SNP. When treated with 5.0 m*M* SNAP, protease activity was completely abolished within 5 min of incubation (Fig. 3B). The pattern of inhibition with SNP was more linear than that observed with SNAP over the concentration ranges tested.

Formation of S-nitrosothiols on HIV-PR

To determine whether NO modified the cysteine residues of HIV-PR, the enzyme was treated with 100 μM NO-gas solution for 5 min at room temperature and subjected to eletrospray ionization mass spectrometry (Mirza *et al.*, 1995). Molecular masses of unmodified and NO-modified species of HIV-PR were obtained from a maximum entropy deconvolution routine (Table 1). The mass spectrometry data show that in solution the protease exists in both monomeric (10,779 Da) and dimeric (21,556 Da) forms. Upon NO treatment, the mass of the monomer shifted to two species of 10,809 and 10,839 Da, differing from the unmodified monomer by 30 and 60 Da, respectively. These differences in masses can be attributed to the addition of one and two nitric oxide molecules, respectively, because the mass of NO is 30 Da. Similarly, NO treatment yielded new species of dimer with masses 21,616 and 21,676 Da, which differed from the native form by 60 and 120 Da (Table 1) respectively. This suggests that the dimer had been modified by two and four nitric oxide molecules. The existence of two cysteine residues per monomer and the appearance of two NO-modified forms of monomer, and the presence of four cysteine residues per dimer and the appearance of four NO-modified forms of dimer strongly suggest that cysteine residues of the protease are targets of nitric oxide.

To confirm that modifications of the protease by NO are due to S-nitrosation of its cysteine residues, we performed a competition experi-

TABLE 1. MODIFICATION OF HIV-PR BY NO AND NEM USING MASS SPECTROMETRY

HIV-PR Treatment	*Observed mass (Da)*			
	Monomer	Δ[a]	*Dimer*	Δ[a]
None	10,779		21,556	
NO	10,809	30	21,616	60
	10,839	60	21,676	120
NEM	11,029	250	22,058	500
NO + NEM	10,809	30	21,616	60
	10,839	60	21,676	120
	10,904	125	21,741	185
	10,934	155	21,806	250
	11,029	250	21,866	310
			21,931	375
			22,056	500

[a]Δ Refers to the difference between the masses of the treated and untreated species. The error in measurement is ± 2 Da.

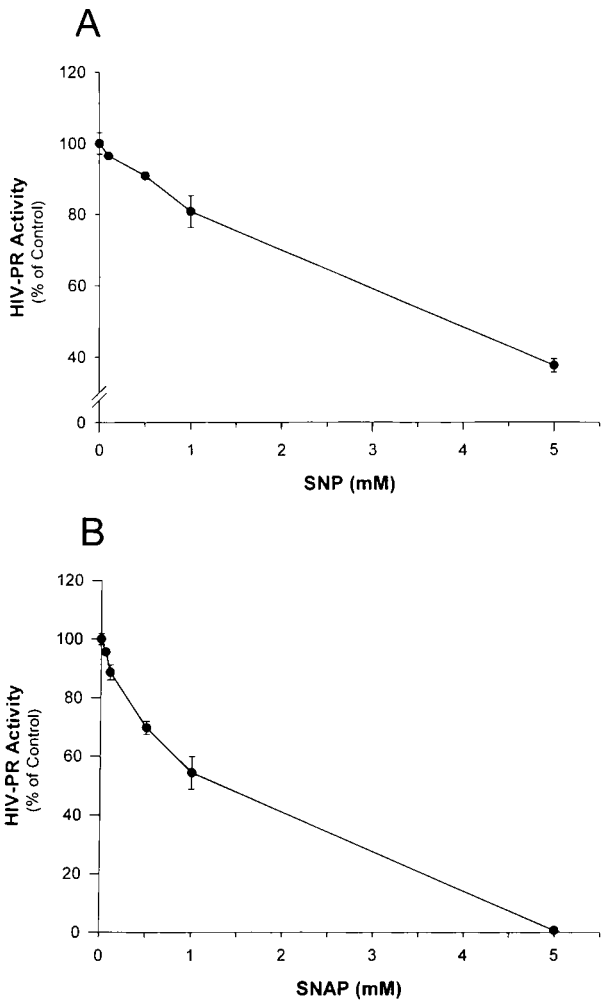

FIG. 3. SNP and SNAP inhibit HIV-PR. The activity of HIV-PR was analyzed in the presence of SNP (**A**) and SNAP (**B**). HIV-PR was incubated with the indicated concentrations of SNP and SNAP for 5 min at 37°C and the fluorescence was measured. Each data point in the concentration curve is an average of three experiments and plotted as the mean ± standard errors.

ment with NEM (mass = 125 Da). Cysteine residues were modified by incubating the enzyme for 1 hr with NEM. On complete modification, the monomer and dimer of HIV-PR acquired extra masses of 250 and 500 Da, respectively, which were due to the addition of two molecules of NEM to the monomer (new mass = 11,029 Da) and four molecules of NEM to the dimer (new mass = MW 22,058 Da). This confirms the specificity of NEM and our ability to measure it. When HIV-PR was allowed to react simultaneously with NO and NEM for 5 min, multiple species corresponding to the monomeric and dimeric forms were generated. The molecular mass of each species is shown in the table along with their modifications (Table 1). The number of new peaks, which appeared during simultaneous treatment, was limited by the number of cysteine residues present in the protease, either modified by NO or NEM. Addition of two NO molecules to the monomer prevented NEM molecules from binding to it and vice versa. Similarly, further addition of NEM molecules to the dimer could not be achieved on a fully NO-modified species and vice versa. These data confirm that NO binds directly to Cys67 and Cys95 of HIV-PR to form *S*-nitrosothiols.

DISCUSSION

The oxidized form of nitric oxide, NO^+, is known to react with the thiolate ion of cysteine residues in proteins resulting in a nitrosothiol adduct ($RSH + NO^+ \rightarrow \mathbf{RSNO} + H^+$). In biological systems, NO reacts with oxygen, superoxide, and transition metals, and products of each of these reactions further facilitate additional nitrosative reactions with proteins (Stamler *et al.*, 1992; Mohr *et al.*, 1994). Nitrosation has been shown to regulate activities of many cytosolic and membrane-bound proteins and enzymes (Stamler, 1994). Here we present evidence indicating that HIV-1 protease can be inhibited by NO by direct modification of its cysteine residues. Using SNP and SNAP as NO donors, we have shown that significant inhibition of HIV-PR activity can be achieved within 5 min at the same time as we detect *S*-nitrosation of cysteine residues. Therefore, it is likely that this enzymatic inhibition is caused by direct modification of cysteine residues.

The kinetics and amount of NO generated from SNP and SNAP are different. However, once produced from either of these sources, NO^+ reacts very fast with free thiol groups of proteins. This was reflected in our inhibition experiments where within 5 min 70% of HIV-PR activity was lost with SNP treatment and SNAP inhibited the enzyme completely. The difference of inhibition of HIV-1 protease by SNP and SNAP can be explained by the amount of NO^+ generated by these congeners. Using a NO probe, it was shown that 5 m*M* SNAP produces almost three-fold more NO than that seen with 5 m*M* SNP in 3 min (Matthews et al., 1996). The amounts varied between ~30 and 100 n*M*, which is within physiological range. Because the total NO generation from these congeners is a mixture of several species (NO, NO^+, NO^-), the amount of NO^+ in solution would be even less.

In a recent communication, Persichini et al. have shown that HIV-PR could be inactivated by the NO donor (*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR-3) (Persichini *et al.*, 1998). However, no analysis was performed to determine if the HIV-PR was modified by NOR-3 treatment. We used electrospray ionization–mass spectrometry to identify the NO-modified species of HIV-PR generated by SNP treatment, thereby providing direct evidence of their existence. In solution, monomer and dimer of HIV-PR exists in equilibrium. When treated with NO gas, modified species of both monomer and dimer are seen, their masses differing by multiples of the mass of NO. These data, supported by competition experiments with NEM, clearly establish that *S*-nitrosation of the HIV-PR occurs at cysteine residues. Complete modification of the enzyme was not achieved and the presence of unmodified forms of HIV-PR explain the residual activity of the protease after SNP treatment. Further studies are required to determine the order of modification of the cysteine residues and to elucidate individual contributions of each modification to the inhibition of HIV-PR.

PERSPECTIVE

NO plays important roles in maintaining host immunity. It exerts microbiostatic and microbicidal activity against a broad range of pathogens, including bacteria, fungi, and some viruses (Nathan and Hibbs, 1991; Torre and Ferrario, 1996). In the case of HIV-1 infection, monocytes/ macrophages play a crucial role in both latency and diffusion of the infection. These cells produce a significant amount (2–5 μM) of NO during HIV-1 infection (Bukrinsky *et al.*, 1995). Cysteine modifications seem to be a common way the activities of cellular proteins are regulated, either under normal physiological conditions or pathological situations, and it is becoming evident that NO is the molecule of choice for cysteine residue modification in cells (Lander, 1997). That HIV-1 protease is susceptible to inactivation by NO, together with the fact that NO produced by dendritic cells impairs the T-cell response (Bonham *et al.*, 1997), suggests that NO production is an important mediator of resistance of dendritic cells to HIV-1 infection.

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