Regulation of Acetylation Restores Proteolytic Function of Diseased Myocardium in Mouse and Human*§

Ding Wang‡$, Caiyun Fang‡¶, Nobel C. Zong‡§, David A. Liem‡, Martin Cadeiras¶, Sarah B. Scruggs‡, Hongxu Yu¶, Allen K. Kim‡, Pengyuan Yang¶, Mario Deng¶, Haojie Lu¶**, and Peipei Ping‡¶**

Proteasome complexes play essential roles in maintaining cellular protein homeostasis and serve fundamental roles in cardiac function under normal and pathological conditions. A functional detriment in proteasomal activities has been recognized as a major contributor to the progression of cardiovascular diseases. Therefore, approaches to restore proteolytic function within the setting of the diseased myocardium would be of great clinical significance.

In this study, we discovered that the cardiac proteasomal activity could be regulated by acetylation. Histone deacetylase (HDAC) inhibitors (suberoylanilide hydroxamic acid and sodium valproate) enhanced the acetylation of 20S proteasomal subunits in the myocardium and led to an elevation of proteolytic capacity. This regulatory paradigm was present in both healthy and acutely ischemia/reperfusion (I/R) injured murine hearts, and HDAC inhibition in vitro restored proteolytic capacities to baseline sham levels in injured hearts. This mechanism of regulation was also viable in failing human myocardium. With 20S proteasomal complexes purified from murine myocardium treated with HDAC inhibitors in vivo, we confirmed that acetylation of 20S subunits directly, at least in part, presents a molecular explanation for the improvement in function. Furthermore, using high-resolution LC-MS/MS, we unraveled the first cardiac 20S acetylome, which identified the acetylation of nine N-termini and seven internal lysine residues. Acetylation on four lysine residues and four N-termini on cardiac proteasomes were novel discoveries of this study. In addition, the acetylation of five lysine residues was inducible via HDAC inhibition, which correlated with the enhancement of 20S proteasomal activity. Taken as a whole, our investigation unveiled a novel mechanism of proteasomal function regulation in vivo and established a new strategy for the potential rescue of compromised proteolytic function in the failing heart using HDAC inhibitors. Molecular & Cellular Proteomics 12: 10.1074/mcp.M113.028332, 3793–3802, 2013.

Proteasome complexes serve as the main proteolytic machinery for cardiomyocytes (1–3). Numerous forms of cardiomyopathies share characteristic perturbations in proteasomal function and a concomitant disarray in protein quality control (4, 5). Pilot studies of genetic intervention (6–8) have demonstrated that altering proteasomal function may yield significant therapeutic benefits. Despite these promising results, undesirable cardiac complications have arisen from reagents that target global proteasome function (9, 10). Thus, an investigation of regulatory pathways targeting subsets of proteasomal populations is warranted prior to the development of therapeutic interventions.

Different subpopulations of proteasomal complexes exhibit distinct proteolytic potencies and substrate selectivities, giving rise to variations in their functional adaptability in the heart. One facet of proteome diversity originates from post-translational modifications, which have been investigated in the heart for four decades (11–13). For instance, phosphorylation (14) and oxidation (15) have been implicated in the regulation of proteolytic activity, demonstrating opportunities for pharmacological intervention with post-translational modifications (16) to target the modulation of protein quality control. In order for these opportunities to be made use of successfully, a comprehensive post-translational modification profile of proteasomal complexes must be obtained.

Proteasomal subunits were recently detected as targets of acetylation from large-scale proteomic investigations in non-cardiac tissues (17, 18). Despite the well-documented effects of acetylation in modulating gene transcription and protein expression (19), its role in protein degradation has only recently begun to be recognized. Acetylation of targeted substrates, pyruvate kinase (20) and PEPCK (21), altered their speed of degradation. However, the effect of acetylation on the proteasomal machinery specifically remains to be investigated.
HDAC Inhibition Promotes Cardiac Proteostasis

In this paper, we report the restoration of mammalian cardiac proteolytic function via alteration of the acetylation of 20S proteasomes. In parallel, a comprehensive acetylation profile of proteasome subunits (acetylation of both N-termini and lysine residues) in the myocardium was delineated via a targeted proteomics workflow. Pharmacological enhancements of acetylation in healthy and diseased myocardium revealed a positive correlation between acetylation and proteolytic function. Importantly, this regulatory mechanism was observed in both murine and human heart, affording novel insights on restoring proteolytic function in the failing human myocardium via therapeutic interventions.

EXPERIMENTAL PROCEDURES

Experimental procedures involving human tissues were approved by the UCLA Human Subjects Protection Committee and the UCLA Institutional Review Boards. All procedures involving animals were performed in accordance with the Animal Research Committee guidelines at UCLA and the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Murine Model of Regional Ischemic Injury—A murine model of ischemia/reperfusion (I/R) injury was created by ligating the left anterior descending coronary artery as previously reported (22, 23). Briefly, the pericardium of a male Hsd:ICR (CD-1) outbred mouse (8 to 10 weeks of age; Harlan Laboratories, Indianapolis, IN) was opened following a left thoracotomy. An 8–0 silk suture was looped under the left anterior descending coronary artery from the tip of the left atrium, which was positioned normally. Ischemia was induced by ligation with the suture for 30 min. The heart was excised at 4 h post-reperfusion for subsequent analyses.

Treatment of Histone Deacetylase Inhibition in Vivo—Histone deacetylase (HDAC) inhibition in vivo was conducted by means of an intraperitoneal injection of an HDAC inhibitor mixture at the following dose-to-body weight ratios: SAHA/vorinostat 25 mg/kg (Cayman Chemicals, Ann Arbor, MI), sodium valproate 200 mg/kg (Sigma, St. Louis, MO), and nicotinamide 250 mg/kg (Sigma). Murine hearts were harvested 6 h post-injection for subsequent biochemical and proteomic analyses.

Cardiac Tissue Collection from Human and Mice—With written consent, human cardiac tissues were obtained from the left ventricular anterior walls of hearts from end-stage heart failure patients during heart transplantation at the UCLA Medical Center. Left ventricular tissues from 10 individuals were collected and used in this study; their relevant disease phenotypes are summarized in the supplemental data. A total of 64 male Hsd:ICR (CD-1) outbred mice (age 10 weeks; Harlan Laboratories, Indianapolis, IN) was opened following a left thoracotomy. An 8–0 silk suture was looped under the left anterior descending coronary artery from the tip of the left atrium, which was positioned normally. Ischemia was induced by ligation with the suture for 30 min. The heart was excised at 4 h post-reperfusion for subsequent analyses.

Cardiac Tissue Collection from Human and Mice—With written consent, human cardiac tissues were obtained from the left ventricular anterior walls of hearts from end-stage heart failure patients during heart transplantation at the UCLA Medical Center. Left ventricular tissues from 10 individuals were collected and used in this study; their relevant disease phenotypes are summarized in the supplemental data. A total of 64 male Hsd:ICR (CD-1) outbred mice (age 10 weeks; Harlan Laboratories, Indianapolis, IN) was opened following a left thoracotomy. An 8–0 silk suture was looped under the left anterior descending coronary artery from the tip of the left atrium, which was positioned normally. Ischemia was induced by ligation with the suture for 30 min. The heart was excised at 4 h post-reperfusion for subsequent analyses.

Purification of Murine Cardiac 20S Proteasome Complexes—Each cardiac tissue was homogenized in buffer (50 mmol/l Tris-HCl, pH 8.0, 100 mmol/l NaCl, 1 mmol/l EDTA, 0.1% Nonidet P-40) and incubated with extraction resin at 25 °C. Additional enzyme was supplemented at an enzyme/protein ratio of 1:100 for an additional 4 h of incubation to complete the proteolysis. Peptides were desalted using a C18 spin column (Thermo Scientific, San Jose, CA), and eluents were dried with a SpeedVac.

Unraveling of Lysine-acetylated Peptides via Immunoprecipitation—The lysine-acetylated peptides were enriched for proteomic analyses (13). The anti-acetyl-lysine antibodies (ImmuneChem, Burnaby, Canada; Cell Signaling, Boston, MA) were mixed 1:1 (w/w) and immobilized onto Protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 4 h to form extraction resin. Following the in-solution trypsin digestion, the resulting peptides were resolved using binding buffer (50 mmol/l Tris-HCl, pH 8.0, 100 mmol/l NaCl, 1 mmol/l EDTA, 0.1% Nonidet P-40) and incubated with extraction resin at 4 °C overnight with gentle rocking. The resin was washed twice with binding buffer and three times with washing buffer (50 mmol/l Tris-HCl, pH 8.0, 100 mmol/l NaCl, 1 mmol/l EDTA). Enriched peptides were eluted with 0.1% trifluoroacetic acid. Salts in the eluents were removed with C18 spin columns (Thermo Scientific).

1 The abbreviations used are: 20S, proteasome proteolytic core particle; HAT, histone acetyltransferase; HDAC, histone deacetylase; I/R, ischemia/reperfusion; MRM, multiple reaction monitoring; SAHA, suberoylanilide hydroxamic acid (Vorinostat).
HDAC Inhibition Promotes Cardiac Proteostasis

LC-MS/MS—HPLC-MS/MS analyses were performed on an LTQ Orbitrap (Thermo Scientific) equipped with a nano-LC instrument (Eksigent, Dublin, CA) (25). A pre-packed column (PicoFrit C18, 75 μm x 10 cm, New Objective, Woburn, MA) containing reverse-phase resin (Biobasic C18, 5-μm particle size, 300-Å pore size, New Objective) was used. The online chromatography flow rate was set at 5 μl/min for peptide loading and 220 nl/min for separation (buffer A: 0.1% formic acid, 2% acetonitrile; buffer B: 0.1% formic acid, 80% acetonitrile). The resolving gradient was set at 5% to 40% buffer B over 70 min, changed to 100% buffer B over 20 min, maintained at 100% buffer B for 10 min, and then returned to 5% buffer B. The LTQ Orbitrap was operated in data-dependent acquisition mode; one full MS scan was followed by five MS² scans. Survey full-scan MS spectra were acquired in the Orbitrap with a mass resolution of 60,000 at m/z 400.

Database Search and Result Analyses—Sequest (Thermo Scientific Bioworks, V3.3.1) was used for the generation of peaklists with default settings. Mass spectra were searched against the International Protein Index (IPI mouse database, V3.87) with partial enzymatic digestion (trypsin, Glu-C, or Lys-C), with four missed cleavages permitted and variable modifications of carboxyamidomethylation (Cys, +57.02 Da), oxidation (Met, +15.99 Da), and acetylation (Lys or N terminus, +42.01 Da). Sequest searched against the HLA A, B, and C alleles. The precursor ion mass tolerance was set at 20 ppm, and the product ion tolerance at 1.0 amu. The spectral files were analyzed independently with Mascot (Matrix Science, Boston, MA, V2.1), permitting two missed cleavages, to validate the search results obtained from Sequest. These results were further filtered using Scaffold (Proteome Software, Portland, OR, V2.01) at confidence levels of ≥95.0% for peptides and ≥99.0% for proteins. Manual spectrum inspection was performed to minimize false positives (26). The representative spectra of acetylated peptides are now deposited in the Cardiac Organellar Protein Atlas Knowledgebase (27).

Statistical Analyses—All data were presented as the mean ± S.E.; Student’s t test was performed, and p values of <0.05 were noted as statistically significant.

RESULTS

HDAC Inhibition in Vitro Enhances Proteolytic Activity of Proteasomes in Acute I/R Injury and in End-stage Ischemic Heart Failure—Interest in our laboratory in the effects of lysine acetylation on the proteolytic activities of proteasomes was first ignited by the physiological observation that the trypsin-like catalytic activity of proteasomes in cytosol extracted from murine hearts was enhanced following a 30-min treatment with two independent HDAC inhibitors, sodium valproate and SAHA. Moreover, after a ∼20% reduction observed in cytosolic extracts from a murine model of acute I/R injury, proteolytic activity was restored to normal levels following either sodium valproate or SAHA treatments (Fig. 1A). To determine whether these observations in I/R injured murine hearts might translate to a human condition of ischemic heart failure, we similarly treated end-stage ischemic heart failure cytosolic extracts with HDAC inhibitors. The results presented in Fig. 1B demonstrate that proteolytic activities were indeed enhanced following a 30-min treatment with either inhibitor. Though no failing human heart controls were included in our analysis, previously published data from Powell’s group (28) showed that end-stage proteolytic activities are reduced by between 20% and 40%, suggesting that the HDAC inhibition performed in this study might indeed restore proteolytic activities to near-normal levels in human heart failure. As numerous components of the ubiquitin proteasome system may be regulated by lysine acetylation, we sought to identify whether the acetylation of 20S subunits specifically was altered. The 20S proteasome complexes were co-immunoprecipitated using an antibody specific for the β2 subunit, in the absence of ATP (to dissociate 19S regulatory particles), and immunoblotted with an anti-acetylated lysine antibody (supplemental Fig. S3). Paired analysis of human cardiac extracts from end-stage heart failure patients before and after HDAC inhibitor treatment suggested that 20S proteasomes are indeed specific targets of lysine acetylation, demonstrating a ∼25% increase in lysine acetylation levels.

Murine Cardiac 20S Proteasomes Are Targets of Lysine Acetylation—To determine which amino acid residues are specifically modified by acetylation on 20S subunits, we analyzed cardiac cytosolic 20S proteasomes using a specialized mass-spectrometry-based proteomic workflow. 20S proteasome subunits are subject to both N-terminal and lysine acetylation, which have an identical mass shift (+42.0106 Da). However, N-terminal acetylation is an irreversible process (29) catalyzed by a group of enzymes that is distinct from that catalyzing lysine acetylation. For this study, it was first necessary to sequence the N-termini of all 17 subunits in order to differentiate these two types of modifications on the same peptides. The primary sequences of proteasome subunits have divergently evolved in order to tailor functional specificity (30, 31), which results in a wide molecular weight range of trypsin-digested N-terminal peptides. Therefore, a panel of endoproteinases (trypsin, Glu-C, and Lys-C) with distinct site specificities was employed to cleave 20S subunits into peptides with MS-detector-compatible lengths, and then the three sets of MS data were compiled for a comprehensive analysis. For example, the N-terminal peptide of the α1 subunit was captured after digestion with Glu-C (Fig. 2A) but not with trypsin (30). Our results indicated that 9 out of 17 20S subunits were acetylated (supplemental Figs. S4–S19). The N termini of 20S catalytic subunits β1, β2, β5, β1, β2, and β5i and noncatalytic subunits β6 and β7 were found to be free of acetylation (supplemental Table S3). This is likely due to the fact that the N-terminal pro-peptides of these subunits are post-translationally removed during complex assembly (32, 33).

Once the baseline of N-terminal acetylation was established, we sought to find sites of internal lysine acetylation in 20S subunits. Lysine acetylation is a reversible modification, and affinity enrichment is often used to target this post-translational modification of low stoichiometric abundance (18, 34). Accordingly, we employed a two-tier enrichment strategy to capture acetylated peptides specifically from cardiac 20S proteasome subunits. 20S proteasomes were first extensively purified (14) and then enriched via immunoprecipitation with anti-acetyl lysine after in-solution trypsin digestion. Precipitated peptides were analyzed using high-resolution LC-MS/MS on an LTQ Orbitrap for high sensitivity and specificity.
Two sites of internal lysine acetylation were identified in 20S proteasome subunits, Lys-104 on the \( H_9251 \) subunit and Lys-203 on the \( H_9251 \) subunit, as shown in Table I, supplemental Fig. S20, and Fig. 2B. Of note, the identifications of acetylation at both sites were novel discoveries in murine cardiac tissue, and the identification of acetylation of \( H_9251 \) Lys-203 residue is the first observation of this in mammalian tissues (Table I).

**Global HDAC Inhibition in Vivo Enhances the Proteolytic Capacity and Redistribution of 20S Proteasomes and Induces Lysine Acetylation of Specific Sites—**

*HDAC inhibition in vitro enhances proteolytic activity of proteasomes in acute I/R injury and in end-stage ischemic heart failure.* A, left-hand panel: murine cytosolic 20S proteasomal activity of the sham group \( n = 5 \) was up-regulated by sodium valproate in a dose-dependent fashion (open circles, \( * \ p < 0.05 \) versus without sodium valproate); a significant increase in activity was reached at 0.04 mM, and the augmentation was maintained through 1 mM of sodium valproate. I/R injury significantly reduced 20S proteolytic activity (solid circles; \( $ \ denotes \ p < 0.05 \) versus sham). Sodium valproate at 0.04 mM restored the compromised 20S activity in the injured myocardium (solid circles; \( # \ denotes \ p < 0.05 \) versus without sodium valproate). This regulatory capacity was consistent with elevated doses of sodium valproate; an adverse effect was observed when 5 mM of sodium valproate was given. Right-hand panel: HDAC inhibition by SAHA up-regulated cytosolic 20S proteasomal activities in the healthy and injured myocardium. The maximal augmentation was achieved by 0.4 \( \mu \)M of SAHA, and the effect was consistent through 50 \( \mu \)M of SAHA. SAHA restored 20S activities in the I/R injured myocardium. \( p < 0.05 \) represents statistic significance; error bar represents standard error.

**Cardiac 20S Proteasomes of End-Stage Heart Failure Patients**

B, left-hand panel: HDAC inhibition by sodium valproate significantly improved the proteolytic activity in diseased myocardium from end-stage heart failure patients (open circles, \( n = 5 \), \( * \ p < 0.05 \) versus without sodium valproate). A range of sodium valproate doses from 0.04 mM to 5 mM consistently achieved this regulatory capacity. Similar therapeutic effects on 20S proteolytic activities were also observed with HDAC inhibitor SAHA (open circles, \( n = 5 \), \( * \ p < 0.05 \) versus without SAHA). \( p < 0.05 \) represents statistic significance; error bar represents standard error.

Fig. 1. **HDAC inhibition in vitro enhances proteolytic activity of proteasomes in acute I/R injury and in end-stage ischemic heart failure.** A, left-hand panel: murine cytosolic 20S proteasomal activity of the sham group \( n = 5 \) was up-regulated by sodium valproate in a dose-dependent fashion (open circles, \( * \ p < 0.05 \) versus without sodium valproate); a significant increase in activity was reached at 0.04 mM, and the augmentation was maintained through 1 mM of sodium valproate. I/R injury significantly reduced 20S proteolytic activity (solid circles; \( $ \ denotes \ p < 0.05 \) versus sham). Sodium valproate at 0.04 mM restored the compromised 20S activity in the injured myocardium (solid circles; \( # \ denotes \ p < 0.05 \) versus without sodium valproate). This regulatory capacity was consistent with elevated doses of sodium valproate; an adverse effect was observed when 5 mM of sodium valproate was given. Right-hand panel: HDAC inhibition by SAHA up-regulated cytosolic 20S proteasomal activities in the healthy and injured myocardium. The maximal augmentation was achieved by 0.4 \( \mu \)M of SAHA, and the effect was consistent through 50 \( \mu \)M of SAHA. SAHA restored 20S activities in the I/R injured myocardium. \( p < 0.05 \) represents statistic significance; error bar represents standard error. B, left-hand panel: HDAC inhibition by sodium valproate significantly improved the proteolytic activity in diseased myocardium from end-stage heart failure patients (open circles, \( n = 5 \), \( * \ p < 0.05 \) versus without sodium valproate). A range of sodium valproate doses from 0.04 mM to 5 mM consistently achieved this regulatory capacity. Similar therapeutic effects on 20S proteolytic activities were also observed with HDAC inhibitor SAHA (open circles, \( n = 5 \), \( * \ p < 0.05 \) versus without SAHA). \( p < 0.05 \) represents statistic significance; error bar represents standard error.
tion is in the clinical spotlight for treating numerous diseases, including cardiac disease, we sought to determine whether global HDAC inhibition in vivo would support our findings in vitro. Mice were administered an HDAC inhibitor mixture of SAHA/vorinostat, sodium valproate, and nicotinamide via intraperitoneal injection for 6 h. To gauge how global HDAC inhibition may be affecting 20S proteasome assembly/redis-

Fig. 2. Murine cardiac 20S proteasomes are targets of lysine acetyla-
tion. A, the mass spectrum of the N-terminal peptide from the α1 subunit of 20S proteasomes in its acetylated form. Digestion by the enzyme Glu-C was used to generate this N-terminal peptide. A cross-correlation score (Xcorr) of 3.83, determined by the Sequest algorithm, demonstrated a confident identification. B, the mass spectrum of peptide containing lysine-203 residue from the α5 subunit of 20S proteasomes. This lysine-203 residue was regulated by acetylation under physiological conditions. An Xcorr score of 3.71 demonstrated a confident identification.

our in vitro findings, cytosolic extracts were assayed for tryp-
tic proteolytic activities. In agreement with our findings above, cytosolic proteasomes displayed a ~20% increase in proteolytic activities following in vivo HDAC inhibition. To further confirm our interpretation that the acetylation of 20S proteasomes specifically was the site of modulatory lysine acetylation, 20S proteasomes were purified from cardiac tissue following global HDAC inhibition in vivo. Upon purifying 20S proteasomes from the complex milieu of the cytosol, we observed a striking 150% increase in tryp tic proteolytic activity (Fig. 3C). It is highly likely that features inherent to the cytosol (e.g., interfering endogenous proteasome substrates) and to native cytosolic proteasomes (e.g., associating partners) can account for this significant difference in proteolytic responses following HDAC inhibition; importantly, in both systems, the direction of the change was the same. To visualize the increase in acetylation of 20S proteasomes following
HDAC Inhibition Promotes Cardiac Proteostasis

Fig. 3. Global in vivo HDAC inhibition enhances the proteolytic capacity and redistribution of 20S proteasomes and induces lysine acetylation at specific sites. A, the impact of HDAC inhibition on the expression of assembled cytosolic 20S complexes. Following 6 h of HDAC inhibition in vivo, the reduced cytosolic expression level of assembled 20S complexes was illustrated via immunoblotting with antibodies against subunit β2 (n = 5, *p < 0.05 versus without HDAC inhibition). B, HDAC inhibition in vivo significantly increased cytosolic proteasomal activities in the murine heart (n = 5, *p < 0.05 versus without HDAC inhibition). C, purified myocardial 20S from mice treated with HDAC inhibitors exhibited significant elevation of proteolytic activities (n = 5 technical replicates, *p < 0.05 versus without HDAC inhibition). Error bar represents standard error (A–C). D, HDAC inhibition in vivo significantly elevated acetylation of purified 20S proteasomal subunits as demonstrated in immunoblots against acetylated lysine residues. Immunoblottings with antibodies against subunits β2 and β7 served as loading controls. E, the mass spectrum of peptide containing lysine-201 residue from the β7 subunit of 20S proteasome. This novel site of acetylation was only detected following HDAC inhibition in vivo. An Xcorr score of 4.92 confirmed a confident identification.

HDAC inhibition, we resolved intact, purified 20S with SDS-PAGE (Fig. 3D) and found a significant enhancement in lysine acetylation following HDAC inhibition. These data strongly suggest that 20S proteasomes are among the specific targets of global in vivo HDAC inhibition, a procedure currently implemented in numerous clinical trials. HDAC inhibition modulates 20S proteasomes by specifically increasing levels of lysine acetylation, which presumably results in the observed

### Table I

Summary of lysine-acetylation sites on murine cardiac 20S proteasomes identified in this study using collision-induced dissociation

| Subunit | Site | Peptide | Charge | Xcorr | Mascot | Inducible | Novelty | Spectrum |
|---------|------|---------|--------|-------|--------|-----------|---------|----------|
| α1      | 104  | YKYGIVEIPDMLCCK | 2      | 4.59  | 34.6   | WT        |         | S20      |
| α5      | 203  | SSILLKQVMEEK    | 2      | 3.71  | 31.7   | WT        |         | 2B and S21 |
| α6      | 30   | IHOIEYAMEAVKQAGS ATVGLK | 3      | 4.49  | 34.2   | Inducible | *        | S22      |
| α6      | 115  | LVSLGSKQTVPTQR   | 2      | 3.36  | 29.0   | Inducible | *        | S23      |
| β3      | 77   | LNLYELKQEGR     | 2      | 2.97  | 33.9   | Inducible | S24      |
| β6      | 203  | LKVQDFISAAER    | 2      | 3.69  | 32.8   | Inducible | S25      |
| β7      | 201  | EVLEKQOPLQTEAR   | 2      | 4.92  | 33.1   | Inducible | *        | 3E and S26 |

Notes: The name of any given proteasomal subunit (“Subunit”), the site of lysine acetylation (“Site”), the sequence of the captured acetyl-peptide (“Peptide”), the charge state of peptide ion (“Charge”), the cross-correlation score for the proteomic identification by the Sequest algorithm (“Xcorr”), the Mascot identification score (“Mascot”), the occurrence of acetylation in physiological (wild-type, untreated) or pharmacological states (“Inducible”), the status of identification in mammals (“Novelty”), and a reference of example mass spectra (“Spectrum”) are provided. Both UniProt and PubMed databases were queried to evaluate whether a particular identification was novel.
enrichments in proteolytic activity. Moreover, lysine acetylation appears to alter proteasome redistribution by shuttling proteasomes out of the cytosolic compartment.

Finally, to discover sites of lysine acetylation 20S subunits that are inducible by global HDAC inhibition in our clinically relevant model, we analyzed lysine acetylation of 20S proteasomes purified from the in vivo model using affinity enrichment and high-resolution LC-MS/MS. Five sites of lysine acetylation that were below the limit of detection at baseline were identified following in vivo HDAC inhibition (Table 1). These sites include Lys-30 of the α6 subunit (supplemental Fig. S22), Lys-115 of the α6 subunit (supplemental Fig. S23), Lys-77 of the β3 subunit (supplemental Fig. S24), Lys-203 of the β6 subunit (supplemental Fig. S25), and Lys-201 of the β7 subunit (Fig. 3E, supplemental Fig. S26). Of these, Lys-30 and Lys-115 on α6 and Lys-201 on β7 were novel observations. These three sites were observed in both acetylated and non-acetylated forms, indicating substoichiometric levels and an endogenous balance of the two coexisting isoforms. These data indicate that the majority of 20S acetylation sites (five out of the seven identified) are undetectable at baseline and are inducible by in vivo HDAC inhibition. This finding suggests that the acetylation of 20S proteasomes may primarily function to tune proteolytic capacity under times of perturbation of basal cardiac function.

In addition to 20S subunits, six mitochondrial proteins and five cytosolic proteins co-purified with 20S and were identified using LC-MS/MS (supplemental Table S4). Many of these proteins are known associating partners (e.g. 14–3-3 protein (36), Alad (37), and Hsp90 (38)) or substrates of the proteasomes (e.g. Ogdh (39) and Pdhb (40)). Ywhaz, Ywhae, and Tpm1 have molecular weights in the range of proteasomal subunit weight; however, none of these three proteins was detected in the acetylated form.

**DISCUSSION**

In this investigation, lysine acetylation was identified as an endogenous regulator of proteolytic activity in the heart. The inhibition of HDACs was effective in altering the acetylation profile of 20S proteasome complexes and enhancing the proteolytic function of healthy and diseased murine and human myocardium. Accordingly, we characterized the first 20S proteasome acetylome using an integrated proteomics workflow (supplemental Fig. S1 and Table I). Acetylation of nine N-termini and seven internal lysine residues was detected; acetylation on five lysine residues was inducible by global HDAC inhibition in vivo. Four of the seven acetylation sites were novel observations in mammals, and the 20S subunit sequence alignments indicated that all regions containing these seven lysine residues are conserved in mouse, human, rat, dog, and cow (supplemental Fig. S30). This study was the first to characterize acetylation as a molecular modulator of the proteasome complex, unveiling a novel mechanism of regulation. Our investigation was the first to link 20S subunit acetylation with alterations in 20S proteolytic capacities, thereby unveiling a potential therapeutic strategy for restoring compromised cardiac proteostasis, which warrants future exploration.

**Importance of the Technical Workflow**—Murine cardiac 20S proteasomes were isolated based on a previously published protocol (14, 41) that has been shown to produce >98% pure, functionally viable 20S proteasomes. The purity and the molecular components of purified 20S proteasomes were displayed via two-dimensional electrophoresis, as discussed in previous publications (14, 41). The fidelity of this preparation was central to our investigation of how acetylation modifies 20S proteasomes specifically, as it has been shown that acetylation of the 11S regulatory subunit also occurs endogenously (35). Because 20S proteasome subunits are targets of acetylation on both N-termini and lysine residues, isobarically, at the onset of this study, the N-termini of all 17 subunits in cardiac 20S proteasomes were characterized using a multi-enzyme digestion approach and mass spectrometry (supplemental Table S3). This ensured accurate differentiation between acetylation modifications on lysine residues and N-termini. Furthermore, the mammalian 20S proteasome is a 750,000-Da protein complex with an assembly combination of 17 subunits. Enzyme digestion of proteasomes leads to a rather complex pool of peptides, which poses a technical challenge in attempts to capture every peptide via traditional LC separation and MS detection. Accordingly, three distinct enzymes were employed to digest 20S proteasomes; however, the combined LC-MS/MS outputs failed to fully detect all 20S peptides (supplemental Table S3). Therefore, affinity enrichment was applied to preferentially target peptides with acetylated lysine residues, which consequently reduced the signal suppression by nonacetylated peptides. This approach was essential for achieving comprehensive characterization of the lysine acetylome on cardiac 20S proteasomes. However, this enrichment approach removed nonacetylated peptides, which made it unfeasible to evaluate stoichiometry via label-free quantification analysis. Furthermore, acetylation of lysine residues renders them indigestible by trypsin, whereas nonacetylated lysine residues are susceptible to trypsin digestion. Thus, without the affinity enrichment procedure, the varying digestion profiles of acetylated versus nonacetylated peptides still preclude the application of a label-free quantitative approach for obtaining a relative abundance ratio. Moreover, the MS dataset of Glu-C digestion (without the affinity enrichment procedure) failed to provide such paired peptides for quantification. We are in the process of developing an MRM-based protocol (42) for quantifying acetylation modifications.

**Physiology of Cardiac Lysine Acetylation**—The oscillation of non-histone protein acetylation is regulated antagonistically by HATs and HDACs. In the murine myocardium, multiple isozymes of HATs and HDACs are co-expressed in distinct subcellular compartments, including the nucleus, mitochon-
dria, and cytosol (supplemental Fig. S2A). These isoforms are also expressed in human cells (see detailed subcellular localizations in supplemental Fig. S2B and corresponding references in supplemental Table S5). Because the specific isoforms governing the acetylation of 20S proteasomes are not yet known, two broad-spectrum HDAC inhibitors, SAHA and sodium valproate, were used in our study. Pharmacological inhibition of endogenous HDACs in vivo triggered the acetylation of five distinct lysine residues. Despite the potent effect on proteolytic activity, lysine acetylation was not detected on catalytically active subunits (e.g. β1, β2, β5, β1i, β2i, and β5i) (Table I). It is conceivable that the noncatalytic subunits are structurally more accessible to HATs and HDACs because of their orientation in the assembled proteasome complexes (43). Thus, the observed functional consequences of lysine acetylation may induce a conformational change in 20S proteasomes that renders the catalytic centers more accessible to substrates. This is supported by a report that acetylation causes the proteasome complexes to be more sensitive to inhibitors (44). We investigated the structure of each acetylated peptide sequence; all acetylated lysine residues were located in an α-helix (supplemental Figs. S2O–S26). This is consistent with the notion that acetylated lysine residues reside more frequently at peptide sequences with ordered secondary structures (34).

Given the fact that the inhibitors used in this study are not specific to particular HDAC isoforms, pinpointing acetylated lysine residues is a necessary step toward the identification of corresponding HDAC and HAT isoforms by which interventions of higher specificity can be formulated. In addition to lysine acetylation, these agents may affect proteasome function via indirect mechanisms. Lysine residues are also subject to other posttranslational modifications. For example, Lys-115 of the α6 subunit (supplemental Fig. S23) has previously been reported as a target of ubiquitination. Acetylation enhances the ubiquitin-independent proteolytic activity of cardiac 20S proteasomes; however, the interplay of lysine acetylation with ubiquitin-dependent pathways of 26S proteasomes remains to be investigated. Another possible impact of HDAC inhibition on 20S proteasomes is that acetylation may induce 20S redistribution among different subcellular compartments. Following HDAC inhibition, cytosolic 20S subunit expression was consistently reduced, whereas 20S abundance in total myocardium remained the same, indicating that acetylation modulates other facets of proteasomal function. (supplemental Figs. S31 and S32).

Clinical Utility of Cardiac Lysine Acetylation—Ischemic injury has previously been associated with the depression of proteasome activities (45, 46), and herein we demonstrated that HDAC inhibition restored 20S proteasome function in acutely injured hearts. A recent study by Li et al. demonstrated that the augmentation of proteasomal function via transgenic up-regulation of the 11S proteasome protects against ischemic injury (47). This supports the notion that the enhancement of proteolytic activities alone may be cardioprotective, and our study offers a clinically feasible avenue for accomplishing this. Indeed, pharmacological reagents altering acetylation via HDAC inhibition have been used to treat neurological (48), malignant (49), and inflammatory (50) diseases, and HDAC inhibitors have shown clinical benefits for certain cardiomyopathies (12). Both broad-spectrum HDAC inhibitors used in this study (SAHA and sodium valproate) have been tested in clinical trials (51, 52); therefore, the pharmacologic parameters acquired from these ongoing trials (e.g. TC50) will undoubtedly expedite the utility of HDAC inhibitors in cardiac trials. Dosage ranges for sodium valproate have been previously reported in a number of cells and tissues (53–57), but we exerted considerable effort to determine the effective range of this inhibitor in murine and human myocardium. HDAC inhibition with sodium valproate elevated 20S proteolytic capacity in a dose-dependent fashion, and maximum proteolytic capacity was achieved at a dosage of 1 mmol/l, which completely restored proteolytic function in injured myocardium. The highest dosage (5 mmol/l) was associated with potential toxicity. In contrast, we did not observe any toxic effect of SAHA (up to 50 μmol/l), and our results regarding the effective regulatory range of SAHA agree with a previous report (56). Importantly, murine and human myocardial 20S capacities exhibited high sensitivity to HDAC inhibition, suggesting that clinical efficacy can be achieved at relatively low doses of HDAC inhibitors. Our study therefore offers new mechanistic insights into the effects of global HDAC inhibition through the identification of 20S catalytic capacities as potent effectors of this therapeutic regime. This is particularly important for pathologies in proteinopathies, in which protein quality control mechanisms have gone awry and damaged proteins accumulate (5).

In summary, we have demonstrated that cardiac proteasomal function can be regulated by a promising class of pharmacological agents, HDAC inhibitors, which are effective both in vitro and in vivo. Our results highlight an exciting potential therapeutic strategy for regulating proteolytic function in the diseased myocardium.

* This work was supported in part by NIH award HL R01-098954 and NHLBI Proteomics Center Award HHSN268201000035C to Dr. Peipei Ping, a Theodore C. Laubisch endowment at UCLA to Dr. Peipei Ping, and NIRS fellowship F32-HL-099020 to Dr. Sarah Scruggs.

** To whom correspondence should be addressed: Peipei Ping, Ph.D., FAHA, FISHR, UCLA School of Medicine, 675 Charles E. Young Drive South, MRL Building Suite 1-609, Los Angeles, CA 90095, Tel.: 1-310-267-5624, Fax: 1-310-267-5623, E-mail: pping@mednet.ucla.edu; Haojie Lu, Ph.D., Department of Chemistry, Fudan University, 138 Yi Xue Yuan Road, Shanghai 200032, China, Fax: 86-21-54237618, E-mail: luhaojie@fudan.edu.cn.

§ These authors contributed to this work equally.
dehydrogenase complex deficiency caused by ubiquitination and proteasome-mediated degradation of the E1 subunit. J. Biol. Chem. 283, 237–243

41. Drews, O., Wildgruber, R., Zong, C., Sukop, U., Nissum, M., Weber, G., Gomes, A. V., and Ping, P. (2007) Mammalian proteasome subpopulations with distinct molecular compositions and proteolytic activities. Mol. Cell. Proteomics 6, 2021–2031

42. Lam, M. P., Lau, E., Scruggs, S. B., Wang, D., Kim, T. Y., Liem, D. A., Zhang, J., Ryan, C. M., Faull, K. F., and Ping, P. (2013) Site-specific quantitative analysis of cardiac mitochondrial protein phosphorylation. J. Proteomics 81, 15–23

43. Unno, M., Mizushima, T., Morimoto, Y., Tomisugi, Y., Tanaka, K., Yasuoka, N., and Tsukihara, T. (2002) The structure of the mammalian 20S proteasome at 2.75 Å resolution. Structure 10, 609–618

44. Dasmahapatra, G., Lembersky, D., Kramer, L., Fisher, R. I., Friedberg, J., Dent, P., and Grant, S. (2010) The pan-HDAC inhibitor vorinostat potentiates the activity of the proteasome inhibitor carfilzomib in human DLBCL cells in vitro and in vivo. Blood 115, 4478–4487

45. Churchill, E. N., Ferreira, J. C., Brum, P. C., Szweda, L. I., and Mochly-Rosen, D. (2010) Ischaemic preconditioning improves proteasomal activity and increases the degradation of deltaPKC during reperfusion. Cardiovasc. Res. 85, 385–394

46. Divald, A., Kivity, S., Wang, P., Hochhauser, E., Roberts, B., Teichberg, S., Gomes, A. V., and Powell, S. R. (2010) Myocardial ischemic preconditioning preserves postischemic function of the 26S proteasome through diminished oxidative damage to 19S regulatory particle subunits. Circ. Res. 106, 1829–1838

47. Li, J., Horak, K. M., Su, H., Sanbe, A., Robbins, J., and Wang, X. (2011) Enhancement of proteasomal function protects against cardiac proteinopathy and ischemia/reperfusion injury in mice. J. Clin. Invest. 121, 3689–3700

48. Guan, J. S., Haggarty, S. J., Giacometti, E., Dannenberg, J. H., Joseph, N., Gao, J., Nieland, T. J., Zhou, Y., Wang, X., Mazitschek, R., Bradner, J. E., DePinho, R. A., Jaenisch, R., and Tsai, L. H. (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459, 55–60

49. Marks, P. A., and Dokmanovic, M. (2005) Histone deacetylase inhibitors: discovery and development as anticancer agents. Expert Opin. Investig. Drugs 14, 1497–1511

50. Adcock, I. M. (2007) HDAC inhibitors as anti-inflammatory agents. Br. J. Pharmacol. 150, 829–831

51. Munster, P., Marchion, D., Bicaku, E., Lacevic, M., Kim, J., Centeno, B., Daud, A., Neuger, A., Minton, S., and Sullivan, D. (2009) Clinical and biological effects of valproic acid as a histone deacetylase inhibitor on tumor and surrogate tissues: phase I/I trial of valproic acid and epirubicin/FEC. Clin. Cancer Res. 15, 2488–2496

52. Friday, B. B., Anderson, S. K., Buckner, J., Yu, C., Giannini, C., Geoffroy, F., Schwerkoske, J., Mazurczak, M., Gross, H., Pajon, E., Jaeckle, K., and Galanis, E. (2012) Phase II trial of vorinostat in combination with bortezomib in recurrent glioblastoma: a north central cancer treatment group study. Neuro Oncol. 14, 215–221

53. Burba, I., Colombo, G. I., Staszewsky, L. I., De Simone, M., Devanna, P., Nanni, S., Avitabile, D., Molla, F., Cosentino, S., Russo, I., De Angelis, N., Soldo, A., Biondi, A., Gambini, E., Gaetano, C., Farsetti, A., Pompilo, G., Latini, R., Capogrossi, M. C., and Pesce, M. (2011) Histone deacetylase inhibition enhances self renewal and cardioprotection by human cord blood-derived CD34 cells. PloS One 6, e22158

54. Zhang, Z., Tong, N., Hong, R., Qiu, B., Liu, L., Xu, X., and Wu, X. (2011) Valproate protects the retina from endoplasmic reticulum stress-induced apoptosis after ischemia-reperfusion injury. Neurosci. Lett. 504, 88–92

55. Papi, A., Ferreri, A. M., Guerra, F., and Orlandi, M. (2012) Anti-invasive effects and proapoptotic activity induction by the rexinoid IIF and valproic acid in combination on colon cancer cell lines. Anticancer Res. 32, 2855–2862

56. Gansmayer, M., Konturek, P., Herold, C., Neurath, M. F., and Zopf, S. (2012) Antitumoral efficacy of four histone deacetylase inhibitors in hepatoma in vitro and in vivo. Anticancer Res. 32, 5263–5269

57. Eikel, D., Lampen, A., and Nau, H. (2006) Teratogenic effects mediated by inhibition of histone deacetylases: evidence from quantitative structure activity relationships of 20 valproic acid derivatives. Chem. Res. Toxicol. 19, 272–278