Cardiac hypertrophy is an independent risk factor for cardiac morbidity and mortality and represents a pathological adaptation for pressure overload and hypertension. Despite its initial compensatory nature to pressure overload, persistent cardiac hypertrophy results in ultimate maladaptation and deteriorated cardiac function en route to heart failure. Cardiac hypertrophy is characterized by increased ventricular wall thickness coinciding with enlarged cardiomyocytes. Mechanical stress attributable to pressure overload, together with a variety of humoral factors (eg, activation of the renin–angiotensin system), has been implicated in the expression of hypertrophic genes, leading to the onset and development of cardiac hypertrophy. Recurrence of fetal genes and other genes with protein synthesis in the heart is associated with hypertrophy.1,2

Cathepsins are a group of lysosomal cysteine proteases belonging to the papain family. Accumulating evidence has implicated a role of cathepsins in the pathogenesis of cardiovascular disease.3,4 Protein levels of cathepsin S and K are elevated in pressure overload–induced cardiac remodeling in rodents and human.5 Along the same lines, pharmacological inhibition of cathepsin attenuated cardiac fibrosis and remodeling in Dahl salt-sensitive hypertensive rats.6,7 Thus, cathepsins represent a viable target to ameliorate cardiac dysfunction associated with pressure overload. Among the cathepsins, cathepsin K possesses the most potent collagenolytic and elastolytic activities.8 Ablation of cathepsin K has been recently shown to improve whole body glucose disposal and prevent adipogenesis in mice.9 We have recently found that knockdown of cathepsin K improves obesity-associated cardiac dysfunction.10 However, it is unclear whether cathepsin K has direct effects on the heart, independent of its metabolic effects. To this end, we hypothesized that ablation of cathepsin K protects against pressure overload–induced cardiac hypertrophy. We took advantage of a unique cathepsin K knockout model and subjected these mice to pressure overload before assessment of myocardial geometry and contractile properties. Because the mammalian target of rapamycin (mTOR) signaling pathway plays a key role in the development of cardiac hypertrophy,11 the expression and phosphorylation levels of mTOR and its downstream signaling molecules 4E-binding protein 1 (4E-BP1), p70S6 kinase, regulatory-associated protein of mTOR (Raptor), and rapamycin-insensitive companion of mTOR (Rictor) were also examined.

Abstract—Evidence from human and animal studies has documented elevated levels of lysosomal cysteine protease cathepsin K in failing hearts. Here, we hypothesized that ablation of cathepsin K mitigates pressure overload–induced cardiac hypertrophy. Cathepsin K knockout mice and their wild-type littermates were subjected to abdominal aortic constriction, resulting in cardiac remodeling (heart weight, cardiomyocyte size, left ventricular wall thickness, and end diastolic and end systolic dimensions) and decreased fractional shortening, the effects of which were significantly attenuated or ablated by cathepsin K knockout. Pressure overload dampened cardiomyocyte contractile function along with decreased resting Ca2+ levels and delayed Ca2+ clearance, which were partly resolved by cathepsin K knockout. Cardiac mammalian target of rapamycin and extracellular signal-regulated kinases (ERK) signaling cascades were upregulated by pressure overload, the effects of which were attenuated by cathepsin K knockout. In cultured H9c2 myoblast cells, silencing of cathepsin K blunted, whereas cathepsin K transfection mimicked phenylephrine–induced hypertrophic response, along with elevated phosphorylation of mammalian target of rapamycin and ERK. In addition, cathepsin K protein levels were markedly elevated in human hearts of end-stage dilated cardiomyopathy. Collectively, our data suggest that cathepsin K ablation mitigates pressure overload–induced hypertrophy, possibly via inhibition of the mammalian target of rapamycin and ERK pathways. (Hypertension. 2013;61:1184-1192.)

Key Words: cardiac hypertrophy • cathepsin K • contractile function • mammalian target of rapamycin

Cathepsin K Knockout Alleviates Pressure Overload–Induced Cardiac Hypertrophy

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Materials and Methods

Please see the expanded methods in the online-only Data Supplement.

Experimental Animals and Surgical Intervention

The experimental protocols have been approved by the animal use and care committee at the University of Wyoming. Cathepsin K global knockout mice (Ctsk−/−) and wild-type control (C57BL/6J) mice were subjected to abdominal aortic constriction as reported previously.13

Human Samples

Left ventricular (LV) tissues from nonfailing and failing hearts were obtained via an Institutional Review Board-approved protocol maintained by the University of Colorado Cardiac Tissue Bank. Hearts donated for research purposes were obtained with written consent from family members or by direct written consent from end-stage patients with heart failure undergoing cardiac transplantation. Patient characteristics are presented in Table 1.

Echocardiographic Assessment

Cardiac geometry and function were evaluated using a 2-dimensionally guided M-mode echocardiography equipped with a 15- to 16-MHz linear transducer. Fractional shortening was calculated from end-diastolic diameter and end-systolic diameter using the following equation: (end-diastolic diameter − end-systolic diameter)/end-diastolic diameter.14 Stroke volume was calculated as (left ventricular end-diastolic dimension) − (left ventricular end-systolic dimension).14 Cardiac output was calculated as the product of stroke volume and heart rate.

Cardiomyocyte Isolation and Mechanics

Mouse cardiomyocytes were isolated as described previously.14 Mechanical properties of myocytes were assessed using an IonOptix soft-edge system. Cell shortening and relengthening were assessed using the following indices: peak shortening, time to peak shortening, time to 90% relengthening, and maximal velocities of shortening/relengthening (±dL/dt).

Intracellular Ca2+ Transients

Myocytes loaded with fura-2-acetoxymethyl ester (0.5 μmol/L) were exposed to lights emitted through either a 360- or a 380-nm filter. Fluorescence emissions were detected between 480 and 520 nm.15

Blood Pressure Measurement

Systolic and diastolic blood pressure values were measured by using a noninvasive blood pressure device (Kent Scientific Co, Torrington, CT).

Histopathologic Analysis

Ventricular tissues were stained with the fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin, and cardiomyocyte cross-sectional area was quantitated from 100 randomly selected cardiomyocytes. Myocardial fibrotic area was assessed using Masson trichrome staining.16

Total RNA Extraction, cDNA Synthesis, Reverse Transcription, and Real-Time Polymerase Chain Reaction

Total RNA extraction, cDNA synthesis, and real-time polymerase chain reaction were carried out as described previously.14

Western Blot Analysis

Proteins were separated on sodium dodecyl sulfate–polyacrylamide gels and were transferred to polyvinylidene difluoride membranes and incubated overnight with specific antibodies. The antigens were detected by the luminescence method.14

In Vitro Hypertrophy, Silencing, and Overexpression of Cathepsin K

H9c2 myoblasts were treated with phenylephrine (PE; 100 μmol/L, 24 hours) to induce hypertrophy.17 Small interfering RNAs against cathepsin K were used to knock out cathepsin K, whereas overexpression of cathepsin K was achieved via plasmid–mediated transfection.

Cell Surface Area Measurement

Immunostaining for α-actin was used for cell surface area measurement as described previously.16

Table 1. Patient Characteristics

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<th>Patient No.</th>
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Mean±SEM 34.7±4.8

7       Failing heart  F  60  IDC  15
8       Failing heart  F  57  IDC  14
9       Failing heart  F  53  IDC  23
10      Failing heart  M  65  IDC  24
11      Failing heart  M  57  IDC  23
12      Failing heart  M  63  IDC  18

Mean±SEM 59.2±1.8 19.29±1.66

IDC: 59.2±1.8 y, n=3 male and 3 female; mean ejection fraction: 19.29±1.66%; NF: 34.7±4.8 y, 3 males and 3 females. CHI indicates closed head injury; IDC, idiopathic dilated cardiomyopathy; MVA, motor vehicle accidents; and NF, nonfailing heart.

Figure 1. Effect of pressure overload on myocardial expression of cathepsin K. A, Representative gel blots exhibiting cathepsin isoforms and GAPDH (loading control) in myocardial tissues from mice subjected to sham or abdominal aortic constriction (AAC) procedure. B, Densitometric quantification of cathepsin K, cathepsin B, cathepsin S, and cathepsin L. Mean±SEM, n=6 mice per group, *P<0.05 vs sham group.
Abdominal Aortic Constriction Resulted in Increased Cathepsin K Expression in the Heart

Protein levels of cathepsin K were significantly elevated in mouse hearts after abdominal aortic constriction procedure compared with sham group, whereas cathepsins B and S remained unchanged. Although cathepsin L displayed a trend toward increase, it failed to reach statistical significance (Figure 1).

Cathepsin K Knockout Attenuates Cardiac Hypertrophy and Remodeling in Response to Pressure Overload

Abdominal aortic constriction resulted in an increase in heart weight and size (normalized to body weight), the effect of which was mitigated by cathepsin K knockout (Table 2 and Figure 2A). Consistently, wild-type mice subjected to aortic constriction exhibited enlarged cardiomyocyte cross-sectional area, and the effect was obliterated by cathepsin K knockout (Figure 2B and 2D). Moreover, pressure overload facilitated interstitial fibrosis in the heart, which was abolished in the cathepsin K knockout mice (Figure 2C and 2E). In addition, myocardial mRNA levels of fetal genes (ANP, BNP, β-MHC) were overtly elevated after abdominal aortic constriction, the effect of which was absent in cathepsin K knockout mice (Figure S1 in the online-only Data Supplement).

Cathepsin K Knockout Alleviates Pressure Overload–Induced Cardiac Dysfunction

Echocardiographic analysis revealed overt cardiac hypertrophy after pressure overload as evidenced by increased ventricular wall thickness as well as enlarged LV end-diastolic and systolic diameters (Figure 3A–3C). Aortic constriction also resulted in compromised cardiac function as seen by reduced fractional shortening (Figure 3D). Cathepsin K knockout reversed these changes induced by aortic constriction. The knockout of cathepsin K did not elicit any notable cardiac phenotype. Furthermore, few changes were noted in cardiac output after aortic constriction in either wild-type or cathepsin K knockout mice. Abdominal aortic constriction significantly enhanced diastolic, systolic, and mean blood pressure values (Figure S2). Cathepsin K ablation did not cause any change in the pressure-overload–induced elevated blood pressure values.
To further elucidate the beneficial effects of cathepsin K knockout on cardiac dysfunction after pressure overload, contractile function and intracellular Ca\(^{2+}\) handling were evaluated in cardiomyocytes. Neither aortic constriction nor cathepsin K deletion, or both, affected resting cell length (Figure 4A). Aortic constriction resulted in decreased peak shortening and +dL/dt in wild-type mice, both of which were reconciled by cathepsin K ablation (Figure 4B and 4C). Although aortic constriction did not elicit any changes in −dL/dt, time to peak shortening, or time to 90% relengthening, a small but significant decrease in these parameters was observed in the cathepsin K knockout mice that were subjected to aortic constriction (Figure 4E and 4F). Consistent with the mechanical data, impairment in intracellular Ca\(^{2+}\) handling was observed in cardiomyocytes from hearts of mice subjected to abdominal aortic constriction as evidenced by suppressed resting intracellular Ca\(^{2+}\) levels and prolonged intracellular Ca\(^{2+}\) decay (Figure 5). Cathepsin K depletion abrogated these changes in Ca\(^{2+}\) handling triggered by pressure overload. Cathepsin K knockout mice subjected to aortic constriction exhibited a lower intracellular stimulated Ca\(^{2+}\) release compared with the ones that underwent sham surgery.

**Cathepsin K Knockout Attenuates Pressure Overload–Induced Upregulation of GATA Binding Protein 4, and Phosphorylation of Akt and AMP-Activated Protein Kinase**

The levels of GATA binding protein 4 (GATA4), a cardiac hypertrophic marker, were significantly upregulated by aortic constriction, which was negated by cathepsin K knockout (Figure 6). Likewise, abdominal aortic constriction resulted in increased phosphorylation of Akt, AMP-activated protein kinase (AMPK), and acetyl-CoA carboxylase (ACC) in the hearts of wild-type mice, whereas such upregulation was

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**Figure 3.** Echocardiographic features in wild-type (WT) and cathepsin K knockout mice subjected to sham or abdominal aortic constriction (AAC). A, Left ventricular wall thickness. B, Left ventricular end-diastolic dimension (LVEDD). C, Left ventricular end systolic dimension (LVESD). D, Fractional shortening (FS). Mean±SEM, n=6 to 9 mice group. *P<0.05 vs WT-sham group, &P<0.05 vs Ctsk\(^{-/-}\)-sham group.

**Figure 4.** Cardiomyocyte contractile properties in wild-type (WT) and cathepsin K knockout mice subjected to sham or abdominal aortic constriction (AAC). A, Resting cell length. B, Peak shortening (PS; normalized to cell length). C, Maximal velocity of shortening (+dL/dt). D, Maximal velocity of relengthening (−dL/dt). E, Time to PS (TPS). F, Time to 90% relengthening (TR\(_{90}\)). Mean±SEM, n=76 to 97 cells per group. *P<0.05 vs WT-sham group, &P<0.05 vs WT-AAC group.

**Figure 5.** Intracellular Ca\(^{2+}\) transients in cardiomyocytes in wild-type (WT) and cathepsin K knockout mice subjected to sham surgery or abdominal aortic constriction (AAC). A–C, Resting fura-2 fluorescence intensity (FFI), electrically stimulated rise in FFI (ΔFFI), single exponential intracellular Ca\(^{2+}\) decay rate, respectively. Mean±SEM, n=94 to 127 cardiomyocytes for intracellular Ca\(^{2+}\) transient detection.
less pronounced in mice devoid of cathepsin K (Figure 6). In contrast, abdominal aortic constriction did not alter the phosphorylation status of GSK3\(\beta\) or p38 mitogen-activated protein kinase (MAPK) in either wild-type or cathepsin K knockout mice (Figure 7).

Cathepsin K Knockout Attenuates Pressure Overload–Induced Phosphorylation of ERK, mTOR, and 4E-BP1

Because mTOR plays a pivotal role in governing protein synthesis and cardiac hypertrophy, the levels of mTOR regulators were scrutinized in hearts from wild-type and cathepsin K knockout mice after pressure overload. Aortic constriction significantly promoted phosphorylation of ERK and components of the mTOR signaling cascade, including mTOR, p70S6 kinase, Raptor, and Rictor (Figure 7). The translational repressor protein 4E-BP1 was also phosphorylated in hearts from mice subjected to pressure overload. Phosphorylation of 4E-BP1 inhibits the interaction of 4E-BP1 with 5'-cap–dependent mRNA resulting in the activation of cap-dependent translation.\(^{19}\) Although cathepsin K knockout itself did not exert any notable effect on the mTOR signaling cascade, it nullified aortic constriction–induced changes in mTOR signaling proteins.

Cathepsin K Is Necessary and Sufficient to Induce Hypertrophy in Cultured Myoblasts

To further validate whether cathepsin K plays a causal role in cardiac hypertrophy, in vitro study was performed using cultured embryonic rat heart–derived H9c2 cells in the presence and absence of the prohypertrophic agent PE. As shown in Figure 8A and 8B, PE elicited a robust hypertrophic response as visualized by immunohistochemical staining of actin. In addition, PE treatment caused a significant increase in protein synthesis in H9c2 cells as shown by \([^{3}H]\) leucine incorporation (Figure 9C). Interestingly, the small interfering RNAs silencing of cathepsin K before PE challenge significantly attenuated the hypertrophic response and leucine incorporation in response to PE. Cathepsin K knockdown itself did not alter either the cell size or the leucine incorporation rate. The gain-of-function approach was used to further evaluate the role of cathepsin K in cardiac remodeling. H9c2 cells were transfected with a cathepsin K plasmid, to achieve a 3- to 4-fold increase in cathepsin K expression.
expression (Figure S3), which resulted in an upregulation of GATA4 (data not shown) and increased leucine incorporation, suggesting hypertrophic response (Figure 8D–8F). Furthermore, the cathepsin K–mediated hypertrophic response and leucine incorporation were abolished by the mTOR inhibitor rapamycin. Cells treated with control plasmid or rapamycin alone did not exhibit any effects on basal cell area and leucine incorporation rate. Furthermore, plasmid–mediated transfection of cathepsin K in H9c2 resulted in elevated levels of phospho-mTOR and phospho–4E-BP1 (Figure 9A). Treating cells with the pharmacological inhibitor of ERK (Erk Inhibitor II, Calbiochem) inhibited the cathepsin K–mediated phosphorylation of mTOR and 4E-BP1 (Figure 9B).

**Figure 8.** Cathepsin K knockdown alleviates phenylephrine (PE)–induced hypertrophy and protein synthesis, whereas cathepsin K overexpression facilitates hypertrophic response in cultured H9c2 cells. A, Representative images of α-actin staining exhibiting H9c2 cells transfected with cathepsin K small interfering RNAs (siRNA) in the presence or absence of phenylephrine (PE, 100 μmol/L, 24 h). B, Quantitative analysis of cell area from α-actin staining images. C, 3H-leucine incorporation in H9c2 cells transfected with cathepsin K siRNA in the presence or absence of phenylephrine. D, Representative images of α-actin staining in H9c2 cells transfected with the cathepsin K plasmid in the presence or absence of rapamycin. E, Pooled data from D. F, 3H-leucine incorporation in H9c2 cells transfected with cathepsin K plasmid in the presence or absence of rapamycin. *P<0.05 vs NT-siRNA group, #P<0.05 vs catK siRNA group, &P<0.05 vs NT-siRNA + PE group in B and C, *P<0.05 vs CONT group, &P<0.05 vs catK cDNA group in E and F.

**Cathepsin K Protein Levels Are Elevated in the Human Failing Heart**

To relate findings from experimental approach to clinical setting, protein levels of cathepsin K were examined in LV tissues obtained from patients with end-stage heart failure and normal healthy controls. As seen in Figure 10, detectable cathepsins were observed in tissues from both nonfailing and failing heart, with much higher cathepsin levels in samples from patients with dilated cardiomyopathy compared with the nonfailing controls (Figure 10).

**Discussion**

Heart failure represents the leading cause of morbidity and mortality. Identifying novel targets to suppress maladaptive
Hypertension constitutes a therapeutic strategy to preempt heart failure. The salient finding of our present study is that mice lacking cathepsin K gene are resistant to pressure overload–induced cardiac hypertrophy, fibrosis, and contractile anomalies. At the molecular level, cathepsin K knockout retards pressure overload–induced cardiac hypertrophic genes. In cardiomyocytes, pressure overload interrupts intracellular Ca²⁺ homeostasis, the effect of which was reconciled by cathepsin K knockout. Cathepsin K knockout failed to alter blood pressure in mice after pressure overload, suggesting a cardiac-specific benefit for cathepsin K ablation. To the best of our knowledge, this is the first study to show that deletion of cathepsin K confers cardioprotection against cardiac hypertrophy. Importantly, cathepsin K inhibitors are currently being developed for their utility in individuals with osteoporosis, and may have potential promises in the management of cardiac hypertrophy and associated comorbidities.

Previous reports have shown that cathepsin K plays a role in the morphogenesis of heart valves, suggesting that this protease may impose a pivotal effect on cardiac development. However, the role of cathepsins in cardiac pathology has gained dramatic attention over the recent years. Our current study showing an upregulation of cathepsin K in mice subjected to pressure overload and in LV tissues from subjects with heart failure is consistent with the notion that cathepsin K plays a causative role in the development of cardiac hypertrophy, inhibition of which attenuates cardiac hypertrophy. It is, however, quite possible that elevated expression of cathepsin K may represent a compensatory mechanism, and that the cathepsin K knockout mice may develop dilated cardiomyopathy with time. However, at 4 weeks of ACC, we did observe a significant decrease in fractional shortening (FS) (which was reconciled by cathepsin K knockout), suggesting apparent transition into decomposition. Furthermore, our in vitro studies suggest that cathepsin K is permissive to cardiac hypertrophy as overexpression of cathepsin K leads to hypertrophy, whereas silencing of cathepsin K inhibits PE–induced hypertrophy. In addition, given that cathepsin K knockout reconciled pressure overload–induced cardiac hypertrophy and contractile dysfunction without altering the pressure-overload–induced elevation in blood pressure, it is likely that the benefits of cathepsin K knockout may have originated from the heart as opposed to systemic circulatory factors.

mTOR signaling has been widely accepted as an initiator of protein synthesis and cardiac hypertrophy. Growth factors and neurohormonal hypertrophic agonists activate mTOR through various pathways. The p70S6K and 4E-BP1 are 2 downstream targets for mTOR. The p70S6K protein phosphorylates the 40S ribosomal protein S6 and the translation initiation factor eIF4B. The pivotal role of mTOR pathway in the generation of cardiac hypertrophy is underscored by the studies which show that rapamycin, an inhibitor for mTOR, attenuates the development of cardiac hypertrophy induced by aortic constriction. Our data show that the mTOR pathway is upregulated in the hypertrophic heart, which is attenuated in the cathepsin K knockout mice. In addition, we found that cathepsin K knockout attenuates AMPK phosphorylation and, to a lesser extent, Akt phosphorylation in the hypertrophic heart. It is possible that AMPK activation may serve as compensatory response in the face of pressure overload, whereas Akt pathway may be responsible for the hypertrophic response, contributing to a greater extent to mTOR activation. Cathepsin K knockout lowered AMPK phosphorylation without altering Akt phosphorylation, suggesting that it may be affecting mTOR signaling independent of these pathways. In cultured myocytes, silencing of cathepsin K resulted in an
inhibition of PE–induced hypertrophic growth and 3H-leucine incorporation, an indicator of de novo protein synthesis, suggesting that cathepsin K may be necessary for protein synthesis and hypertrophy. In addition, plasmid–mediated transfection of cathepsin K was sufficient to promote hypertrophic cell growth and protein synthesis in cultured cardiomyocytes. Cathepsin K–induced hypertrophic response and protein synthesis were inhibited by rapamycin, an inhibitor of the mTOR pathway. Cells transfected with cathepsin K also exhibited an increased level of GATA4, p-mTOR, and p–4E-BP1, providing further support for a role of cathepsin K in prohypertrophic response through mTOR signaling. Furthermore, mTOR phosphorylation consequent to cathepsin K overexpression was negated by a pharmacological inhibitor of ERK. Although ERK signaling has been implicated in the development of cardiac hypertrophy9,10 and inhibition of cathepsin K has been shown to suppress ERK phosphorylation in other cell types,11 the mechanisms by which it does so are yet unclear.

In summary, findings from our present study indicate that targeting cathepsin K may represent an attractive therapeutic approach to curtail hypertrophy and improve cardiac function during hemodynamic stress. Our data show that the antihypertrophic effect of cathepsin K deletion is attributable to decreased protein synthesis via the inhibition of mTOR signaling. Furthermore, cathepsin K expression mimics prohypertropic growth and protein synthesis in cultured cardiomyocytes, which were inhibited by rapamycin. Based on these observations, it should be worthwhile to explore the clinical utility of pharmacological inhibitors of cathepsin K (currently in clinical trial for osteoporosis) in the management of cardiac hypertrophy and the transition from hypertrophy to failure.

Perspectives
Cysteine protease cathepsin K is upregulated in the failing heart. Knockdown of cathepsin K mitigates pressure overload–induced pathological cardiac hypertrophy and contractile dysfunctions. Pharmacological inhibitors of cathepsin K are under clinical trials for the treatment of osteoporosis. It is likely that these selective cathepsin K inhibitors may have potential utility in the treatment or control of cardiac dysfunction.

Acknowledgments
Y. Hua researched data and wrote the first draft of the manuscript. X. Xu researched data and assisted with abdominal aortic constriction model. G. Shi reviewed the article, contributed to discussion, and provided transgenic mice. A. Chicco reviewed the article, contributed to discussion, and provided human tissue samples. J. Ren reviewed/editied the article, assisted with echocardiography, and contributed to discussion. S. Nair planned the study, wrote the article, and contributed to discussion.

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Disclosures
None.

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Novelty and Significance

What Is New?
• Cathepsin K protein levels are elevated in failing human heart and mouse heart subjected to pressure overload.
• Cathepsin K knockout attenuates pressure overload–induced cardiac hypertrophy, fibrosis, and contractile dysfunction.

What Is Relevant?
• Chronic pressure overload–induced pathological cardiac hypertrophy and contractile dysfunction are mitigated by cathepsin K knockout.

Summary
Cathepsin K is a novel therapeutic target for pathological cardiac hypertrophy.
Cathepsin K Knockout Alleviates Pressure Overload–Induced Cardiac Hypertrophy
Yinan Hua, Xihui Xu, Guo-Ping Shi, Adam J. Chicco, Jun Ren and Sreejayan Nair

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CATHEPSIN K KNOCKOUT ALLEVIATES PRESSURE OVERLOAD-INDUCED CARDIAC HYPERTROPHY

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Short title: CatK-KO Mitigates Cardiac Hypertrophy

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Supplement Methods

**Experimental Animals and Surgical Intervention** All procedures performed in animals were approved by the University of Wyoming Institutional Animal Care and Use Committee. To induce cardiac hypertrophy, 3 months-old male C57BL/6 and whole-body cathepsin K knockout (Ctsk\(^{-/-}\))\(^1\) mice were anesthetized (phenobarbital sodium, 50 mg/kg) and placed in a supine position. Abdominal aorta at the superior mesenteric artery level was dissected free of surrounding adventitial adipose tissues and muscles and the aorta was constricted by a 6-0 silk suture ligature between the celiac and superior mesenteric arteries to yield a 1/3 narrowing of luminal diameter. Sham operation included all procedures except the suture ligature. Operative incisions were sutured and mice were allowed to recover on warm pads. Four weeks following surgery, animals were used for studies.\(^2\)

**Human Samples** Left ventricular (LV) tissues were obtained from hearts explanted from patients diagnosed with idiopathic dilated cardiomyopathy (IDC; 59.2±1.8 years; n = 3 male, 3 female; mean ejection fraction, 19.29 ± 1.66%) or patients who had no history of cardiac, pulmonary or metabolic disease (NF; 34.7±4.8 years; 3 males and 3 females). Patient characteristics are presented in Table 1. Non-failing and failing hearts were obtained via an Institutional Review Board-approved protocol maintained by the University of Colorado Cardiac Tissue Bank. Hearts donated for research purposes were obtained with written consent from family members or by direct written consent from end-stage HF patients undergoing cardiac transplantation. Tissues were homogenized in lysis buffer containing a protease inhibitor cocktail and electrophoresed as described below on 15% gels loading 20µg protein/lane.

**Echocardiographic Evaluation** Cardiac geometry and contractile function were evaluated in anesthetized mice using a two-dimensional (2-D) guided M-mode echocardiography (Sonos 5500, Phillips Medical System, Andover, MA) equipped with a 15-6 MHz linear transducer as reported\(^3\). Anterior and posterior wall thickness, diastolic and systolic left ventricular dimension were recorded from M-mode images. Left ventricular fractional shortening (FS) was calculated from LV end diastolic diameter (LVEDD) and LV end systolic diameter (LVESD) using the following formula: (LVEDD-LVESD)/LVEDD × 100.\(^4\)

**Cardiomyocyte Isolation and Mechanics** Mouse cardiomyocytes were isolated using liberase enzymatic digestion and the mechanical properties were evaluated by using an IonOptix\(^{\text{TM}}\) soft-edge system (IonOptix Milton, MA)\(^5\). Cell shortening and relaxation were assessed using peak shortening (PS), maximal velocities of shortening/relengthening (± dL/dt), time-to-PS (TPS) and time-to-90% relengthening (TR\(_{90}\)).\(^4\)

**Intracellular \(Ca^{2+}\) Transients** A cohort of cardiomyocytes was incubated with fura2/AM (0.5 µM) for 15 min, and fluorescence intensity was recorded by a dual-
excitation fluorescence photomultiplier system (IonOptix). Cells were exposed to light emitted by a 75W lamp, while being stimulated to contract at a frequency of 0.5Hz. Fluorescence emissions were detected between 480 and 520 nm; qualitative change in fura-2 fluorescence intensity (FFI) was inferred from the FFI ratio at the two wavelengths (360/380). Fluorescence decay rate was calculated as an indicator of intracellular Ca\textsuperscript{2+} clearing.\textsuperscript{4}

**Blood Pressure Measurement** Systolic and diastolic blood pressure was measured by using a CODA semi-automated non-invasive blood pressure device (Kent Scientific Co, Torrington, CT).

**Histopathological Analysis** Ventricular tissues were stained with the FITC-conjugated wheat germ agglutinin and cardiomyocyte cross-sectional area was quantitated from 100 randomly selected cardiomyocytes. Myocardial fibrotic area was assessed using Masson’s trichrome staining.\textsuperscript{6}

**Quantitative real-time reverse-transcription PCR (qRT-PCR) for mRNA expression** Total RNA was isolated from left ventricles followed by DNase digestion to clear up genomic DNA contamination. cDNA was synthesized using a iScript\textsuperscript{TM} cDNA synthesis kit (Bio-rad, Hercules, CA) from 1\textmu g RNA. qRT-PCR analysis was performed for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), \alpha- and \beta-myosin heavy chain (NHC) as well as GAPDH (housekeeping gene). The primer sequences were as the following: ANP -FWD: 5’ - ACA GCC AAG GAG GAA AAG GC – 3’; ANP -REV: 5’ – CCA CAG TGG CAA TGT GAC CA – 3'. BNP –FWD: 5’ – TCC AGA GCA ATT CAA GAT GCA – 3'; BNP –REV: 5’ – CTT TTG TGA GGC CTT GGT CC – 3’. \alpha-MHC –FWD: 5’ – CCA CCT GGG CAA GTC TAA CAA – 3’; \alpha-MHC –REV: 5’ – TGT AGT CCA CGG TGC CAG C – 3’. \beta-MHC –FWD: 5’ – GAT GTT TTT GTG CCC GAT GA – 3'; \beta-MHC –REV: 5’ – ACC GTC TTG CCA TTC TCC G – 3'. GAPDH –FWD: 5’ – TGA AGC AGG CAT CTG AGG G – 3'; GAPDH –REV: 5’ – CGA AGG TGG AAG AGT GGG AG – 3’.\textsuperscript{4}

**Western Blot Analysis** Protein was extracted using a RIPA lysis buffer from mouse and human left ventricular tissues or myoblasts and immunoblotted against cathepsins (K, B, S, L), GATA binding protein-4 (GATA4), phospho-Akt, Akt, phospho-glycogen synthase kinase 3\textbeta{} (GSK3\textbeta{}), GSK3\textbeta{}, phospho-AMP-activated protein kinase (AMPK), AMPK, phospho-acetyl-CoA carboxylase (ACC), ACC, phospho-p38 mitogen-activated protein kinases (MAPK), p38 MAPK, phospho-extracellular signal-regulated kinases (ERK), phospho-mTOR, mTOR, phosphor-4EBP1, 4EBP1, phospho-p70S6K, p70S6K, phospho-Raptor, Raptor, Rictor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as loading control).\textsuperscript{4}

**In vitro Hypertrophy, Silencing and Overexpression of cathepsin K** Phenylephrine (PE, 100 \textmu M, 24h) was used to induce hypertrophy *in vitro*.\textsuperscript{7} In brief, H9c2 myoblasts were grown up to 80% confluence, and small interfering RNAs
(siRNAs) against cathepsin K or non-target (NT) siRNA were transfected using DharmaFECT® transfection reagent according to manufacturer’s instructions. Cathepsin K plasmid was transformed in competent E. coli cells by using heat shock method, followed by purified using a Qiagen plasmid mini kit (Qiagen, Germantown, MD). Cathepsin K plasmid was then transformed in H9c2 cells with the help of Lipofectamine® LTX & Plus reagent according to manufacturer’s instructions (Invitrogen, Grand Island, NY).

**Cell Surface Area Measurement** Immunostaining for α-actin was employed for cell surface area measurement. In brief, H9c2 cells were fixed in 4% paraformaldehyde for 15 min, followed by permeabilized with 0.2% Triton X-100 for 15 min. The cells were blocked in 5% BSA for 30 min prior to incubation with an antibody against muscle specific α-actin at 4°C overnight, followed by incubated in an anti-rabbit IgG-FITC antibody at 37°C for 60 min. The cells were counterstained by DAPI and viewed under an Olympus fluorescent microscope. Cell area was measured by the ImageJ® Software.

[^3H] Leucine incorporation Cells were incubated with [^3H] leucine (1.0µCi/ml) for 24h prior to harvest. Cells were then incubated with 10% trichloroacetic acid for 20 min at 4°C and lysed in 0.5 M NaOH overnight. The precipitate was measured using a scintillation counter.

**Online Supplement References:**


Figure S1. mRNA levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC) and α-myosin heavy chain (α-MHC) of sham or abdominal aortic constriction (AAC) hearts from wild-type (WT) and Cathepsin K knockout (Ctsk⁻/⁻) mice. *p < 0.05 vs. WT-sham group, &p < 0.05 vs. WT-AAC group. n= 6 per group.
Figure S2. Blood pressure in wild-type and cathepsin K knockout mice subjected to sham surgery or abdominal aortic constriction (AAC). A-C: diastolic blood pressure, systolic blood pressure and mean blood pressure, respectively. Mean ± SEM, n = 6-9 mice per group for blood pressure measurement.
Figure S3. Quantification graph of the protein expression of cathepsin K in control-cDNA and Cathepsin K-cDNA transfection group. *p < 0.05 vs. Control-cDNA group. The experiment was repeated by four times.