In situ dynamically monitoring the proteolytic function of the ubiquitin-proteasome system in cultured cardiac myocytes

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Dong, Xin, Jinhao Liu, Hanqiao Zheng, Joseph W. Glasford, Wei Huang, Quan Hai Chen, Niels R. Harden, Faqian Li, A. Martin Gerdes, and Xuejun Wang. In situ dynamically monitoring the proteolytic function of the ubiquitin-proteasome system in cultured cardiac myocytes. Am J Physiol Heart Circ Physiol 287: H1417–H1425, 2004. First published April 22, 2004; 10.1152/ajpheart.01233.2003.—The ubiquitin-proteasome system (UPS) is responsible for turnover of most cellular proteins in eukaryotes. Protein degradation by the UPS serves quality control and regulatory functions. Proteasome inhibition showed great promise in effectively treating cancer and restenosis. UPS dysfunction in cardiac hypertrophy and failure has recently been suspected but remains to be investigated. A system capable of monitoring dynamic changes in proteolytic function of the UPS in cardiac myocytes in situ would offer a powerful tool to further investigate UPS dysfunction in the heart and to evaluate the effect of proteasome inhibition on cardiac myocytes. We successfully established such a system in cultured cardiac myocytes by delivering and expressing a modified green fluorescent protein (GFPu) gene using recombinant adenoviruses. GFPu contains a ubiquitination signal sequence fused to the COOH terminus. Fluorescence microscopy and Western blots revealed that protein abundance of modified green fluorescent protein (GFPu), but not wild-type green fluorescent protein, in cultured cardiac myocytes was incrementally increased when function of the proteasomes was inhibited in various degrees by specific inhibitors. The increase in GFPu protein levels and fluorescence intensity is paralleled by a decrease in the in vitro peptidase activity of the proteasomes. Our results demonstrate that GFPu can be used as a surrogate marker to monitor dynamic changes in proteolytic function of the UPS in cultured cardiac myocytes. Application of this novel system reveals that moderate levels of H2O2, a reactive oxygen species generator, impair proteolytic function of the UPS in cultured cardiac myocytes.

protein degradation; proteasome inhibition; adenoviruses; tissue culture; rat

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the Ubc6 and/or Ubc7 subpathways of the UPS in yeast (7, 8). It was recently proven that the CL1 degron destabilizes a stable green fluorescent protein (GFP) in a UPS-dependent fashion in human embryonic kidney (HEK) cells when fusing to the COOH terminus of GFP (2). This suggests that mammalian cells possess types of Ubc that function similarly to Ubc6 and Ubc7 in yeast.

UPS function has been implicated in a diverse set of cellular processes, including turnover of oxidized, gap junction, and myofibrillar proteins, regulation of NF-κB-dependent stress response pathways, and, more recently, apoptosis (24). Proteasome impairment has shown great promise in effectively treating cancer and preventing restenosis (21, 26, 36). Meanwhile, UPS malfunction has been implicated in neural degenerative diseases (2). Coronary occlusion-reperfusion was shown to lead to the dynamic changes in the proteolytic function of the UPS and inactivation of the proteasome, especially (2). Coronary occlusion-reperfusion was shown to lead to the dynamic changes in the proteolytic function of the UPS and inactivation of the proteasome, especially (2). Oxidative modification and inactivation of the proteasome, suggesting that acquired suppression of the proteasome might be involved in the pathogenesis of cardiac ischemia-reperfusion injury (4). UPS dysfunction in cardiac hypertrophy and failure has recently also been suspected but remains to be investigated (11, 18). Therefore, a system capable of monitoring the dynamic changes in the proteolytic function of the UPS in cardiac myocytes in situ would no doubt benefit significantly the efforts to decipher the pathogenic significance of UPS dysfunction in the heart and to evaluate the effect of proteasome inhibition on the heart. We recently successfully established such a system in cultured neonatal rat cardiac myocytes by delivering and expressing a modified GFP (GFPu) gene using recombinant adenoviruses. Using this novel system, we further demonstrated that administration of moderate levels of a reactive oxygen species (ROS) generator, H$_2$O$_2$, led to impairment of proteolytic function of the UPS in cultured cardiac myocytes.

**MATERIALS AND METHODS**

*Recombinant adenoviruses.* A plasmid harboring a modified enhanced GFP (EGFP) coding sequence behind a cytomegalovirus promoter was generously provided by Dr. Ron Kopito (Stanford University). In this modified EGFP, a ubiquitination signal sequence (CL1 degron, ACKNWFSSLSHFVIHL) was fused to the COOH terminus of a conventional EGFP (2). This modified EGFP is referred to as GFPu. Recombinant adenoviruses harboring the GFPu expression cassette (Ad-GFPu) were created using the AdEasy adenoviral vector system (Stratagene, Cedar Creek, TX) and accompanying protocol (10). Recombinant adenoviruses that harbor β-galactosidase or wild-type EGFP (Ad-β-Gal or Ad-EGFP, respectively) were generously gifts from Qiangrong Liang (Cardiovascular Research Institute, University of South Dakota School of Medicine). Infection of cultured cardiac myocytes with any of these recombinant adenoviruses at an indicated multiplicity of infection (MOI) was generally started 48–72 h after myocytes were plated.

*Neonatal rat ventricular myocyte culture.* A method originally described by Simpson et al. (29) has been adopted with modifications. Briefly, neonatal rat ventricular myocytes (NRVMs) were isolated using the neonatal cardiac myocyte isolation system (Worthington Biochemical, Lakewood, NJ). Hearts were collected from 1- to 2-day-old Harlan Sprague Dawley rat neonates. The ventricles were minced into 1- to 2-mm pieces and sequentially digested with trypsin (50 μg/ml) overnight at 4°C and collagenase (94 U/ml) for 45 min. The digestion was followed by tituration and filtration. Cells were collected by centrifugation at 80 g for 5 min at 4°C and resuspended in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U/ml; Invitrogen, Carlsbad, CA). Subsequently, the cells were differentially plated for 1 h on uncoated cell culture dishes for removal of nonmyocytes. The enriched cardiac myocytes were plated on fibronectin-precoated cell culture dishes and chamber slides at 1,000 cells/mm$^2$. Institutional guidelines were followed in the care and use of experimental animals.

*Detection of cell death.* An important feature of transition from reversible to irreversible cell injury (i.e., cell death) is loss of plasma membrane integrity. Propidium iodide (PI) is an exclusion fluorescent dye that binds to chromatin and fluoresces on loss of membrane integrity (1). Cell viability was quantified with PI nuclear stain as previously described with minor modifications (20). Briefly, 5 μM isotonic PI (Molecular Probes, Eugene, OR) was added to cultured NRVMs in chamber slides, and the slides were incubated for 30 min. The medium containing PI was then removed, and the chamber slides were rinsed three times with cold PBS for 2 min to completely remove residual PI. The cells were then fixed with 4% paraformaldehyde for 10 min and subsequently rinsed with PBS. After incubation with 1% Triton X-100 in PBS for 30 min, the nuclei of all cells were stained with 4,6-diamidino-2-phenylindole (DAPI). The stained cells were visualized with an epifluorescence microscope (model IX-70, Olympus) and filters optimal for detection of PI (red-) and DAPI (blue)-bound DNA (Leeds Precision Instruments, Minneapolis, MN). For each chamber, 5 random fields and a total of 300 nuclei were examined. The percentage of PI-stained nuclei (dead cells) over DAPI-positive nuclei (all the cells examined) was defined as the cell death index, and data from three independent experimental repeats are presented.

*Lactate dehydrogenase activity assay.* Release of lactate dehydrogenase (LDH) from the cytoplasm of NRVMs to the culture medium indicates a loss of plasma membrane integrity. Culture medium was collected from individual dishes or wells, and cell debris was removed by centrifugation. LDH activity in the collected culture medium was measured using the cytopotoxicity detection kit (Roche, Penzberg, Germany) by the reduction of lactate to pyruvate in the presence of NAD. The resultant NADH reduces tetrazolium to a red formazan product that is detectable at 490 nm. Briefly, 100 μl of reaction reagent were added to each well of a 96-well plate containing 100 μl of optimally titrated culture medium, and the mixture was incubated for 30 min at room temperature. Absorbance of the samples at 490 nm was read on a Thermas microplate reader (Molecular Devices, Sunnyvale, CA). The reference wavelength was 620 nm. LDH release of an experimental group is expressed as a percentage of the mean LDH activity of the corresponding control group. Results from at least four separate experiments are presented.

*Proteasome inhibition.* At ~65 h after recombinant viral infection, control or adenovirus-infected cardiac myocytes in individual dishes were treated with DMSO (as mock treatment), proteasome-specific inhibitors (MG-132 at an indicated concentration, 6 μM lactacystin, and 10 μg/ml ALLN), or protease inhibitors (50 μg/ml apronitin, 100 μM leupeptin, and 100 μM PMSE) (2, 21). After 6 h of incubation, cells were collected and washed twice with PBS. The total proteins were extracted for Western blots and the soluble proteins for proteasome peptidase activity assays. The concentration of proteins for each sample was determined using the bicinchoninic acid protein assay reagent kit (Pierce Biotechnology, Rockford, IL).

*Western blots.* Ten micrograms of total protein from each sample were loaded in each lane, fractionated by 12% SDS-PAGE, and electrophoretically transferred onto polyvinylidene difluoride membranes (34). Mouse monoclonal anti-GFP (B2, Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal anti-α-actinin (sarcomeric; Sigma-Aldrich) were used as primary antibodies, and horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) was used as secondary antibody to detect GFP (or GFPu) and sarcomeric α-actinin simultaneously. For detection of ubiquitinated proteins, a polyclonal antiubiquitin antibody (Sigma-Aldrich) was used as primary antibody. ECL-Plus detection reagents (Amersham Bioscience, Piscataway, NJ) and a VersaDoc imaging system (model 3000, Piscataway, NJ).
Bio-Rad, Hercules, CA) were used to visualize and digitalize the Western blot images. The density of Western blots was determined with Quantity One software. The same-lane α-actinin level was employed as protein loading control (32).

**Immunolabeling and fluorescence microscopy.** The chamber slides carrying cultured NRVMs were washed with PBS, fixed in 4% paraformaldehyde-PBS, and permeabilized with 1% Trition X-100-PBS. Immunolabeling was performed as previously described (33). After they were blocked with 1% BSA-PBS for 1 h, the cells were incubated with a mouse monoclonal antibody (1:500; Sigma) for sarcomeric α-actinin overnight at 4°C. The cells were then washed with PBS and incubated with Alexa 568-conjugated goat anti-mouse IgG. Fluorescence microscopy was performed subsequently. The green fluorescence from GFP or GFPu was visualized directly with an epifluorescence microscope (model IX-70, Olympus) and a filter optimal for detection of GFP (Leeds Precision Instruments).

**Proteasome peptidase activity assays.** The synthetic fluorogenic peptide substrate III [Suc-LYY-aminomethylcoumarin (AMC), 25 μM; Calbiochem, San Diego, CA] was used to assay chymotrypsin-like activities. Assays were carried out using 10 μg of cellular protein in a total volume of 200 μl. The assay buffer consisted of 25 mM Tris-HCl and 2 mM ATP at pH 7.5 (2, 4). After incubation at 37°C for 90 min, the reaction was quenched by addition of 300 μl of ethanol. A 2.0-ml aliquot of H2O was then added, and the fluorescence intensity of the samples was evaluated using a luminescence spectrometer (model LS 50B, Perkin-Elmer). The excitation and emission wavelengths are 350 and 440 nm, respectively, for AMC products.

Peptide cleavage reactions were carried out in the absence or presence (20 μM) of the proteasome-specific inhibitor MG-132 (N-Cbz-LLL), and the difference between the two rates is attributed to the proteasome. As determined in our pilot control experiments (data not shown), peptidase activities are linear for the first 150 min under the conditions described.

**H2O2 treatments.** At 65 h after cultured NRVMs were infected with Ad-GFPu at 5 MOI or 72 h after myocytes were plated without adenoviral infection, each individual dish was treated with 100 μM H2O2 or an equal volume of H2O (as control) for 2 or 4 h. After H2O2 treatment, the cells were collected and washed twice with cold PBS. The total proteins were extracted for Western blots and the soluble proteins for proteasome peptidase activity assays as described above.

**Statistical analysis.** For LDH activity, chymotrypsin-like activity, protein levels in the culture medium, and GFPu protein levels shown by Western blots, the values of experimental groups were normalized to the control group as described above. Differences between experimental and control groups were compared using one-way ANOVA and Fisher’s protected least-significant difference test. P < 0.05 was considered significant.

**RESULTS**

Adenoviral introduction of moderate levels of GFPu into cultured NRVMs does not affect cell viability or inhibit proteasomal function and protein secretion. The goal of this study is to develop a system to monitor UPS function in cultured cardiac myocytes in situ by introduction of a surrogate UPS-specific substrate through recombinant adenovirus-mediated gene delivery. The surrogate marker that we chose to test was GFPu. A prerequisite for such a system to be valid is that introduction of a sufficient level of the surrogate marker should not affect the viability of cultured NRVMs. It had been shown in pilot studies that infection with Ad-GFPu at 5 MOI was sufficient to monitor changes in the proteolytic function of the UPS. Therefore, the effects of adenoviral introduction of GFPu at 5, 10, or 15 MOI on the viability of cultured NRVMs compared with adenoviral introduction of wild-type GFP, as well as MG-132-induced proteasome inhibition were determined. Two complementary parameters were employed to assess the viability of cultured NRVMs: LDH release and cell death index. Infection with Ad-GFP or Ad-GFPu at 5, 10, or 15 MOI did not significantly alter the cell death index or LDH release from cultured cells to the culture medium, whereas administration of MG-132 at a final concentration of 20 μM increased significantly the cell death index as well as LDH activity in the culture medium (P < 0.01; Fig. 1).

To validate GFPu as a surrogate marker for UPS function in cultured NRVMs, it is also important to determine whether introduction of GFPu at an appropriate level affects the proteasomal function and general protein synthesis. None of the three recombinant adenoviruses (Ad-β-Gal, Ad-GFP, or Ad-GFPu) tested at 5, 10, or 15 MOI decreased the chymotrypsin-like activity, although MG-132 inhibited the activity in a dose-dependent manner, indicating that introduction of GFPu at ≤15 MOI does not inhibit the peptidase activity of the proteasome (Fig. 2A). Interestingly, infections with Ad-GFP at 10 or 15 MOI or Ad-GFPu at 15 MOI actually increased chymotrypsin-like activities in cultured NRVMs.

Because LDH activity in the culture medium was not significantly altered by adenoviral introduction of GFP or GFPu at each of the three tested MOIs (Fig. 1A), changes in the protein concentration in the culture medium should reflect the changes in the amount of protein actively secreted by the cells. The protein concentration in the culture medium was not affected by infection with Ad-β-Gal at 5, 10, or 15 MOI or Ad-GFPu at 5 or 10 MOI but was increased significantly by infection with Ad-GFPu at ≤15 MOI or Ad-GFPu at 15 MOI (Fig. 2B).

Specific inhibition of the proteasome results in accumulation of GFPu, but not GFP, protein expressed in cultured NRVMs. It has previously been shown in cultured HEK cells that conventional GFP is not degraded by the proteasome but that fusion of the CL1 degron to the COOH terminus of GFP renders GFPu a specific substrate for the UPS (2). To test whether GFPu can be used as a specific surrogate substrate to monitor UPS function in cultured NRVMs, we created replication-incompetent recombinant adenoviruses (Ad-GFPu) that harbor a GFPu expression cassette and used the viruses to infect cultured NRVMs to express GFPu. Western blots and fluorescence microscopy showed that infection with Ad-GFPu gives rise to expression of GFPu in NRVMs (Figs. 3 and 4). To test whether the degradation of GFP and GFPu in NRVMs is UPS dependent, 10 μM MG-132, a proteasome-specific inhibitor, was employed to inhibit the proteasome, and the protein abundances of GFP and GFPu in NRVMs in the presence and absence of MG-132 treatment were compared. Western blots (Fig. 3A) and fluorescence microscopy (Fig. 4) consistently showed that GFPu, but not wild-type GFP, protein was considerably increased by proteasomal inhibition, suggesting that GFPu, but not GFP, can be degraded by the UPS.

To further test whether GFPu protein is degraded exclusively through the UPS, proteasome-specific inhibitors (10 μM MG-132, 6 μM lactacystin, and 10 μg/ml ALLN) and various protease inhibitors (50 μg/ml aprotinin, 100 μM leupeptin, and 100 μM PMSF) were respectively applied to NRVM cultures 65 h after Ad-GFPu infection. Compared with untreated and mock treatment groups, only proteasome-specific inhibitor groups showed significant increases in protein abundance of GFPu as assessed by Western blots (Fig. 3B). These findings
demonstrate that the degradation of GFPu, but not wild-type GFP, is exclusively UPS dependent in NRVMs. Accumulation of GFPu protein inversely correlates to proteasomal activities. To determine whether increases in protein abundance of GFPu correlate linearly to reduction of the proteolytic function of the proteasome, we inhibited the proteasomal function at various degrees using various concentrations of MG-132. Because MG-132 inhibits the proteasome mainly through inhibition of the chymotrypsin-like peptidase of the 20S proteasome complex, chymotrypsin-like peptidase activity was measured in vitro as an indicator of proteasomal function. At 6 h after application of 0.5, 5, and 10 μM MG-132, the chymotrypsin-like peptidase activities decreased on average by 17%, 30%, and 40%, respectively, whereas the abundance of GFPu protein increased by 1.6-, 2.8-, and 3.9-
fold, respectively (Fig. 5). An inverse linear correlation between proteasomal function and GFPu protein levels was evident and statistically significant ($r^2 = 0.92$, $P < 0.001$), demonstrating that changes in GFPu protein levels sensitively and quantitatively reflect proteasomal function.

**ROS inhibit UPS function.** Myocardial reperfusion injury has been shown to oxidize and inhibit proteasomal function, and ROS is one of the major mediators for reperfusion injury (4). To apply our newly established UPS function-monitoring system to a biologically relevant problem, we treated cultured NRVMs with an ROS donor, H$_2$O$_2$, and examined the function of the UPS with conventional assays as well as the GFPu system. Notably, the chymotrypsin-like peptidase activities were decreased by ~45% at 2 and 4 h after treatment with 100 $\mu$M H$_2$O$_2$ (Fig. 6A). Meanwhile, ubiquitinated proteins were significantly increased in the cultured cardiac myocytes, whereas the abundance of free ubiquitin was not changed with 2 and 4 h of treatment compared with the mock-treatment group (Fig. 6B). Consistently, the GFPu protein levels as measured by Western blots were increased by nearly sixfold at both time points when Ad-GFPu was introduced before H$_2$O$_2$ treatments (Figs. 6C and 7). As expected, the H$_2$O$_2$ treatment did not increase wild-type GFP protein expressed in cultured NRVMs (data not shown).

To verify whether increased GFPu protein is in cardiac myocytes and to determine the subcellular distribution of increased GFPu, H$_2$O$_2$-treated and control cells were immunolabeled with anti-α-actinin and a red fluorescence probe to identify cardiac myocytes. The red fluorescence-labeled α-actinin, as well as green fluorescence emitted from GFPu, was visualized with an epifluorescence microscope. As expected, the green fluorescence in the control NRVMs was nearly invisible, whereas GFPu green fluorescence was bright. Nearly all the cells that showed green fluorescence are sarcomeric α-actinin positive, indicative of their cardiac myocyte identity (Fig. 7e). Interestingly, accumulated GFPu protein in H$_2$O$_2$-treated NRVMs appeared to have the tendency to form aggregates (Fig. 7e). These findings demonstrate that ROS can directly impair the proteolytic function of the proteasomes, and our newly developed GFPu system is sensitive enough to reveal the impairment.

**DISCUSSION**

Evaluation of proteasomal function largely relies on in vitro peptidase assay (2), which has at least two inherent pitfalls: 1) these assays estimate only the peptidase activities of individual components of the proteasome, not the proteolytic function of the 20S or 26S proteasome complex as a whole, and 2) in vitro conditions used for the assays differ tremendously from conditions in a real living cell. The goal of the present study was to establish and validate a system capable of monitoring dynamically changes in the proteolytic function of the UPS in cardiac myocytes in situ, and we have accomplished this goal by adenoviral introduction of a surrogate substrate for the UPS. As mentioned earlier, this system is expected to meet the needs to decipher the pathogenic significance of UPS dysfunction in the heart and to evaluate effects of altered proteasomal function on heart muscle cells.

The majority of proteins in cardiac myocytes are degraded through the UPS, and changes in their levels may sometimes reflect the functional status of the UPS. However, none of these endogenous proteins are really suitable to serve as a marker for UPS function, because the levels of each of these endogenous proteins are subject to not only the function status of the UPS but also a variety of intrinsic mechanisms that regulate the synthesis and degradation of the protein. In this regard, a biologically inert surrogate-specific substrate for the UPS should serve as a better marker. Conventional GFP is not a specific substrate for the UPS, with a half-life of >10 h when expressed in cultured HEK cells, but fusion of the CL1 degron, a ubiquitination signal sequence isolated from yeast, to the expressed in cultured HEK cells, but fusion of the CL1 degron, a ubiquitination signal sequence isolated from yeast, to the surrogate substrate for the UPS in HEK cells with a half-life of 30 min (2). Therefore, GFPu has proven to be a dynamic indicator for UPS function in HEK cells. However, it was unknown whether GFPu is rapidly degraded specifically by the UPS in cardiac myocytes. Because it is technically mature and less challenging, the NRVM culture system is extensively employed by many investigators in the cardiac research community. Thus we decided to determine whether GFPu can be used as a surrogate indicator to monitor in situ UPS function in cultured NRVMs.

Because it is extremely difficult to transfect NRVMs with regular plasmids, recombinant adenoviruses harboring the GFPu-expressing cassette were successfully created and employed to deliver the GFPu gene into cultured NRVMs. Using this system, we found that GFPu, but not wild-type GFP, expressed in cultured NRVMs behaved as a substrate for the UPS. This is because Western blot and fluorescence micro-
scopic examinations showed that GFPu, but not GFP, was significantly increased when the proteasomal function was inhibited by a specific inhibitor (MG-132) in cultured NRVMs (Figs. 3A and 4). To further determine whether GFPu is a specific substrate for the UPS, various specific inhibitors (MG-132, lactacystin, and ALLN), as well as nonspecific protease inhibitors (aprotinin, leupeptin, and PMSF), were tested, and the results demonstrate that only proteasome inhibition accumulated GFPu in cultured NRVMs (Fig. 3B). This indicates that GFPu is a specific substrate for the UPS in NRVMs. A statistically significant inverse linear correlation between protein abundance of GFPu and proteasomal peptidase activities (Fig. 5) clearly demonstrated that GFPu is a sensitive dynamic indicator to monitor proteasomal function.

It appears that minor cleaved forms of α-actinin protein were detectable, and the cleavage was apparently inhibited by all three proteasome inhibitors (MG-132, lactacystin, and ALLN) and by aprotinin, which is not proteasome specific (Fig. 3B), suggesting that the cleavage of α-actinin is proteasome dependent and independent. Also, a minor GFPu band is visible below the major GFPu band in the Western blot, and this shorter GFPu responded to proteasome inhibition in the same way as the long form of GFPu (Fig. 3B). We observed the same phenomenon when we used GFPu plasmids to transfect 293 cells (unpublished data). This shorter form of GFPu might result from 1) the use of an alternative translational starting site (e.g., the ATG at position of 853–855 of the GFPu coding sequence), which would result in a protein that is 78 amino acids shorter at the NH2 terminus than the full-length GFPu, and 2) posttranslational modifications, such as cleavage at the NH2 terminus by a nonproteasomal protease, which would result in a cleaved GFPu with the CL1 sequence remaining at the COOH terminus. Whatever causes the presence of two forms of GFPu, our data clearly demonstrate that degradation of both forms of GFPu is proteasome dependent, because, identical to the full-length form of GFPu, the abundance of the shorter form increased only when the proteasome is inhibited (Fig. 3B).

It is important to note that infection with recombinant adenoviruses harboring wild-type GFP or GFPu at ≤15 MOI did not alter the viability of cultured NRVMs, whereas treatment of MG-132 at a higher dose (20 μM) showed cytotoxicity as measured by the cell death index and LDH leakage (Fig. 1). To address a legitimate concern that adenoviral introduction of GFPu into cultured NRVMs might affect proteasomal function, infections with Ad-GFPu viruses were tested and compared with Ad-β-Gal and Ad-GFP at 5, 10, or 15 MOI (Fig. 2A).

Although infection with Ad-GFPu at 15 MOI slightly, but
The means by which proteins are released from cultured cells to the medium conceivably includes two categories: passive leakage and active secretion. The latter is often proportional to the synthesis of the secreted proteins. Because infection with Ad-GFP or Ad-GFPu at 5, 10, or 15 MOI did not increase LDH activity in the culture medium, changes in protein concentration in the medium should indicate changes in statistically significantly, increased the peptidase activity, administration of 5 or 10 MOI Ad-GFPu did not affect peptidase activity. Compared with application to uninfected cells (Fig. 2A), application of a given dose of MG-132 to Ad-GFPu (5 MOI)-infected NRVMs (Fig. 5) produced essentially the same degree of proteasomal inhibition. These findings consistently suggest that the use of Ad-GFPu at ≥10 MOI is safe and accurate in terms of detection of proteasomal malfunction. It has been demonstrated in this study that use of Ad-GFPu at these safe levels is adequate for detection of proteasomal malfunction. Interestingly, infections with Ad-GFP started to increase the peptidase activity at 10 MOI, whereas Ad-β-Gal did not alter the peptidase activity, even at 15 MOI (Fig. 2A). The cause of the differences is not clear. A reasonable hypothesis is that it may be related to the difference in the half-lives among the three overexpressed proteins. However, it should be cautioned that use of Ad-GFPu at ≥15 MOI may potentially underestimate proteasomal malfunction, because infection with Ad-GFPu at this higher level stimulated the function of the proteasomes in cultured NRVMs.

Fig. 5. Accumulation of GFPu protein sensitively reflects linearly proteasomal malfunction. At 7 h after treatment with DMSO (mock treatment) or various doses of proteasome-specific inhibitor MG-132 (0.5, 5, or 10 μM), cultured NRVMs of each dish were harvested and split into 2 fractions: one for extracting total protein for Western blots to estimate GFPu levels (A and B) and the other for collecting cell lysates for chymotrypsin-like activity assays (B). Results similar to those in A were obtained from 3 independent experiments and are presented in B. Chymotrypsin-like activity of mock-treatment control was set as 100 AU, and its GFPu protein band intensity on Western blot was set as 1 AU. These control values were used as references to normalize MG-132-treated groups. An inverse linear correlation between GFPu abundance and proteasomal activities was statistically significant.

Fig. 6. H2O2 treatment inhibits proteasomal activity (A) and leads to accumulation of ubiquitinated proteins (B) and GFPu (C) in cultured NRVMs. Cultured NRVMs without (A and B) or with Ad-GFPu (5 MOI) infection (C) were treated with 100 μM H2O2 or an equivalent volume of H2O2 and collected and split into 2 fractions: one for collecting cell lysates for chymotrypsin-like activity assays (A) and the other for extracting total protein for detection of ubiquitinated proteins (B). A: chymotrypsin-like activities at 2 and 4 h of H2O2 treatment from 3 rounds of repetitive experiments were decreased on average by 40% compared with mock-treatment group. *P < 0.05. B: parallel to decreases in proteasomal activities, ubiquitinated proteins detected by Western blots with an antibody for free ubiquitin (Ub) and conjugated Ub were increased considerably in H2O2-treated cells. C: when cells were infected with Ad-GFPu (5 MOI) before H2O2 treatment, similar degrees of decreases in chymotrypsin-like activity were observed (data not shown) and accompanied by significant increases of GFPu levels in total protein extracts 2 and 4 h after H2O2 was administered. The same-lane α-actinin signal serves as a loading control for comparison of GFPu levels among different lanes. Experiments were repeated 3 times with essentially the same results.
protein secretion from the cultured cells to the medium. Compared with noninfected controls, infection with Ad-GFPu at 5 and 10 MOI did not affect protein secretion, but at 15 MOI it increased protein secretion (Fig. 2B). Similar to their effects on peptidase activities, Ad-GFP, but not β/Gal, at 5, 10, and 15 MOI affected the amount of protein secretion. Although the effect of adenoviral introduction of GFPu on general protein synthesis was not directly analyzed, protein secretion data indirectly suggest that use of Ad-GFPu at a level adequate for the purpose of monitoring proteasomal function does not appear to significantly affect cellular protein synthesis.

Ischemia-reperfusion injury has recently been shown to impair proteasomal function (4). ROS and reactive nitrogen species generated by ischemia-reperfusion events are believed to be at least partially responsible. To verify that ROS impairs the UPS, we applied our newly established monitoring system to investigate effects of administration of moderate levels of H2O2, an ROS generator, on the UPS. Indeed, treatment of H2O2 quickly caused accumulation of GFPu in NRVMs as measured by Western blots and fluorescence microscopy (Figs. 6 and 7). Consistently, a decrease in peptidase activities and accumulation of ubiquitinated proteins were found in H2O2-treated NRVMs in the absence of Ad-GFPu infection (Fig. 6, A and B). These findings demonstrate impairment of the UPS by ROS in cardiac myocytes. Notably, the abundance of free ubiquitin protein in H2O2-treated NRVMs was not affected, whereas ubiquitin conjugates were significantly increased. A similar observation was made by Bence et al. (2), who found that abnormal protein aggregation in HEK cells impaired the UPS but did not deplete free ubiquitin. A recent study with C2C12 myotubes showed that a similar level of H2O2 treatment stimulated ubiquitination (19). Therefore, the increase in ubiquitinated proteins in H2O2-treated NRVMs is potentially attributed to increased ubiquitination and decreased proteasome-mediated degradation. Increases in ROS have been associated with not only reperfusion injury but also cardiac hypertrophy and failure (23, 28). Modulation of protein degradation via inhibition of the UPS may be a mediating mechanism for ROS to lead to cardiac failure. The mechanism by which ROS impairs the UPS is unknown and is not in the scope

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**Figure 7.** Representative fluorescence micrographs of H2O2-treated NRVMs. NRVMs grown on fibronectin-coated chamber slides were infected by Ad-GFPu (5 MOI). At 65 h after infection, cells were treated with 100 μM H2O2 (d-f) or an equal volume of H2O2 (a-c) for 4 h; then they were fixed with 4% paraformaldehyde and immunolabeled for sarcomeric α-actinin (red). Green fluorescence (from GFPu) is barely visible in control NRVMs (b) but is fairly bright in the H2O2-treated NRVMs (e). Accumulated GFPu appeared to form aggregates in H2O2-treated cells. Overlay image shows accumulation of GFPu in α-actinin-positive cells. Scale bar, 10 μm.
of the present report but is certainly worthy of further investigation.

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