Nitric Oxide Inhibits Dystrophin Proteolysis by Coxsackieviral Protease 2A Through S-Nitrosylation
A Protective Mechanism Against Enteroviral Cardiomyopathy

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Background—Infection with enteroviruses like coxsackievirus B3 (CVB3) as well as genetic dystrophin deficiency can cause dilated cardiomyopathy. We recently identified cleavage and functional impairment of dystrophin by the viral protease 2A during CVB3-infection as a molecular mechanism that may contribute to the pathogenesis of enterovirus-induced cardiomyopathy. Nitric oxide (NO) is elevated in human dilated cardiomyopathy, but the relevance of this finding is unknown. In mice, NO inhibits CVB3 myocarditis. Therefore, we investigated the effects of NO on the coxsackieviral protease 2A.

Methods and Results—In vitro, NO donors like PAPA-NONOate inhibited the cleavage of human and mouse dystrophin by recombinant coxsackievirus B protease 2A in a dose-dependent manner (IC50, 51 μmol/L). In CVB3-infected HeLa cells, addition of the NO donor SNAP inhibited protease 2A catalytic activity on dystrophin. Because this inhibitory effect was reversed by the thiol-protecting agent DTT, we investigated whether NO S-nitrosylates the protease 2A. In vitro, NO nitrosylated the active-site cysteine (C110) of the coxsackieviral protease 2A, as demonstrated by site-directed mutagenesis. Within living COS-7 cells, SNAP-induced S-nitrosylation of this site was confirmed with electron spin resonance spectroscopy.

Conclusions—These data demonstrate inactivation of a coxsackieviral protease 2A by NO through active-cysteine S-nitrosylation in vitro and intracellularly. Given that the enteroviral protease 2A cleaves mouse and human dystrophin, NO may be protective in human heart failure with an underlying enteroviral pathogenesis through inhibition of dystrophin proteolysis. (Circulation. 2000;102:2276-2281.)

Key Words: cardiomyopathy ■ nitric oxide ■ viruses

Congestive heart failure and enlargement of ventricular dimensions are hallmarks of dilated cardiomyopathy, a multifactorial disease of inherited and acquired pathogenesis.1-3 Many forms of familial dilated cardiomyopathy are due to defects of the extrasarcomeric myocyte cytoskeleton, whereas in acquired cardiomyopathy there is evidence of enteroviral infection, in particular with coxsackievirus B serotypes, in up to 30% of patients.3-5 By analogy to other virus-mediated illnesses, both the host’s immune response and direct viral effects play an important role in the pathogenesis of enteroviral heart disease.6 In mice, however, transgenic expression of coxsackievirus B3 (CVB3) proteins in the heart is sufficient to induce dilated cardiomyopathy.7

Among the coxsackieviral proteins are 2 proteases, protease 2A and protease 3C, both of which are essential for the viral life cycle.8 The viral protease 2A resembles serine-like proteases in overall folding, although it has a cysteine at its catalytic core.9 We have previously identified a molecular mechanism that may contribute to the pathogenesis of enterovirus-induced cardiomyopathy: dystrophin, a large cytoskeletal protein that connects the internal F-actin–based cytoskeleton to the plasma membrane-integrated dystrophin-associated glycoproteins and the extracellular laminin-2,10 is cleaved by the enteroviral protease 2A in the hinge 3 region.11 Dystrophin is functionally and morphologically disrupted in the hearts of mice infected with CVB3.12 Because genetic dystrophin deficiency causes familial dilated cardiomyopathy,13-15 the dystrophin cleavage in enteroviral cardiomyopathy suggests that both inherited and acquired forms of heart failure can occur secondarily either to defects in the transmission of mechanical force from the sarcomere to the extracellular matrix or to increased sarcolemmal permeability.10,16

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Nitric oxide (NO), an important regulator of multiple cardiovascular responses, is synthesized from l-arginine by 3 different isoforms of NO synthase (NOS). In contrast to the neuronal (NOS 1) and endothelial (NOS 3) isoforms, the inducible (NOS 2) isoform synthesizes large amounts of NO in a calcium-independent manner. In human dilated cardiomyopathy, increased systemic NO production, increased myocardial NOS 2 mRNA, and enzymatic activity have been reported. However, the pathophysiological role of NO in this context remains controversial. Increased cardiac NO can be myocytotoxic and reduce contractility. Conversely, NO inhibits CVB3 replication in vitro, and targeted disruption of the NOS 2 gene in mice leads to aggravated myocardial damage after infection with CVB3.

The mechanisms of the protective effect of NO in enteroviral cardiomyopathy are incompletely understood. Although NO exerts many of its physiological functions in a cGMP-dependent fashion, it can directly S-nitrosylate the sulfhydryl group of cysteine residues in proteins. This leads to enzyme inactivation in the case of cysteine proteases. Caspase-3, HIV protease, and the enteroviral protease 3C are inhibited by NO through this mechanism. Because the enteroviral protease 2A has a catalytic cysteine residue, we investigated the effects of NO on this protease.

Methods

Recombinant Coxsackievirus Proteases 2A

For determination of catalytic activity in vitro, recombinant purified protease 2A from coxsackievirus B4 was used as previously described. For the in vitro nitrosylation experiments, the VP1-2A region of a cardioptic H3 strain of CVB4 (protease 2A) was amplified by polymerase chain reaction from the infectious cDNA. The polymerase chain reaction product was cloned into the BamHI/EcoRI sites of pTrcHis2B (Invitrogen). Expression in Escherichia coli and purification with Ni-NTA-agarose beads were performed as described.

For expression in COS-7 cells, the VP1-2A region of CVB3 was similarly amplified (sense, 5'-GCGATGGATCCCTAC-GAGAAGGCAAGAAGGTCG-3'; antisense, 5'-CTTCGAATCTTGATGATGGATC-3'). The polymerase chain reaction product was cloned into the BamHI/EcoRI sites of pTrcHis2B (Invitrogen). Expression in COS-7 cells was performed as described. For expression in COS-7 cells, the VP1-2A region of CVB3 was similarly amplified (sense, 5'-ATGGGAATTTCTACGAGAGGCAAGAACGTG-3'; antisense, 5'-ATTCCTCGAGTCCGCCATGGATC-3') and cloned into the EcoRI/XbaI sites of pcDNA3.1 mycHisA (Invitrogen). Between the cytomegalovirus promoter and the start of the VP1 coding sequence, either a consensus mammalian translational initiation sequence or the internal ribosomal entry site (IRES) from encephalomyocarditis virus (EMC) virus located in plasmid pCTE4b (Novagen) (nucleotides 16 to 530) was cloned into the BamHI/EcoRI sites. Site-directed mutagenesis of the active-site cysteine was performed with the Quick Change Mutagenesis Kit (Stratagene). All sequences were verified by DNA sequencing.

NO Donors and In Vitro Dystrophin Cleavage Assay

Various concentrations of PAPA-NONOate, spermine-NONOate, and SNAP (all Cayman Chemical) were incubated with protease 2A (500 ng) in modified protease 2A cleavage buffer (mmol/L: KCl 100, NaCl 50, Tris-HCl 80 [pH 7.3], CaCl2 1, and EDTA 0.1, and 0.1% NP-40) at 37°C for 1 hour to allow release of NO from the donors. Subsequently, a human (amino acids 2091 to 2603) or mouse (amino acids 2093 to 2759) dystrophin miniprotein containing the hinge 3 region, synthesized in vitro (TnT T7, Promega) with 35S-labeled methionine, was added. After incubation for 60 minutes at 30°C, cleavage was detected by autoradiography.

Cell Culture and Virus Infection

HeLa and COS-7 cells were cultured and infected with CVB3 as described. CVB3 was derived from the infectious cDNA of the cardiotropic H3 strain of CVB3 and was titered by plaque-forming assay. SNAP was added 1 hour after infection as described. Dystrophin cleavage activity was measured in HeLa cell extracts prepared in protease 2A cleavage buffer as above.

Western Blotting

Immunoblotting of HeLa cell extracts was performed as previously described with the following primary antibodies: monoclonal anti-myc (9E10), polyclonal anti-myc (Santa Cruz), or polyclonal anti-protease 2A. Bound antibodies were detected by chemiluminescence (Amersham).

Detection of S-Nitrosylation

In vitro, S-nitrosylation was measured with the Saville assay. In COS-7 cells, S-nitrosylation was determined by immunoprecipitation of transfected protease 2A, followed by NO spin-trap and cryogenic electron-spin-resonance (ESR) spectroscopy as described in detail elsewhere.

Results

NO Inhibits Dystrophin Proteolysis by Coxsackievirus Protease 2A In Vitro

To measure the susceptibility of the coxsackievirus protease 2A to NO, we preincubated different NO donors, PAPA-NONOate, spermine-NONOate, and SNAP, with the protease 2A before the addition of a recombinant dystrophin substrate. As the dystrophin protein substrate, we used a mouse or human dystrophin miniprotein with the mapped protease 2A cleavage site in the hinge 3 region.

PAPA-NONOate (Figure 1A), spermine-NONOate, and SNAP (data not shown) all inhibited the cleavage of mouse and human dystrophin by protease 2A with a similar dose-dependency. Quantitative densitometric assessment of the protease 2A cleavage activity relative to the control without NO donor (Figure 1B) showed that half-maximal inhibition of protease 2A catalytic activity (IC50) occurred with 51 μmol/L NO donor. The control substances without nitroso groups, PAPA, spermine, and N-acetylpenicillamine, did not have an inhibitory effect (data not shown). Interestingly, DTT, a thiol-protecting agent, completely reversed the NO inhibition of protease 2A (Figure 1A, right lane). Because S-nitrosylation reactions are characterized by their reversibility, this suggests that NO may inhibit coxsackieviral protease 2A by S-nitrosylating and thereby inactivating cysteine residues such as the essential catalytic C110.

NO S-Nitrosylates the Catalytic Cysteine of Coxsackievirus Protease 2A In Vitro

To determine whether NO S-nitrosylates coxsackieviral protease 2A, the protease was expressed in E coli as a precursor protein attached to the end of the capsid protein VP1 with a carboxy-terminal (His6) tag for purification (Figure 2A). Active protease 2A cleaves itself away from VP1, yielding the mature enzyme that migrates faster on SDS-PAGE. Figure 2B shows that purified wild-type protease 2A (top) is catalytically active, whereas replacement of the active-site cysteine by serine (C110S) renders the protease nearly inactive (bottom). Thus, perturbation of this cysteine severely inhibits protease 2A cleavage activity.
Both PAPA-NONOate (Figure 2C) and spermine-NONOate (data not shown) led to the formation of \(1 \text{ mmol S-nitroso groups/mmol wild-type protease 2A}\) in the presence of a molar excess of NO. This indicates that a single cysteine residue out of the total 6 cysteine residues in the protease 2A is the primary target of S-nitrosylation. Because the amino acids surrounding the active-site cysteine fit the S-nitrosylation consensus motif\(^{33}\) (Figure 2A, bottom), we hypothesized that the active-site cysteine might be the target of S-nitrosylation. Consistent with this hypothesis, the C110S mutant is S-nitrosylated only slightly above background levels (Figure 2C). These data show that NO S-nitrosylates the active-site cysteine of the enteroviral protease 2A in vitro.

**NO Inhibits Protease 2A Activity in CVB3-Infected HeLa Cells**

Next, we investigated whether NO also has an inhibitory effect on the viral protease 2A in CVB3-infected cells. We elected to use HeLa cells, an established cell culture model of CVB3 infection.\(^{23,30}\) Because HeLa cells do not express dystrophin, protease 2A cleavage activity on dystrophin was measured in HeLa cell extracts with the mouse dystrophin miniprotein.\(^{11}\)

As shown in Figure 3, lysate from uninfected HeLa cells did not contain any dystrophin cleavage activity or coxsackieviral protease 2A. In contrast, lysate prepared 8 hours after infection with CVB3 cleaved dystrophin and showed expression of coxsackieviral protease 2A. The dystrophin fragments obtained after incubation with HeLa lysates are identical to the ones produced by addition of purified coxsackievirus protease 2A (Figure 1B), demonstrating that the dystrophin cleavage activity in coxsackievirus-infected cells is due to the viral protease 2A. Addition of the NO donor SNAP to the HeLa cells 1 hour after CVB3 infection led to a dose-dependent decrease of dystrophin proteolysis by the viral protease 2A. Substantial inhibition of the dystrophin proteolysis was obtained with 125 \(\mu\text{mol/L}\) SNAP, a dose well below cytotoxic levels. At this concentration, the inhibitory effect of SNAP on the dystrophin cleavage was clearly greater than the...
reduction of protease 2A expression (Figure 3B). This suggested a direct inhibition of protease function by SNAP. Higher SNAP concentrations also significantly reduced protease 2A expression, and this mechanism may contribute to the inhibitory effect of SNAP on the dystrophin cleavage.

These results demonstrate that NO inhibits the dystrophin cleavage activity of the viral protease 2A during CVB3 infection of cultured HeLa cells. Furthermore, these results suggest a direct effect of NO on protease 2A function during viral infection.

NO S-Nitrosylates the Catalytic Cysteine of CVB3 Protease 2A in COS-7 Cells

Next, we investigated whether the active-site cysteine of coxsackie B virus protease 2A can be S-nitrosylated by NO within cells. To determine the effects of NO on coxsackievirus protease 2A independent of confounding factors stemming from the viral infection, we expressed recombinant protease 2A in COS-7 cells, in which high expression levels can be achieved.

Expression vectors of the VP1-2A region of CVB3 were generated containing the myc-tagged protease 2A behind a consensus mammalian translational initiation sequence or the IRES from EMC virus as alternative translation initiation signals (Figure 4A). Figure 4A shows that in contrast to the inactive mutant (C110S), the active wild-type protease 2A cannot be expressed from a consensus mammalian translational initiation sequence in COS-7 cells, in which high expression levels can be achieved.

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In contrast, cap-independent translation of the wild-type protease 2A from the EMCV IRES results in expression in COS-7 cells.

Using this strategy, we investigated S-nitrosylation of coxsackievirus protease 2A in COS-7 cells. A, COS-7 cells were transiently transfected with constructs indicated, and at 24 or 36 hours later, cell extracts separated by SDS-PAGE were immunoblotted for myc-tagged protease 2A (top) or actin (to demonstrate equal loading, bottom). Expression of IRES-driven wild-type protease 2A is possible in COS-7 cells. B, Wild-type (wt) and mutant (C110S) CVB3 protease 2A were transiently transfected into COS-7 cells, and 24 hours later, cells were incubated with 500 μmol/L SNAP for 3 hours in some cases. myc-tagged protease 2A constructs were then immunoprecipitated (IP) with a mouse monoclonal antibody and detected (Western blot, WB) with a rabbit polyclonal antibody directed against myc tag. Equal levels of protease 2A from treated vs untreated cells were immunoprecipitated. C, ESR spectra (same samples as in B). Note triphasic signal demonstrating S-nitrosylation of wild-type coxsackievirus protease 2A. Representative result from 3 independent experiments.
S-nitrosylated within living cells and confirm the active-site cysteine as the primary residue of intracellular S-nitrosylation.

Discussion

The main finding of the present study is that NO inhibits dystrophin proteolysis by the coxsackieviral protease 2A in vitro and during CVB3 infection in cell culture. NO S-nitrosylates the active-site cysteine in vitro and within cells, suggesting a molecular mechanism for the NO-mediated inhibition of coxsackievirus protease 2A.

The transfer of NO to cysteine sulfhydryl groups inactivates a number of cysteine proteases. Because the viral protease 2A is also a cysteine protease, we hypothesized that it might be a target of S-nitrosylation as well. Indeed, NO inhibits dystrophin proteolysis by purified coxsackieviral protease and S-nitrosylates the active-site cysteine (C110) in vitro. Because C110 is critical for protease 2A activity, nitrosylation at this site represents a molecular mechanism by which NO inactivates coxsackieviral protease 2A. Because the amino acids surrounding the active-site cysteine are entirely conserved among all known enteroviral proteases 2A,9 the protease 2A from all enteroviruses should be subject to cysteine nitrosylation.

During CVB3 infection of cultured HeLa cells, NO dose-dependently inhibited the protease 2A cleavage activity on dystrophin. Furthermore, the active-site cysteine of coxsackievirus protease 2A was found to be S-nitrosylated within COS-7 cells by NO. To the best of our knowledge, the enteroviral 2A is only the second protease (after caspase-3)7.26) for which S-nitrosylation has been demonstrated to occur intracellularly in mammalian cells. The same effects of NO on coxsackieviral protease 2A may be expected to occur in other virally infected cells, such as cardiomyocytes.

NO has been shown to inhibit coxsackieviral replication in cultured cells23 and mice,24,37 and this effect has been attributed, at least in part, to inhibition of the viral protease 3C.30 Given the facts that NO inhibits protease 2A catalytic activity on dystrophin during viral infection and that protease 2A is 20-fold more sensitive to NO inhibition than protease 3C in vitro,30 we propose that inhibition of the viral protease 2A may participate in the antiviral effect of NO during coxsackievirus infection. Because both enteroviral proteases are inactivated by S-nitrosylation, it appears that NO has a dual protective role against enteroviral infection.

We have previously demonstrated cleavage and functional disruption of dystrophin in mouse hearts infected with CVB3 and proposed that this may contribute to the pathogenesis of enterovirus-induced dilated cardiomyopathy.12 NO inhibits dystrophin proteolysis in the hinge 3 region and thus prevents functional dystrophin impairment.11 Intact dystrophin is required for physiological sarcolemmal integrity and is thought to contribute to the transmission of mechanical force from the sarcomere to the extracellular matrix.10 NO may prevent disruption of these important dystrophin functions during CVB3 infection. Inhibition of the dystrophin proteolysis by enteroviral protease 2A represents a molecular mechanism through which NO may protect against enteroviral cardiomyopathy. Consistent with this concept, pharmacological inhibition of endogenous NO production or NOS 2 gene deletion in mice results in aggravated myocardial damage.23,37 Our data indicate that increased NO levels in human dilated cardiomyopathic hearts may be beneficial in cases with an underlying enteroviral pathogenesis.

In summary, active-site cysteine nitrosylation of the enteroviral protease 2A inhibits dystrophin proteolysis in vitro and in virally infected cells. These data suggest a molecular mechanism by which NO may protect against enterovirus-induced dilated cardiomyopathy.

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