

Cathepsin K Knockout Mitigates High-Fat Diet–Induced Cardiac Hypertrophy and Contractile Dysfunction

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The cysteine protease cathepsin K has been implicated in pathogenesis of cardiovascular disease. We hypothesized that ablation of cathepsin K protects against obesity-associated cardiac dysfunction. Wild-type mice fed a high-fat diet exhibited elevated heart weight, enlarged cardiomyocytes, increased left ventricular wall thickness, and decreased fractional shortening. All these changes were reconciled in cathepsin K knockout mice. Cathepsin K knockout partly reversed the impaired cardiomyocyte contractility and dysregulated calcium handling associated with high-fat diet. Additionally, cathepsin K knockout alleviated whole-body glucose intolerance and improved insulin-stimulated Akt phosphorylation in high-fat diet–fed mice. High-fat feeding increased the expression of cardiac hypertrophic proteins and apoptotic markers, which were inhibited by cathepsin K knockout. Furthermore, high-fat feeding resulted in cathepsin K release from lysosomes into the cytoplasm. In H9c2 myoblasts, silencing of cathepsin K inhibited palmitic acid–induced release of cytochrome c from mitochondria and expression of proapoptotic signaling molecules. Collectively, our data indicate that cathepsin K contributes to the development of obesity-associated cardiac hypertrophy and may represent a potential target for the treatment to obesity-associated cardiac anomalies. *Diabetes* 62:498–509, 2013

Obesity is an emerging health problem worldwide and is an independent risk factor for the pathogenesis of cardiovascular disease (1–3). Uncorrected obesity predisposes individuals to myocardial damage characterized as cardiac hypertrophy and contractile dysfunction (4–6). Although several theories have been postulated to explain obesity-associated cardiac anomalies, including alterations in myocardial substrate utilization, neurohumoral activation, mitochondrial dysfunction, oxidative stress, impaired insulin signaling, and lipotoxicity, the underlying pathological mechanisms remain elusive (7). Obesity is known to trigger a number of adverse cellular signaling processes in the heart, such as re-expression of fetal genes, intracellular Ca²⁺ mishandling, derangement in proteins responsible for excitation-contraction coupling, and cardiac fatty acid oxidation (8,9). Obesity also triggers changes in extracellular matrix (10) and apoptosis of cardiomyocytes (11), which also can contribute to heart failure.

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Cathepsins are cysteine proteases that are ubiquitously expressed in various tissues and are implicated in the pathogenesis of cardiovascular diseases (12–14). Primarily located in lysosomes, cathepsins are transported between different cellular organelles and are released into the circulation under pathological conditions such as diabetes and atherosclerosis (15,16) or after lysosomal leakage caused by reactive oxygen species. Once released from lysosomes, cathepsins trigger apoptotic cell death by activating caspases and releasing proapoptotic factors from the mitochondria (17). Elevated levels of cathepsins S and K have been reported in atherosclerotic plaques, neointimal lesions, and in the failing heart in both humans and animals (18–21). Elevated levels of myocardial cathepsin B has been reported in individuals with dilated cardiomyopathy (22). However, deficiency of cathepsin L has been associated with cardiomyopathy whereas overexpression of this protease was shown to retard cardiac hypertrophy (23), suggesting distinct regulation of cardiac tissue by various cathepsins. Recent studies have shown that cathepsin K is elevated in adipose tissues of obese humans and mice and inhibition of cathepsin K attenuated body weight gain and serum glucose and insulin levels in obese mice (24). Given that cardiac dysfunction is a major complication of obesity, this study was designed to test the hypothesis that ablation of cathepsin K protects against high-fat diet–induced cardiac dysfunction. Our studies reveal that deletion of cathepsin K attenuates obesity-associated cardiac hypertrophy and contractile dysfunctions.

RESEARCH DESIGN AND METHODS

Experimental animals. The University of Wyoming Institutional Animal Care and Use Committee approved this study. Six-week-old male cathepsin K knockout mice (*Ctsk*^{−/−}) (25) and wild-type littermates were subjected to a normal diet (10% calories from fat, 3.91 kcal/g) or high-fat diet (45% calories from fat, 4.83 kcal/g; Research Diets, New Brunswick, NJ) for 20 weeks. Mice were housed in a climate-controlled environment (22.8 ± 2.0°C, 45–50% humidity) with a 12/12 light/dark cycle with free access to designated diet and water ad libitum. Body weight and food intake were monitored weekly.

Intraperitoneal glucose tolerance test. After 5 months of high-fat diet, mice were fasted for 12 h and were challenged with glucose (2 g/kg, intraperitoneally). Blood samples were drawn from the tail vein immediately before glucose challenge, as well as 30, 60, 90, and 120 min thereafter. Serum glucose levels were determined using a glucometer.

Insulin, triglyceride, cholesterol, and free fatty acid detection. Serum insulin levels were detected by enzyme-linked immunosorbent assay (Millipore). Serum triglyceride, cholesterol, and free fatty acid and cardiac and liver triglycerides were detected by kits from BioVision (Milpitas, CA).

Echocardiographic analysis. Cardiac geometry and function were evaluated in anesthetized mice using a two-dimensional guided M-mode echocardiography (Sonos 5500; Phillips Medical System, Andover, MA) equipped with a 15–6 MHz linear transducer as reported previously (26). Anterior and posterior wall thicknesses and diastolic and systolic left ventricular dimensions were recorded from M-mode images. Left ventricular fractional shortening was calculated from left ventricular end diastolic diameter (LVEDD) and left ventricular end systolic diameter (LVESD) using the following formula: (LVEDD – LVESD) / LVEDD × 100.

Blood pressure measurement. Mouse systolic and diastolic blood pressures were measured by a CODA semiautomated noninvasive blood pressure device (Kent Scientific, Torrington, CT).

Cardiomyocyte isolation and mechanics. Mouse cardiomyocytes were isolated using liberase enzymatic digestion; mechanical properties were assessed using an IonOptix soft-edge system (IonOptix, Milton, MA) as described previously (27). For experiments involving the inhibition of cathepsin K, isolated cardiomyocyte from high-fat diet-fed wild-type mice were incubated for 2 h with various concentration of cathepsin K inhibitor II (Calbiochem). Cell shortening and relengthening were assessed using peak shortening (PS), time to PS, time to 90% relengthening, and maximal velocities of shortening/relengthening (\pm dL/dt).

Intracellular Ca^{2+} transients. A cohort of myocytes was loaded with fura-2/AM (0.5 μ mol/L) for 15 min, and fluorescence intensity was recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). Cells were exposed to light emitted by a 75-W lamp while being stimulated to contract at a frequency of 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm; qualitative change in fura-2 fluorescence intensity was inferred from the fura-2 fluorescence intensity ratio at the two wavelengths (360/380). Fluorescence decay rate was calculated as an indicator of intracellular Ca^{2+} clearing (27).

Histopathological analysis and TUNEL staining. Ventricular tissues were stained with FITC-conjugated wheat germ agglutinin and cardiomyocyte cross-sectional area was quantitated by measuring 60 random cardiomyocytes. Fibrotic area was measured on Masson trichrome-stained sections. Apoptotic cardiomyocytes were detected by using the In Situ Death Detection Kit (Roche, Branchburg, NJ), with myocytes counterstained by Desmin antibody (Cell Signaling Technology, Beverly, MA) and observed using a Zeiss confocal microscope. Adipose tissues were stained with hematoxylin and eosin; adipocyte sizes were quantitated by measuring 60 random adipocytes.

Assessment of mRNA expression by quantitative real-time PCR. Total RNA was isolated from left ventricles followed by DNase digestion to eliminate genomic DNA contamination. Synthesis of cDNA was performed at 37°C for 60 min using 1 μ g total RNA in a 20- μ L system by Superscript III. Quantitative real-time RT-PCR analysis was performed for cathepsins B, D, H, K, and L, cystatin-C, fatty acid transport protein-1, muscle carnitine palmitoyl transferase-1, long-chain acyl CoA dehydrogenase, CD36, succinyl-CoA:3-ketoacid CoA transferase, acetyl-coA carboxylase- α , peroxisome proliferator-activated receptor- α , and 18S (housekeeping gene). The primer sequences are shown in Supplementary Table 1.

Western blot analysis. Protein was extracted using a RIPA lysis buffer and Western blotted against antibodies for GATA binding protein-4 (GATA4), NFATc3, atrial natriuretic peptide (ANP), cathepsins B, K, L, and S, phospholamban, phospho-phospholamban, sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase-2 (SERCA2), phospho-Akt, Akt, insulin receptor- β , cytochrome C, BAX, cleaved caspase-3, BCL-XL, cleaved nuclear enzyme poly (ADP-ribose) polymerase (PARP), catD, glucose transporter-4, COXIV (used as the loading control for mitochondrial proteins), and GAPDH (loading control) (27).

RNA silencing. H9c2 myoblasts were grown up to 80% confluence, and small interfering RNAs (siRNAs) of cathepsin K (25 nmol/L) or control nontarget siRNA were transfected using DharmaFECT transfection reagent per manufacturer's instructions.

Cell viability. H9c2 myoblast viability was assessed after treatment with palmitic acid (0.4 mmol/L, 6 h) by measuring formazan. Palmitate-containing media was prepared by conjugating palmitic acid with fatty acid-free bovine serum albumin (28).

Lysosome and cathepsin K staining. Lysosomes were stained with Cell Navigator lysosomal staining kit (ATT Bioquest, Sunnyvale, CA). Intracellular cathepsin K was detected by Magic Red-cathepsin K reagent staining (ImmunoChemistry Technologies, Bloomington, MN).

Statistical analysis. Data are presented as mean \pm SEM. Statistical comparison was performed by the two-way ANOVA followed by a Bonferroni post hoc test using the SigmaPlot software (Jandel Scientific, San Rafael, CA) for comparison of multiple groups. The Student *t* test was used to compare two groups. The null hypothesis was rejected with $P < 0.05$.

RESULTS

Increased expression of cathepsin K in the heart after high-fat feeding. As illustrated in Supplementary Fig. 1A and B, cardiac protein levels of cathepsins K and B were significantly elevated in mice fed the high-fat diet compared with the normal diet (Supplementary Fig. 1A and B), whereas other cathepsins remained unaltered (cathepsins S and L are shown). In contrast, the high-fat

diet did not alter the message levels of any cathepsins or cystatin C (the endogenous inhibitor of cathepsin) (Supplementary Fig. 1C). To determine the cellular source of cathepsin K, we performed immunohistochemical analysis on isolated cardiomyocytes. Supplementary Fig. 1D and E demonstrate that high-fat diet feeding resulted in increased cathepsin K-positive staining (puncta) in the cardiomyocytes. The extralysosomal cathepsin K-positive puncta indicate that high-fat feeding results in cathepsin K release from lysosomes to the cytoplasm.

Cathepsin K knockout does not affect high-fat diet-induced changes in body lipid content. High-fat diet feeding resulted in a significant elevation in serum triglycerides, cholesterol, and free fatty acids, as well as cardiac and liver triglyceride levels (Table 1). None of these parameters were altered by cathepsin K knockout.

Cathepsin K knockout does not alter blood pressure. The systolic and mean blood pressures were unchanged after high-fat diet feeding in the wild-type and cathepsin K knockout mice, nor did the high-fat diet per se increase diastolic blood pressure. A slightly lower diastolic blood pressure was recorded in cathepsin K knockout mice fed the normal diet. Furthermore, cathepsin K knockout mice on a high-fat diet exhibited a comparable diastolic blood pressure with that of the wild-type mice fed a high-fat diet (Supplementary Fig. 2).

Cathepsin K knockout alleviates high-fat diet-induced glucose intolerance. In wild-type mice fed the normal diet, after acute glucose challenge serum glucose peaked at 30 min and approached baseline after 120 min (Fig. 1A and B). In contrast, postchallenge glucose levels remained at much higher levels between 30 and 120 min in high-fat diet-fed wild-type mice, indicating glucose intolerance. In high-fat diet-fed cathepsin K knockout mice, postchallenge blood glucose peaked at 30 min (similar to high-fat diet-fed wild types), but glucose levels were significantly lower at 60, 90, and 120 min. However, the total area under the glucose disposal curve did not show a significant effect of cathepsin K knockout in the integrated postglucose challenge glucose disposal (Fig. 1B). In addition serum glucose and insulin levels that were significantly elevated in the high-fat diet-fed mice were attenuated in the cathepsin K knockout mice (Table 1).

Cathepsin K knockout attenuates weight gain and cardiac hypertrophy in response to high-fat diet feeding. Whereas food intake did not differ by cathepsin knockout or diet, wild-type mice fed the high-fat diet gained significantly more weight than those fed the normal diet (Table 1 and Fig. 1C). High-fat feeding also resulted in a significant increase in organ weight (heart and liver, but not kidneys) compared with normal diet-fed mice (Table 1, Fig. 1B). Cathepsin K knockout and wild-type mice fed a normal diet did not differ in body or organ weight gain. Although cathepsin K knockout mice also gained weight after high-fat diet feeding, the weight gain was significantly less than that of wild-type mice. However, the high-fat diet-induced accumulation of epididymal fat and adipocyte hypertrophy was unaltered in the cathepsin K knockout mice (Table 1, Supplementary Fig. 3, respectively).

In high-fat diet-fed cathepsin K knockout mice, heart weight and heart weight to tibia length were significantly reduced compared with high-fat diet-fed wild-type mice and did not differ significantly from the mice fed a normal diet (Table 1). Histological analysis revealed that the heart size and cardiomyocyte cross-sectional area were significantly increased in high-fat diet-fed wild-type mice,

TABLE 1
General features of wild-type and cathepsin K knockout mice after 20 weeks of a normal diet or a high-fat diet

Parameter	WT-ND	WT-HFD	<i>Ctsk</i> ^{-/-} -ND	<i>Ctsk</i> ^{-/-} -HFD
Food intake, g/mouse/day	3.60 ± 0.03	3.60 ± 0.03	3.57 ± 0.01	3.55 ± 0.01
Body weight, g	30.20 ± 0.29	53.32 ± 0.77*	30.98 ± 0.87	44.05 ± 1.81†‡
Epididymal fat pad, g	0.378 ± 0.06	2.069 ± 0.99*	0.581 ± 0.95	2.497 ± 0.16†
Heart weight, mg	167 ± 7	214 ± 10*	165 ± 10	154 ± 4‡
Liver weight, g	1.57 ± 0.1	2.79 ± 0.2*	1.56 ± 0.1	1.67 ± 0.2‡
Kidney weight, g	0.41 ± 0.02	0.47 ± 0.02	0.42 ± 0.01	0.43 ± 0.03
Tibia length, mm	20.98 ± 0.68	20.67 ± 0.96	22.7 ± 0.2	22.7 ± 0.18
Heart weight/tibia length, mg/mm	7.99 ± 0.41	10.53 ± 0.75*	7.27 ± 0.49	6.77 ± 0.17‡
Heart rate, bpm	496 ± 31	458 ± 21	472 ± 34	414 ± 48
Plasma insulin, ng/mL	0.20 ± 0.08	2.64 ± 0.19*	0.31 ± 0.07	0.67 ± 0.04†‡
Plasma glucose, ng/mL	71.57 ± 2.7	118.75 ± 12.4*	74.20 ± 4.3	89.40 ± 10.76
Serum triglyceride, nmol	0.94 ± 0.05	1.36 ± 0.03*	0.75 ± 0.09	1.22 ± 0.04†
Serum cholesterol, μg	0.49 ± 0.13	2.03 ± 0.26*	0.38 ± 0.05	1.88 ± 0.08†
Serum free fatty acid, nmol	0.27 ± 0.05	0.61 ± 0.07*	0.29 ± 0.05	0.46 ± 0.04†
Cardiac triglyceride, nmol	0.40 ± 0.09	1.89 ± 0.19*	0.40 ± 0.05	2.12 ± 0.40†
Liver triglyceride, nmol	0.48 ± 0.09	6.23 ± 0.55*	0.56 ± 0.11	6.55 ± 0.91†

Values are mean ± SEM, *n* = 6–7 mice per group. WT, wild-type; ND, normal diet; HFD, high-fat diet. **P* < 0.05 vs. WT-ND group. †*P* < 0.05 vs. *Ctsk*^{-/-}-ND. ‡*P* < 0.05 vs. WT-HFD.

changes attenuated in cathepsin K knockouts (Fig. 1D–F). Similarly, markers of hypertrophy (NFATc3, GATA4, and ANP) were significantly upregulated after high-fat diet, changes that are attenuated in cathepsin K knockouts (Fig. 1G and Supplementary Fig. 4). Additionally, cardiomyocytes isolated from high-fat diet–fed cathepsin K knockout mice were significantly smaller compared with those obtained from the high-fat diet–fed wild-type mice (Fig. 1H), supporting the notion that the observed increase in heart mass is attributable to myocyte hypertrophy, not increased interstitial content. The high-fat diet increased the extent of interstitial fibrosis, but cathepsin K knockout did not lower the extent of fibrosis (Supplementary Fig. 5). **Cathepsin K knockout ameliorates obesity-induced compromised cardiac performance.** Baseline heart rates did not differ between genotypes or diet groups (Table 1). High-fat feeding led to compromised echocardiographic function, as evidenced by a significant increase in left ventricular wall thickness without affecting the diameters (LVEDD and LVEDS), indicating a concentric pattern of cardiac hypertrophy (Fig. 2A–C). In addition, high-fat diet feeding elicited a significant decrease in fractional shortening. Cathepsin K knockout ameliorated the high-fat diet–induced wall thickening and impaired fraction shortening but, by itself, did not affect myocardial geometry or fraction shortening (Fig. 2D).

Cathepsin K knockout reconciles high-fat diet–induced impaired cardiac contractility and intracellular Ca²⁺ handling. To further evaluate the effects of cathepsin K knockout on cardiac function, we assessed contractile function and intracellular Ca²⁺ handling in isolated cardiomyocytes (see Fig. 4). Neither high-fat diet nor cathepsin K ablation affected resting cell length (Fig. 3C). In wild-type mice, high-fat intake significantly reduced PS, reduced maximum velocity of shortening/relengthening (±dL/dt), and prolonged time to 90% relengthening without affecting time to PS (Fig. 3D–H). Strikingly, cathepsin K knockout rescued these high-fat diet–induced cardiomyocyte mechanical anomalies in PS, ±dL/dt, and time to 90% relengthening.

Intracellular calcium changes associated with the high-fat diet were nullified in cathepsin K knockouts. Cardiomyocytes from high-fat diet–fed wild-type mice displayed significantly elevated baseline intracellular Ca²⁺ and peak intracellular

Ca²⁺ levels, reduced intracellular Ca²⁺ increase in response to electrical stimulus (Δfura-2 fluorescence intensity), and delayed intracellular Ca²⁺ clearance (Fig. 4A–D). Interestingly, however, cathepsin K knockout mice that received a normal diet also exhibited elevated baseline and peak intracellular Ca²⁺. Figure 4E to 4H illustrates that cardiac expression of the Ca²⁺-handling proteins SERCA2a and phospholamban are both enhanced in the high-fat diet–fed mice, although the levels of phosphorylated phospholamban and the ratio of SERCA2a to phospholamban remained unaltered. In contrast, SERCA2a, phospholamban, and phospholamban-to-SERCA2a ratio were all elevated in cathepsin K knockout mice under basal conditions, suggesting a phenotypic difference in terms of Ca²⁺-handling proteins. Consistent with our results from the cathepsin K knockout mice, pharmacological inhibition of cathepsin K partially reconciled the impaired contractile parameters in cardiomyocytes from high-fat diet–fed mice (Fig. 5).

Cathepsin K knockout does not alter genes involved in cardiac fatty acid metabolism. Because the cardiac substrate utilization is known to switch from predominantly oxidation of fatty acids to carbohydrate metabolism in response to nutritional stress, we evaluated the effect of cathepsin K knockout on the expression of genes involved in fatty metabolism. Wild-type mice subjected to high-fat diet exhibited decreased expression of genes associated with cardiac fatty acid utilization and transport (fatty acid transport protein1, acetyl-coA carboxylase-α, and peroxisome proliferator-activated receptor-α) compared with those that received a normal diet; this was not observed in the cathepsin K knockout mice. No changes were observed in the message levels of fatty acid transport protein-4, muscle carnitine palmitoyl transferase-1, long-chain acyl CoA dehydrogenase, CD36, and succinyl-CoA:3-ketoacid CoA transferase levels among any of the treatment groups (Supplementary Fig. 6).

Cathepsin K deficiency inhibits cardiomyocyte apoptosis. Consistent with previous reports (11,29), chronic high-fat feeding caused a significant elevation in cardiomyocyte apoptosis as evidenced by increased TUNEL-positive nuclei (Fig. 6A and B) and increased expression of proapoptotic proteins cytochrome C, BAX, cleaved caspase-3,

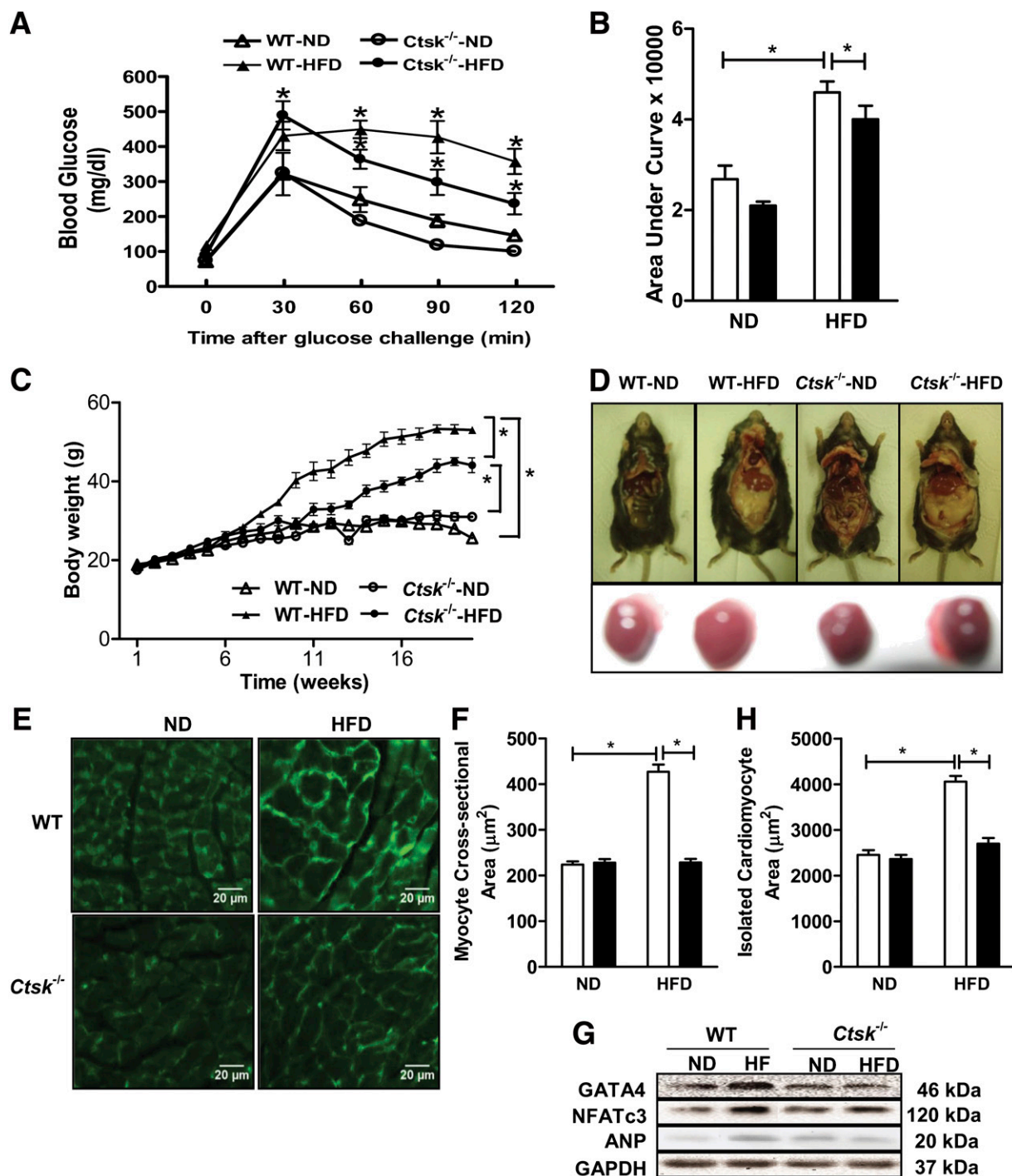


FIG. 1. *A* and *B*: Effect of cathepsin K knockout on glucose disposal in mice fed a normal diet (ND) or a high-fat diet (HFD) after an intraperitoneal glucose (2 g/kg, body weight) challenge (*A*). Integrated area under the postglucose challenge, glucose-disposal curve (*B*). *C*: Effect of ND and HFD, respectively, on body weight gain over a 20-week period in wild-type and cathepsin K knockout mice. *D*: Representative images of wild-type (WT) and cathepsin K knockout mice fed ND or HFD and representative hearts from each group. *E–H*: Effect of cathepsin K knockout on HFD-induced cardiac hypertrophy. Representative images using wheat germ agglutinin staining of the left ventricular tissue (*E*), quantitation of cardiomyocyte cross-sectional area (*F*), and representative gel blots for GATA4, NFATc3, ANP, and GAPDH (loading control) (*G*), and size of isolated cardiomyocytes (*H*). Data are represented as mean \pm SEM, $n = 5–7$ mice per group, * $P < 0.05$ between the notated groups. (A high-quality color representation of this figure is available in the online issue.)

and cleaved PARP, and also a reciprocal decrease in the antiapoptotic BCL-XL (Fig. 6C, quantification of the blots provided in Supplementary Fig. 7), which was reconciled by cathepsin K knockout. Additionally, rat cardiac H9c2 cells transfected with cathepsin K siRNA exhibited reduced levels of apoptotic protein expression when challenged with

the saturated fatty acid, palmitic acid (Fig. 6D and Supplementary Fig. 8A). Apoptosis induced by palmitic acid was characterized by a significant increase in cytosolic cytochrome C with a reciprocal reduction of the mitochondrial cytochrome C (Fig. 6D and Supplementary Fig. 8B–D). Cathepsin K silencing attenuated palmitic acid-induced

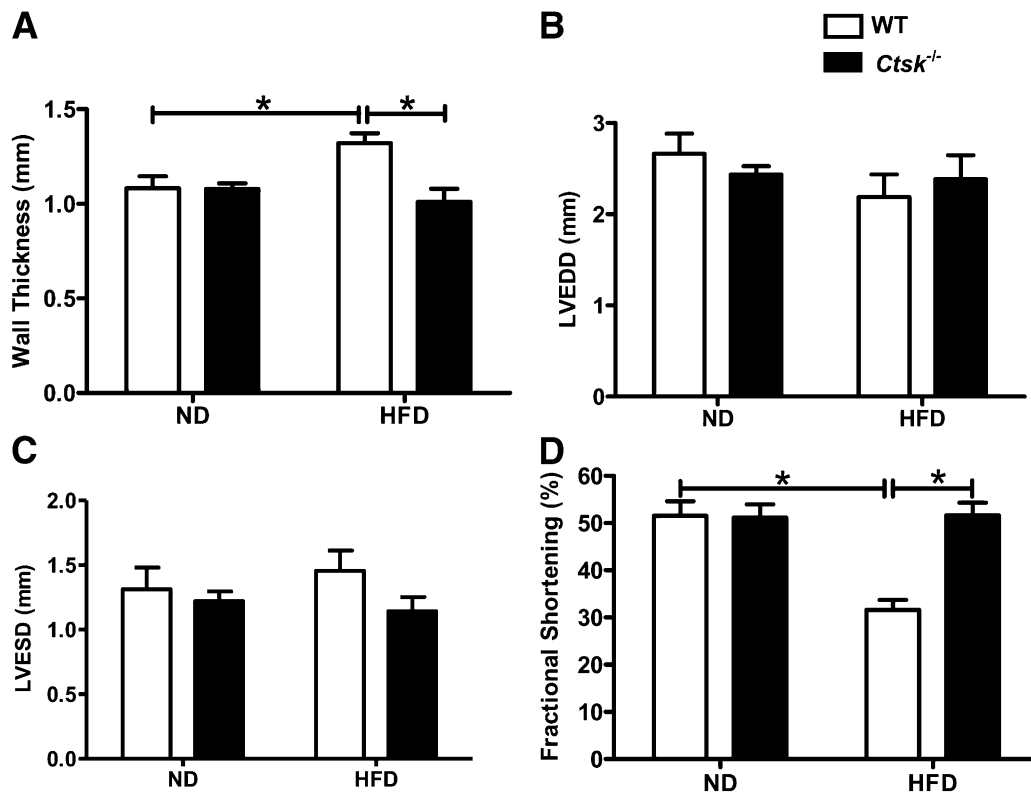


FIG. 2. Echocardiographic features in wild-type (WT) and cathepsin K knockout mice fed normal diet or high-fat diet. *A*: Left ventricular wall thickness. *B*: LVEDD. *C*: LVESD. *D*: Fraction shortening [(LVEDD – LVESD)/LVEDD × 100]. Mean ± SEM, *n* = 6–7 mice per group, **P* < 0.05 between the notated groups.

cell death in H9c2 cells (Supplementary Fig. 8E). Costaining of H9c2 cells for cathepsin K and lysosomes demonstrated that cathepsin K is predominantly localized in the lysosomes under basal condition (Fig. 7A). On stimulation with palmitic acid, cathepsin K staining was observed in the cytoplasm as well, suggesting that palmitic acid challenge causes the release of cathepsin K from the lysosomes into the cytoplasm (Fig. 7A).

Cathepsin K knockout alleviates cardiac insulin resistance. Because whole-body glucose disposal was facilitated in cathepsin K knockout, we investigated the effect of high-fat diet feeding and cathepsin K knockout on key proteins in insulin signaling pathways. We transiently challenged the mice with insulin before removing the heart. Hearts from high-fat diet-fed wild-type mice demonstrated a significant blunting of insulin-stimulated Akt-phosphorylation; this was reversed in cathepsin K knockout mice (Fig. 7). In contrast, basal levels (without insulin injection) of phospho-Akt and insulin receptor-β were elevated in hearts from high-fat diet-fed wild-type mice hearts compared with those fed the normal diet; this was attenuated by cathepsin K knockout (Fig. 7B,D,E).

High-fat diet feeding results in compensatory upregulation of cathepsin L in cathepsin K knockout mice. We determined if ablation of cathepsin K causes a compensatory upregulation of other cathepsins in hearts from normal diet-fed and high-fat diet-fed mice. Although cathepsin K knockout per se did not result in the upregulation of other cathepsins, a significant upregulation of cathepsin L was seen in the hearts of cathepsin K knockout mice subjected to high-fat diet feeding (Fig. 7F and G).

DISCUSSION

Several studies have consolidated the role of obesity as an independent risk for cardiac hypertrophy and contractile dysfunction. In this study, we examined the role of cathepsin K in obesity-induced cardiac morphometric and functional changes. Our data indicated that cathepsin K was upregulated in the myocardium in response to high-fat diet feeding and cathepsin K deletion effectively attenuated or mitigated high-fat diet-induced cardiac hypertrophy, contractile dysfunctions, and intracellular Ca²⁺ derangements. High-fat diet-induced cardiac geometric and functional anomalies were associated with the increased cardiac expression of prohypertrophic proteins GATA4, NFATc, and ANP, as well as elevated levels of apoptosis, all of which were negated by cathepsin K deletion. Furthermore, ablation of cathepsin K resulted in reduced high-fat diet-induced body weight gain and improved whole-body glucose disposal subsequent to a glucose challenge. Additionally, the cathepsin K knockout attenuated the elevated serum insulin and glucose levels and blunted cardiac insulin signaling associated with high fat. In cultured cardiac myoblasts palmitic acid (a saturated fatty acid and an essential component of the high-fat diet) upregulated cathepsin K, stimulated cathepsin K release from lysosomes into the cytoplasm, and induced apoptosis. These untoward effects of palmitic acid were inhibited by cathepsin K silencing. Collectively, these data show that cathepsin K ablation mitigates obesity-associated cardiac dysfunctions, and these effects may be mediated by the attenuation of high-fat diet-induced weight gain, alleviation of insulin resistance, and inhibition of apoptosis.

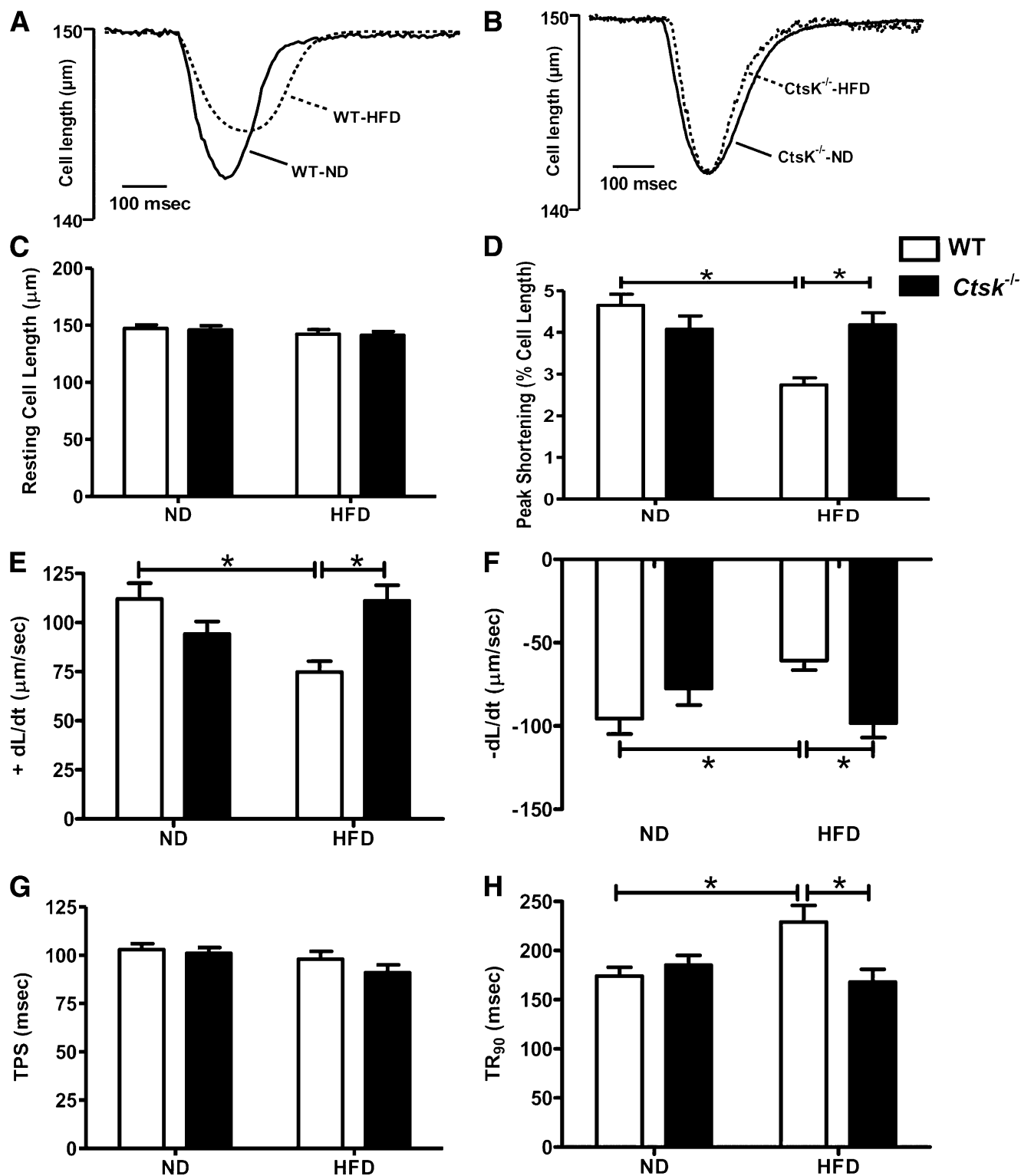


FIG. 3. Cardiomyocyte contractile properties in wild-type (WT) and cathepsin K knockout mice fed normal diet (ND) or high-fat diet (HFD). *A* and *B*: Representative traces from cardiomyocytes isolated from wild-type and cathepsin K knockout mice. *C–H*: Resting cell length, peak shortening (normalized to cell length), maximal velocity of shortening (+dL/dt), maximal velocity of relengthening (–dL/dt), time-to-peak shortening (TPS), and time to 90% relengthening (TR₉₀), respectively. Mean ± SEM, *n* = 76–87 cells per group. **P* < 0.05 between the notated groups.

Several studies have demonstrated altered expression levels of cathepsins in the failing heart, which has been recently reviewed by Cheng et al. (14). Whereas elevated levels of myocardial cathepsins K and S have been reported in cardiac hypertrophy and heart failure in both rats

and humans (21), overexpressing cathepsin L has been shown to improve cardiac function via retarding cardiac hypertrophy (23). In our studies, protein levels of cathepsins K and B were upregulated in the hearts after high-fat diet, without significant alterations in other cathepsins.

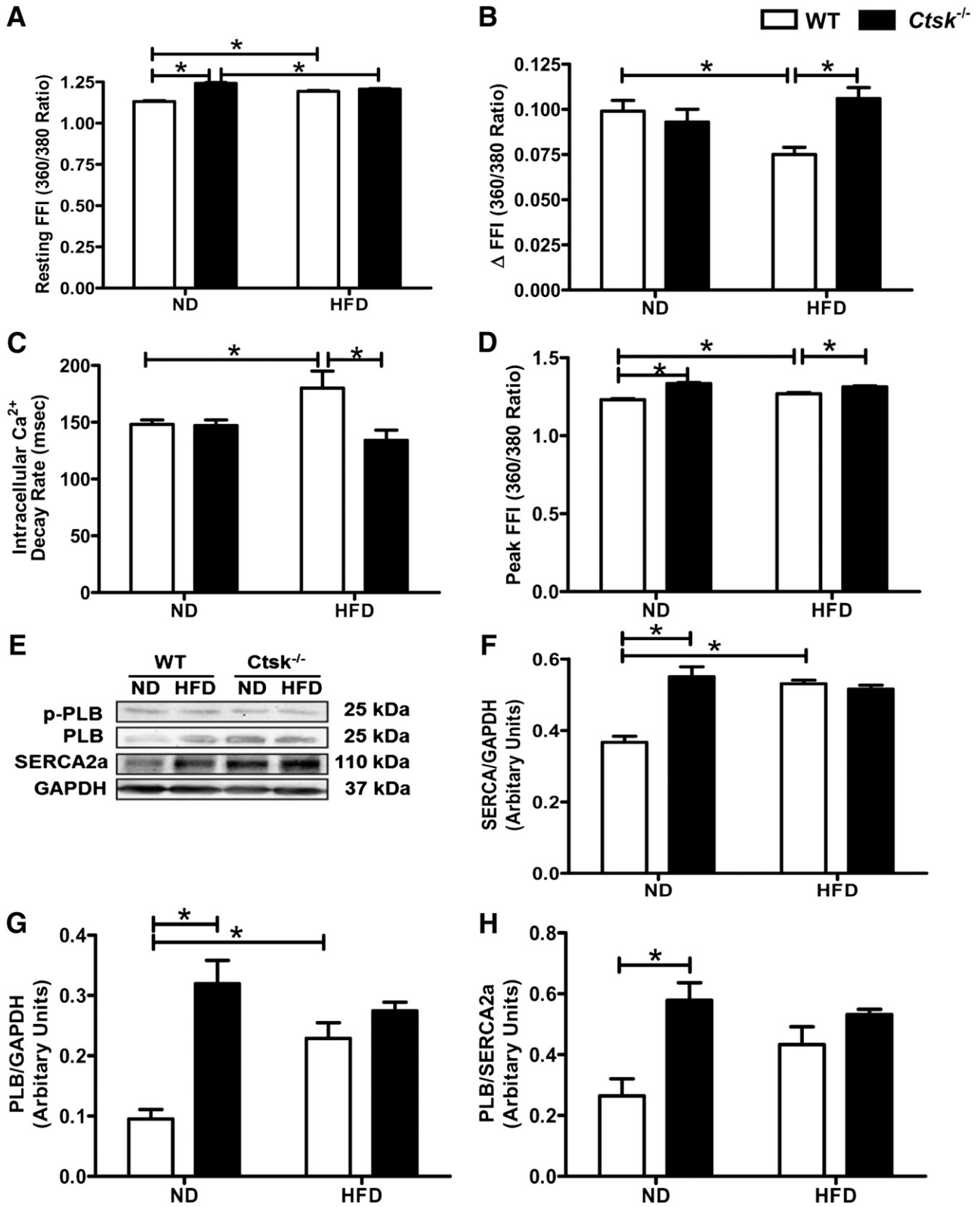


FIG. 4. Intracellular Ca²⁺ transients in cardiomyocytes and expression levels of proteins related to intracellular Ca²⁺ handling in wild-type (WT) and cathepsin K knockout mice fed a normal diet (ND) or high-fat diet (HFD). *A–D*: Resting fura-2 fluorescence intensity (FFI), electrically stimulated increase in FFI (Δ FFI), single exponential intracellular Ca²⁺ decay, and peak FFI, respectively. *E*: Representative Western blot images for phospho-phospholamban, phospholamban (PLB), SERCA2, and GAPDH (loading control). *F–H*: Densitometric quantitation of SERCA normalized to GAPDH, PLB normalized to GAPDH, and PLB-to-SERCA ratio, respectively. Mean \pm SEM, $n = 94$ –100 cells or 5–7 mice per group. * $P < 0.05$ between the notated groups.

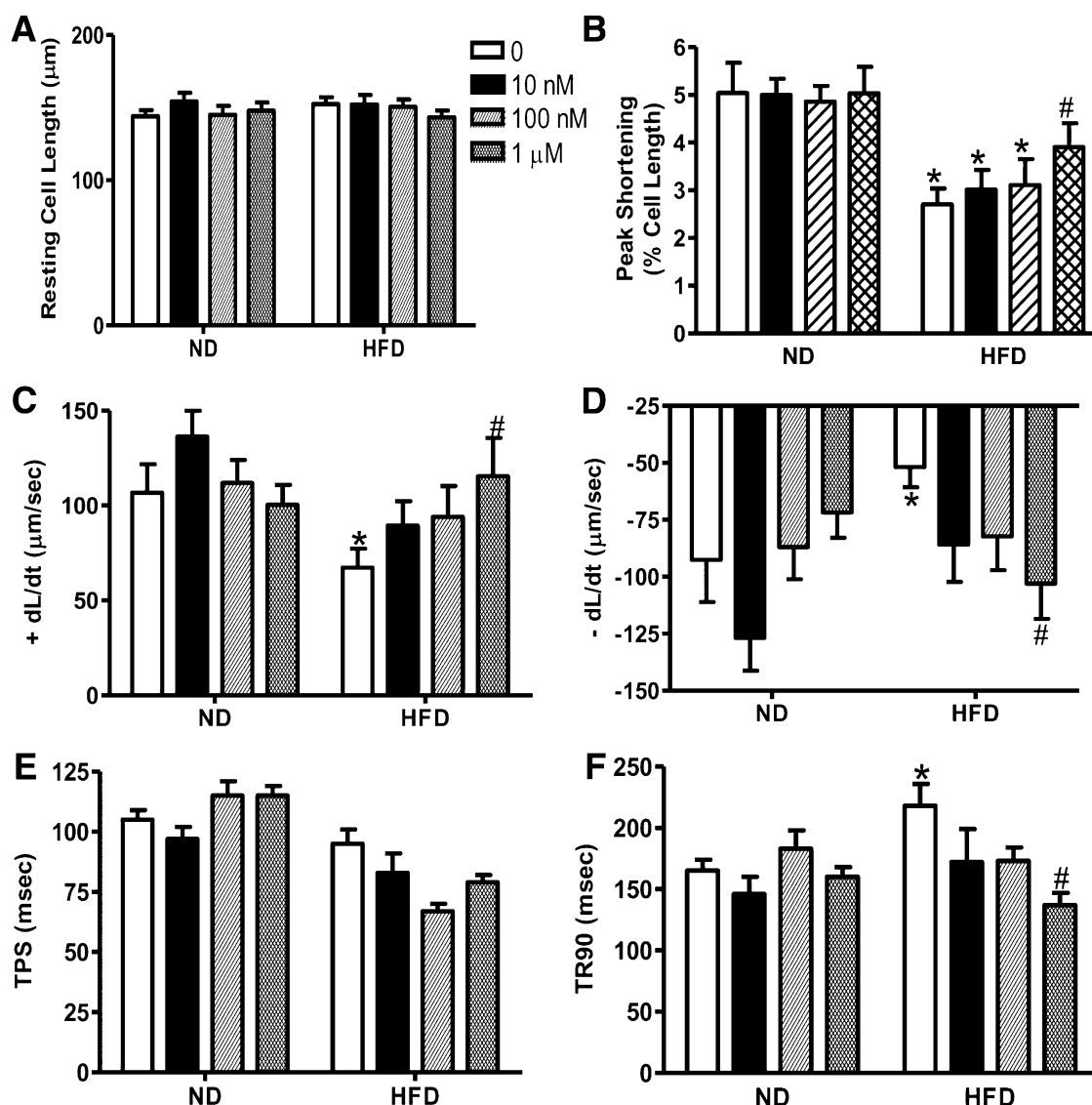


FIG. 5. Effect of cathepsin K inhibitor II on resting cell length (A), PS (B), and time to 90% relengthening (C) of cardiomyocytes isolated from normal diet (ND)-fed and high-fat diet (HFD)-fed wild-type mice. Data are means \pm SEM, $n = 50$ cardiomyocytes per group. * $P < 0.05$ vs. ND control group, # $P < 0.05$ vs. HFD control group.

The fact that we did not observe changes in the mRNA levels of these proteins is consistent with the observations made by previous studies that cathepsin K expression is regulated at the translation level (18). Although knock-down of the cathepsin K gene did not alter the expression of other cathepsins at basal levels, high-fat diet feeding resulted in an induction of cathepsin L in the cathepsin K knockout mice, suggesting a compensatory effect. This is significant because cathepsin L has been shown to counteract cardiac dysfunctions (30,31), thus suggesting a reciprocal regulation of cardiac structure and function by these cathepsins.

Yang et al. (24) found a reduction in weight gain and body fat accumulation in female cathepsin K knockout mice subjected to high-fat diet. Consistent with these findings, cathepsin K ablation attenuated body weight gain in our studies. However, we did not see any changes in fat accumulation consequent to cathepsin K knockout. This discrepancy may be attributed to gender differences because we used only male mice in our study. Neither

message levels of the transcription factors involved in fatty acid transport and utilization nor serum lipid levels provided an explanation of the benefits of cathepsin K knockout, because these parameters, although altered by high-fat diet, were not affected by knockout of cathepsin K. Lower levels of peroxisome proliferator-activated receptor- α and acetyl-coA carboxylase- α in the knockout mice compared with wild-type mice under high-fat diet conditions indicate improved fatty acid utilization in cathepsin K knockout mice, which, however, fails to explain its lack of effect on fat accumulation.

Numerous studies have shown that high-fat diets worsen cardiac remodeling and cause contractile dysfunction (32). In the current study, we found the high-fat diet was associated with impaired cardiomyocyte contractility and intracellular Ca^{2+} handling, which were reconciled by cathepsin K knockout. Additionally, pharmacological inhibition of cathepsin K reversed palmitic acid-induced impaired cardiomyocyte contractility, suggesting a direct role of cathepsin K in regulating cardiac contractility.

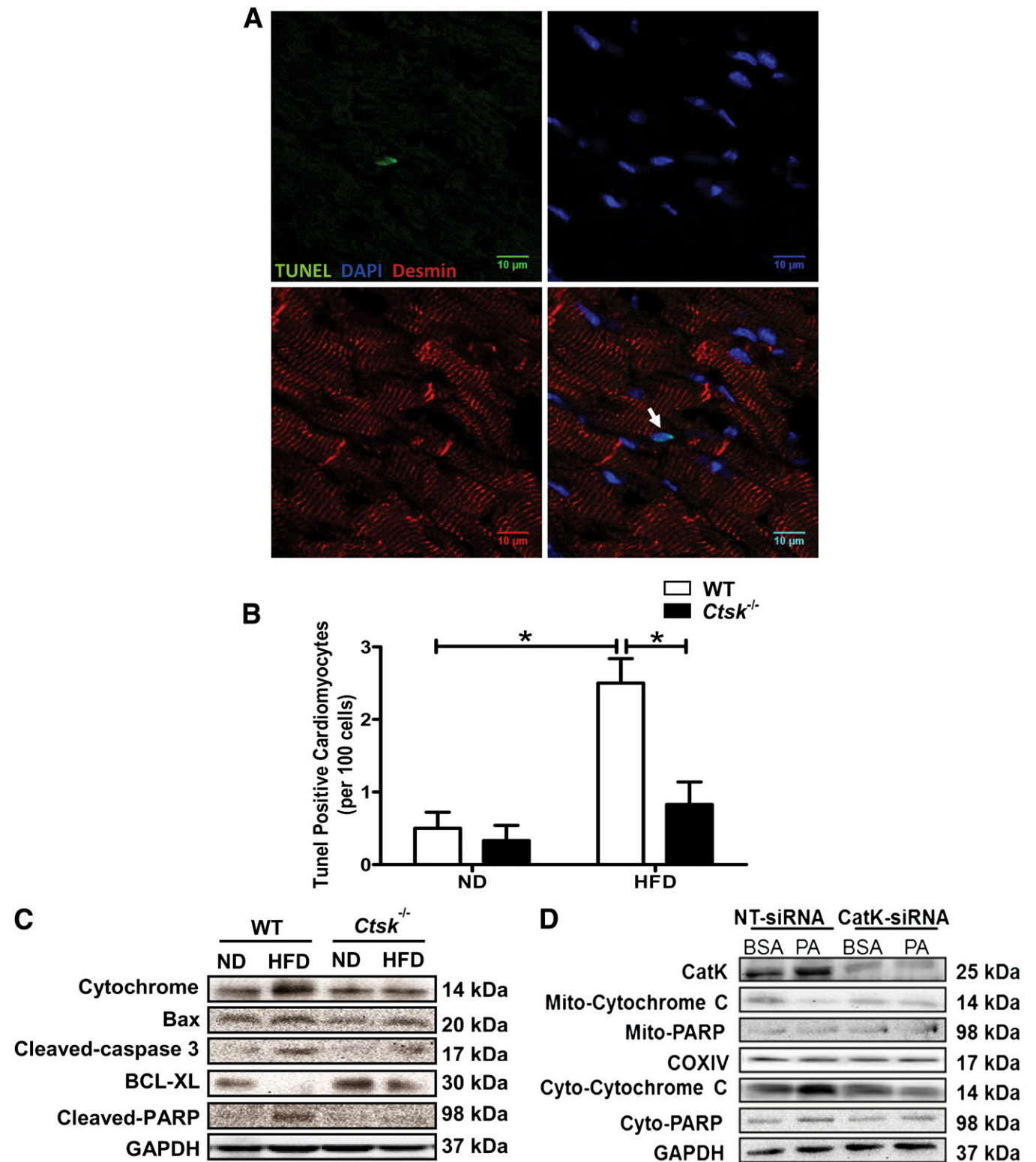


FIG. 6. A and B: Representative TUNEL staining of cardiomyocytes from heart sections of wild-type (WT) mice fed a high-fat diet (HFD). Apoptotic nuclei stained by TUNEL (green), counterstained with DAPI (blue) to mark nuclei, and cardiomyocytes (desmin, red) were imaged by confocal microscopy, arrow indicates TUNEL-positive nuclei in the cardiomyocyte (A); and quantitation of TUNEL-positive cardiomyocytes (B). ND, normal diet. **P* < 0.05 between the notated groups. **C:** Effect of cathepsin K knockout on HFD-induced cardiac apoptosis. Representative Western blot image of proapoptotic proteins cytochrome C, BAX, cleaved caspase-3, cleaved nuclear enzyme poly (ADP-ribose) polymerase (PARP), and the antiapoptotic protein BCL-XL are shown. **D:** H9c2 myoblasts were transfected with nontarget siRNA (NT-siRNA) or cathepsin K siRNA in the presence of palmitic acid (0.4 mmol/L, 6 h) or vehicle BSA. Representative gel blots of total cathepsin K, mitochondrial cytochrome C, mitochondrial PARP, COXIV (loading control), and cytosolic cytochrome C, and cytosolic PARP, and GAPDH (loading control). (A high-quality digital representation of this figure is available in the online issue.)

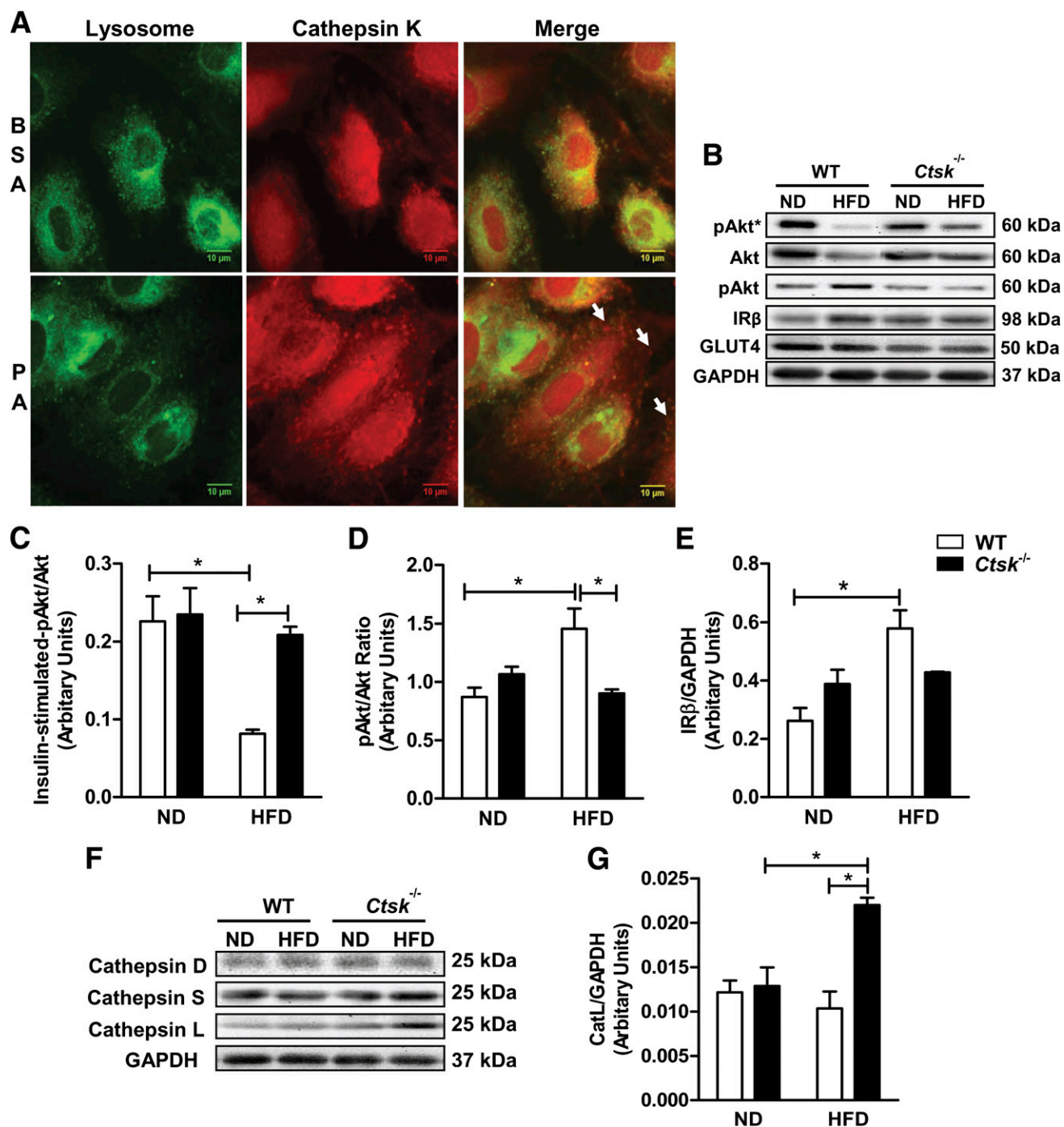


FIG. 7. *A*: Immunolocalization of cathepsin K in cultured cells. H9c2 myoblasts were labeled using lysosomal dye and fluorogenic substrate MR-(LR)₂, which detects active cathepsin K. Upper panel (merged figure) shows colocalization of cathepsin K in lysosomes under basal conditions. On stimulation with palmitic acid, active cathepsin K was released from lysosomes to the cytoplasm (indicated by arrows). *B–E*: Effect of cathepsin K knockout on cardiac insulin signaling molecules in normal diet (ND)-fed and high-fat diet (HFD)-fed mice. Mice were challenged with insulin (1.5 U/100 g body weight, intraperitoneally) for 10 min before isolation of the heart tissue for Western blot. Insulin-stimulated phosphorylation of Akt (indicated by an asterisk), total Akt, basal phospho-Akt, basal levels of insulin receptor- β , and glucose transporter-4 (Glut4) were assessed by Western blotting (*B*) and quantitated by densitometry (*C–E*). *F* and *G*: Effect of cathepsin K knockout on the protein levels of other cathepsins in ND-fed and HFD-fed mice. Mean \pm SEM, $n = 3–4$ per group. WT, wild-type. * $P < 0.05$ between the notated groups. (A high-quality digital representation of this figure is available in the online issue.)

Elevated SERCA2a has been postulated to reduce cardiac hypertrophy (33), whereas an increase in its inhibitory subunit phospholamban (nonphosphorylated, monomeric) induces hypertrophy (34). Contrary to this notion, we found an upregulation of SERCA2a and phospholamban after high-fat feeding. More importantly, cathepsin K knockout

resulted in elevated SERCA2a and phospholamban and the ratio of phospholamban to SERCA2a. Although the significance of these findings are difficult to explain in the context of improved contractility, the elevated phospholamban-to-SERCA2a ratio may partly explain the increased resting Ca²⁺ in the myocytes from cathepsin K knockout mice.

The most significant finding of our study is that cathepsin K knockout attenuates high-fat diet-induced cardiac hypertrophy. Previous studies have shown that inhibition of proteases such as the matrix metalloproteinases ameliorates myocardial remodeling by inhibiting both cardiomyocyte hypertrophy and interstitial fibrosis (35). In contrast, cardiac fibrosis was unaltered by cathepsin K knockout, suggesting that attenuation of heart weight was a consequence of reduction in individual myocyte size. This was further confirmed by comparing the size of the individual cardiomyocytes isolated from high-fat diet-fed wild-type and cathepsin K knockout mice. Akt has pleiotropic effects on the heart (36); short-term activation of Akt in the heart can cause physiological hypertrophy (37), whereas sustained activation of Akt can result in pathological hypertrophy (38). Activation of fetal genes ANP, GATA4, and NFATc3 suggests pathological hypertrophy in our high-fat diet-fed mice. Some of the beneficial effects of cathepsin K knockout on cardiac hypertrophy may be attributed to its ability to alleviate insulin resistance, as evidenced by inhibition of high-fat diet-induced elevated cardiac phospho-Akt and insulin receptor- β , reversal of the blunting of cardiac insulin-stimulated Akt phosphorylation, and increased insulin-stimulated Akt phosphorylation and improved serum glucose and insulin levels.

Cardiac hypertrophy is an adaptive response to a variety of intrinsic and extrinsic stimuli. Paradoxically, myocyte apoptosis plays a critical role in causing cardiac hypertrophy and heart disease (39,40). Because myocytes are nondividing cells, apoptosis of cardiomyocytes causes the workload on the remaining cardiomyocytes to increase, eventually leading to hypertrophy. Recent studies have implicated cathepsins in the regulation of apoptosis (17,41). Our studies demonstrate that a high-fat diet induces cardiomyocyte apoptosis that is nullified by cathepsin K ablation. Furthermore, silencing of cathepsin K inhibited apoptotic responses to palmitic acid, and pharmacological inhibition of cathepsin K abrogated apoptotic response to staurosporine (data not shown). The benefits of cathepsin K inhibition may be partly mediated by blocking the proapoptotic actions of cathepsin K.

In summary, our finding that cathepsin K ablation inhibits obesity-associated cardiac hypertrophy and contractile dysfunction without affecting basal cardiac function provides a novel target for intervention in obesity-associated heart disease. Given that selective pharmacological inhibitors of cathepsin K are being designed for clinical use in the treatment of osteoporosis and other diseases, the role of cathepsin K in cardiac dysfunction may assume new significance.

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Y.H., Y.Z., and J.D. researched data. Y.H. wrote the first draft of the manuscript. G.-P.S. and J.R. reviewed the manuscript. G.-P.S., J.R., and S.N. contributed to discussion. J.R. edited the manuscript and assisted with echocardiography. S.N. planned the study and wrote the manuscript. S.N. is the guarantor of this work and, as such, had full access to all the data in the study and takes

responsibility for the integrity of the data and the accuracy of the data analysis.

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