Short Communication

Proteasome Inhibition Reduces Coxsackievirus B3 Replication in Murine Cardiomyocytes

Honglin Luo, Jingchun Zhang, Caroline Cheung, Agripina Suarez, Bruce M. McManus, and Decheng Yang

From the McDonald Research Laboratories/The iCAPTURE⁴E Center, Department of Pathology and Laboratory Medicine, St. Paul's Hospital/Providence Health Care, University of British Columbia, Vancouver, British Columbia, Canada

Coxsackievirus is the most prevalent virus associated with the pathogenesis of myocarditis and its sequela dilated cardiomyopathy. We have previously shown that coxsackievirus infection facilitates the ubiquitin/proteasome processing of the cell-cycle protein cyclin D1 and the tumor suppressor p53, which raises the possibility that the ubiquitin/proteasome pathway may be used by virus to promote viral replication. In this study, we examined the interplay between coxsackievirus replication and the ubiquitin/proteasome pathway in murine cardiomyocytes. We found that treatment of cells with the proteasome inhibitors MG132 or lactacystin significantly decreased virus titers in the supernatant and prevented virus-induced cell death. We further examined the effects of proteasome inhibitor on different stages of coxsackievirus life-cycle. We showed that inhibition of the ubiquitin/proteasome pathway did not affect virus entry and had no influence on viral protease proteolytic activities. However, viral RNA transcription and protein translation were markedly reduced after addition of proteasome inhibitors. We further demonstrate that ubiquitin/proteasome pathway-mediated viral replication does not appear to be related to changes in proteasome activities. Taken together, our data suggest that proteasome inhibitor reduces coxsackievirus replication through inhibition of viral RNA transcription and protein synthesis. Thus, proteasome inhibition may represent a novel therapeutic approach against myocarditis. (*Am J Pathol* 2003, 163:381-385)

Coxsackievirus B3 (CVB3), a member of the *Picornaviridae* family, is the primary causative agent of viral myo-

carditis.¹ Viral myocarditis leads to dilated cardiomyopathy, which may result in sudden, unexpected death in children and adolescents or end-stage congestive heart failure in adults.² The only definitive treatment currently available for this condition is heart transplantation. Understanding the interplay between virus and target host signaling will provide novel insights into the pathogenic mechanisms involved in viral myocarditis.

The ubiquitin/proteasome pathway is a major intracellular protein degradation pathway in eukaryotic cells.³ Proteasomes are large intracellular protein complexes that catalyze the rapid degradation of abnormal proteins or short-lived regulatory proteins. Substrates are first conjugated to multiple units of the polypeptide ubiquitin, and then degraded by the proteasome. In addition to disposal of damaged, misfolded, or unnecessary proteins, this pathway has been found to be involved in various intracellular functions, including cell-cycle regulation, apoptosis, antigen processing, and transcriptional regulation, processes important in the progression of many diseases.^{4,5} For example, several cell-cycle proteins, including cyclins, cyclin-dependent kinase inhibitors (p21, p27), and tumor suppressors (p53) are all substrates of the ubiquitin/proteasome pathway.^{3,4,5}

In addition to the well-known role of polyubiquitination in protein degradation, monoubiquitination of some proteins, such as calmodulin, histones, actin and some membrane receptors, has been suggested to regulate protein function, including membrane envelopment and histone-mediated transcriptional regulation, and monoubiquitination is not evidently involved in cytosolic degradation.⁶

We have previously shown that the cell-cycle protein cyclin D1 and the tumor suppressor p53 are down-regulated during CVB3 infection of HeLa cells.⁷ This reduction is abrogated when specific inhibitors of the ubiquitin/

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Address reprint requests to Honglin Luo, McDonald Research Laboratories/The iCAPTURE⁴E Center, University of British Columbia-St. Paul's Hospital, 1081 Burrard St., Vancouver, B.C., Canada V6Z 1Y6. E-mail: hluc@mrl.ubc.ca.

proteasome pathway are used. Perhaps most interestingly, we have shown evidence that CVB3 facilitates the ubiquitination of cyclin D1, which suggests a direct mechanism of protein degradation during CVB3 infection. Our results raise the possibility that the ubiquitin/proteasome pathway may be used during CVB3 infection to promote viral replication and infectivity. Thus, in the present study, we attempted to explore the role of proteasome inhibition in CVB3 infectivity and the mechanisms contribute to proteasome inhibitor suppression of CVB3 replication in a murine cardiomyocyte culture model.

Materials and Methods

Cell Culture

The immortalized cardiomyocyte HL-1 cell line, generously provided by Dr. William C. Claycomb (Louisiana State University Health Science Center), was established from mouse atrial cardiomyocyte tumors.⁸ These cells proliferate and can be serially passaged, while maintaining the ability to contract and retaining the differentiated cardiomyocyte phenotype. HL-1 cells were grown in Claycomb medium (JRH Biosciences, Lenexa, KS) containing 10% fetal bovine serum, 0.1 mmol/L norepinephrine, and 2 mmol/L L-glutamine.

Virus Infection

HL-1 cells were infected at a multiplicity of infection (MOI) of 50 to 100 with CVB3 (Kandolf strain) or sham-treated with phosphate-buffered saline (PBS) for 1 hour. Cells were washed with PBS and cultured in fresh Claycomb medium containing 10% fetal calf serum. For inhibitor experiments, HL-1 cells were preincubated with proteasome inhibitor MG132 (BIOMOL, Plymouth Meeting, PA) or lactacystin (Calbiochem, San Diego, CA) for 30 minutes. Cells were then infected with CVB3 for 1 hour, washed with PBS, and placed in Claycomb media containing fresh inhibitor.

In Situ Hybridization

In situ hybridization was performed as previously described with slight modification.⁹ Fixed cells were hybridized with digoxigenin-labeled CVB3 antisense riboprobes, which were prepared from the full-length CVB3 cDNA using an *in vitro* transcription kit according to the manufacturer's instructions (Promega, Madison, WI). Hybridized riboprobes were detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche, Basel, Switzerland) and a color substrate Vector Red (Vector Laboratories, Burlingame, CA). Cells were counterstained with hematoxylin.

Western Blot Analysis

Western blot was performed as previously described.⁷ Equal amounts of protein were subjected to SDS-PAGE

and then transferred to nitrocellulose membranes. The membrane was blocked with 5% nonfat dry milk solution containing 0.1% Tween 20 for 1 hour. The blot was then probed for 1 hour with the rabbit polyclonal anti-CVB3 VP1 antibody (Accurate Chemical & Scientific, Westbury, NY), followed by incubation for 1 hour with horseradish peroxidase-conjugated secondary antibody. VP1 protein expression was visualized by chemiluminescence (Amersham Biosciences Inc.).

Plaque Assay

The amount of CVB3 produced was measured on monolayers of HeLa cells by agar overlay plaque assay of supernatant cultures as previously described.⁹ Cell supernatant was serially diluted and overlaid on monolayers of HeLa cells. Following 1 hour of incubation, medium was removed and complete Dulbecco's modified Eagle's medium containing 0.75% agar was overlaid. Three days post-infection, cells were fixed with Carnoy's fixative (25% acetic acid, 75% ethanol) and then stained with 1% crystal violet. Viral titer was determined as plaque forming unit (PFU) per milliliter.

Cell Viability Assay

A modified 3, 4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay, which measures mitochondrial function, was used to determine cell viability according to the manufacturer's instruction (Promega). Sixteen hours post-infection, cells were incubated for 2 hours in MTS solution, and absorbance was measured at 490 nm with an ELISA reader. CVB3-infected HL-1 cells were also examined for morphological changes by phase-contrast microscopy.

Viral Proteolytic Processing

Viral proteolytic activity was determined by pulse-chase metabolic labeling as described previously.¹⁰ Briefly, HL-1 cells were labeled with 150 μ Ci/ml of [³⁵S] methionine for 1 hour, and the synthesis of viral proteins was analyzed by SDS-PAGE with subsequent autoradiography. Proteolytic activity was quantitated by measuring the ratio of viral precursor polyprotein to processed protein products.

Proteasome Activity

Fresh cytoplasmic extracts were used to measure proteasome activity as described previously.¹¹ Briefly, cytoplasmic proteins were incubated with assay buffer (20 mmol/L Tris-HCl, pH 8.0, 1 mmol/L adenosine triphosphate, and 2 mmol/L MgCl₂) in the presence of the synthetic fluorogenic substrate for the proteasome (Suc-Leu-Leu-Val-Tyr-AMC; Calbiochem) at 30°C for 30 minutes. The fluorescence product AMC in the supernatant was measured at 460-nm emission, using a fluorometer.

Results and Discussion

Low molecular weight inhibitors of the proteasome have been widely used in basic research and in the clinical trials of several diseases. Of appreciable promise is a potential therapeutic use in cancer treatment. In fact, phase II evaluation of proteasome inhibitors as anti-tumor agents for human cancer therapy is currently underway.¹² A number of distinct mechanisms might contribute to the anti-neoplastic or pro-apoptotic effects of proteasome inhibitors, such as the ability for accumulation of p53, p27, activation of c-Jun N-terminal kinase, inhibition of NF κ B nuclear translocation and activation of the receptor-mediated apoptotic pathway.^{3,4,5} However, thus far the potential role of proteasome inhibitor in viral infection remains largely unknown. In this study, we used two chemically distinct proteasome inhibitors, MG132 and lactacystin, to explore the effect of proteasome inhibition on CVB3 replication. The peptide-aldehyde MG132 is a highly potent and reversible inhibitor of the chymotryptic-like activity of the proteasome.¹³ In contrast, lactacystin is highly specific and inhibits the proteasome irreversibly by covalent modification of the proteasome β -subunits.¹³ Murine cardiomyocytes, HL-1 cells, were incubated with varying concentrations of proteasome inhibitors (MG132 and lactacystin) and then infected with CVB3. Sixteen hours post-infection, supernatant was collected and plaque assay was performed to determine the effects of these proteasome inhibitors on viral progeny release. As shown in Figure 1A, proteasome inhibition reduced the titer of released virus in the supernatant in a dose-dependent manner. Consistent with previous reports, the effect of MG132 on reducing viral replication seems to be more potent than lactacystin.¹³ It should also be noted that throughout all doses of proteasome inhibitors used in this study, cell viability assays and morphological assessment showed no detectable cell death in HL-1 cells. In contrast, CVB3-induced cytopathic effect and cell death were dramatically blocked after the addition of MG132 (Figure 1, B and C). Our data suggest that proteasome inhibitors decrease CVB3 infectivity and the anti-apoptotic effect of the proteasome inhibitor may be due to reduced viral replication.

The CVB3 life-cycle consists of several steps: viral attachment to the host cells, internalization, translation and proteolytic processing of the viral polyprotein, replication of the RNA genome via a RNA intermediate, virion assembly, and release from the host cells. To further determine which step(s) in the virus life-cycle the proteasome inhibitors target. We first examined whether the reduction of viral titers in the supernatant by proteasome inhibitors is due to the blockade of virus entry, MG132 was given either 30 minutes before virus infection or 1 hour post-infection (after virus entry into host cells). Figure 2A shows that exposure to proteasome inhibitor MG132 1 hour post-infection significantly inhibited viral protein production, almost to the same extent as that observed when MG132 was added before infection, suggesting that inhibition of viral replication by a proteasome inhibitor is likely not dependent on the blockade of viral entry into host cells.

Figure 1. Proteasome inhibition reduces CVB3 infectivity. **A:** HL-1 cells were preincubated with different concentrations of proteasome inhibitors, MG132 and lactacystin, for 30 minutes and then infected with CVB3 (MOI = 50) for 1 hour. Medium was collected from CVB3-infected HL-1 cells at 16 hours post-infection and virus titers were determined by plaque assays on HeLa cell monolayers. Values are means $\pm$ standard errors (SE) of three independent experiments. **B:** HL-1 cells were pretreated with various concentrations of MG132 and then infected with CVB3 as described above. Cell viability was assessed by the MTS assay at 16 hours post-infection. The values of MTS in sham-infected cells in the absence of MG132 were defined as 100% survival. Values are means $\pm$ SE ($n = 4$). **C:** Representative morphological changes of HL-1 cells treated with MG132 (20 μ mol/L) or vehicle (DMSO) at 16 hours post-infection by phase-contrast microscopy.

We further assessed the effect of proteasome inhibitors on viral RNA transcription and protein translation by *in situ* hybridization and Western blot. Treatment with proteasome inhibitors MG132 or lactacystin dose-dependently decreased viral RNA (Figure 2B) and protein expression (Figure 2C), suggesting that proteasome inhibitors decreased viral replication via suppression of viral RNA transcription and protein translation.

Although our data demonstrate that proteasome inhibitors reduce both viral RNA and protein levels, we have not yet determined whether the reduction in viral RNA is due to a direct inhibition of viral RNA transcription or due to a decrease in viral protein translation which leads to reduced synthesis of viral polymerase. Meanwhile, we do not yet know whether the reduction in viral proteins is secondary to a decrease in viral RNA transcription or due to a direct inhibition of viral protein translation.

The ubiquitin/proteasome pathway has been implicated in a wide variety of cellular processes. Most re-

cently, studies by three independent groups have found that ubiquitin is involved in a late stage of the retroviral life-cycle, specifically, viral budding and release.^{14–16} They showed that proteasome inhibition interferes with the processing of viral Gag polyproteins and decreases release and infectivity of secreted virions. They further demonstrated that proteasome inhibition reduced the

Figure 3. Proteasome activity following CVB3 infection of HL-1 cells. At different times after CVB3 infection (MOI = 100) in the present or absence of proteasome inhibitor MG132, cell lysates were collected and proteasome activity was measured using the fluorogenic substrate SLLVY-AMC. Results are expressed as the amount of AMC formed by the enzymatic cleavage of substrate. Results are mean $\pm$ SE of three independent experiments.

level of free ubiquitin in human immunodeficiency virus type-1 infected cells and prevented monoubiquitination of p6^{Gag}. However, to our knowledge, our results are the first to demonstrate that the ubiquitin/proteasome pathway is involved in virus replication.

Viral proteases 2A and 3C are responsible for the cleavage of viral precursor polyproteins into structural and enzymatic proteins, which are essential for viral replication. To determine whether the inhibitory effects of proteasome inhibitors on CVB3 replication depend on viral protein 2A and 3C protease activities, we examined the effect of proteasome inhibitors on the proteolytic processing. As shown in Figure 2D, addition of proteasome inhibitors (MG132 and lactacystin) led to a dramatic reduction of processed viral protein products (VP1, 3D, and 3CD). However, we did not observe an accumulation of viral precursor polyproteins (P1) which has been previously shown to occur with the protease 3C inhibitor.¹⁰ These data suggest that it is unlikely that proteasome inhibitors target the proteolytic activities of viral proteases.

The molecular mechanism underlying regulation of the ubiquitin-proteasome system can occur at two levels: the

Figure 2. Proteasome inhibition decreases viral RNA transcription and protein translation in CVB3-infected HL-1 cells. **A:** HL-1 cells were either preincubated 30 minutes before infection (lane 3) or 1 hour post-infection (lane 4) with MG132. Six hours post-infection (MOI = 100), cell lysates were harvested and Western blot was performed using a polyclonal antibody that recognizes CVB3 capsid protein VP1. These same blots were stained with an antibody to α -tubulin to illustrate protein loading. **B:** HL-1 cells were treated and infected as described above. Six hours post-infection, positive-strand viral RNA was determined by *in situ* hybridization using anti-sense riboprobes for CVB3 (red). Cell nuclei were counterstained with hematoxylin (blue). Scale bar, 50 μ m. **C:** HL-1 cells were preincubated with various concentrations of proteasome inhibitors, MG132 (**top**) and lactacystin (**bottom**), in an identical manner as described above. Cell lysates were collected 6 hours post-infection and Western blot detection for viral capsid protein VP1 was performed. These same blots were stained with an antibody against α -tubulin to illustrate equal protein loading. **D:** HL-1 cells were preincubated with proteasome inhibitors, MG132 (10 μ mol/L) and lactacystin (20 μ mol/L) for 30 minutes and then infected with CVB3 (MOI = 100). Viral proteolytic activity was determined by pulse-chase metabolic labeling at 6 hours post-infection as described in Materials and Methods. The masses of protein markers are indicated.

ubiquitination process and/or the proteasome degradation.^{3,4,5} We first examined the proteasome activity following CVB3 infection and to determine the effect of proteasome inhibitors on its activity. As shown in Figure 3, as compared to sham-infected samples, proteasome activity was unchanged in CVB3-infected cells. In contrast, proteasome inhibitor MG132 dramatically inhibited proteasome activity in both sham- and CVB3-infected cells. This observation suggests that a precursor step, eg, ubiquitination, may play a role in the ubiquitin/proteasome pathway-mediated CVB3 replication or protein degradation. Indeed, we have previously shown that CVB3 infection facilitated ubiquitination of cyclin D1⁷ and our studies using Affymetrix microarray technology has demonstrated that the expression of several ubiquitin-related genes were up-regulated following CVB3 infection, such as human ubiquitin-like protein (unpublished data).

How does the ubiquitin/proteasome pathway regulate CVB3 viral replication? Perhaps the ubiquitination of viral proteins is required for ubiquitin/proteasome-mediated viral replication, such as viral polymerase 3D which is essential for initiating viral RNA replication. Alternatively, ubiquitination and degradation of certain intracellular components may target specific aspects of the viral replication process controlling viral replication. For example p53, which is largely regulated through the ubiquitin/proteasome pathway, has been suggested to be involved in viral replication in a number of viruses.^{17,18} In addition, we have previously shown that inhibition of the extracellular signal-related kinases (ERK1/2) signaling pathway reduces CVB3 replication.¹⁹ Therefore, it may also be speculated that proteasome inhibitor-induced inactivation of ERK1/2 contribute in part to its antiviral effect.²⁰

It has been reported that cells arrested at G1 or G1/S phase produce high levels of CVB3.²¹ Dysregulation of cell-cycle progression may affect CVB3 replication. Proteasome inhibitors prevent cell-cycle progression and induce G1 arrest by increasing the cellular levels of p53 and its target gene product p21.^{22,23} Thus, it is unlikely that the inhibition of viral replication by proteasome inhibitors is due to cell-cycle dysregulation.

In conclusion, we have demonstrated here that inhibition of the ubiquitin/proteasome pathway effectively reduces CVB3 replication in a murine cardiomyocyte culture model as determined by decreases in both viral RNA production and protein synthesis. Our results suggest that the ubiquitin/proteasome pathway may be a novel therapeutic target by which to mitigate viral myocarditis.

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