Cathepsin K Deficiency Prevented Kidney Damage and Dysfunction in Response to 5/6 Nephrectomy Injury in Mice With or Without Chronic Stress

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Supplementary Materials

Antibodies and reagents

The following commercially available antibodies were used. Anti-p38MAPK (cat. no. 9212), anti-phospho-p38mitogen-activated protein kinase (p-p38MAPK; #4511), cleaved caspase-3 (#9661), and anti-Sirt1 (#2028) were from Cell Signaling Technology (Danvers, MA). Anti-gp91phox (clone: 53) was from BD Transduction Laboratories (San Jose, CA). Antibodies for p16 INK4A (CDKN2A, cat. no. 10883-1-AP) were from Proteintech (Rosemont, IL). The single-stranded deoxyribonucleic acid (ssDNA) rabbit IgG was from IBL (Gunmaken, Japan). Anti-desmin (DE-U-10, ab6322) and anti-p21 (#2947) were purchased from Abcam (Cell Signaling Technology). Anti-NLRP3/NALP3 (Cryo-2) was from Adipogen Life Science (Cell Signaling Technology). FITC-conjugated anti-mouse immunoglobulin (Ig) G was from Medical & Biological Laboratories (Nagoya, Japan).

The enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor-alpha (TNF-α, cat no. MTA00B) and interleukin (IL)-17 (cat. no. KE1700) were from BD (Franklin Lakes, NJ). The RNeasy Micro Kits and SYBR™ Green Master Mix were from Qiagen (Hilden, Germany). The ImmPACT™ DAB peroxidase substrate (cat. no. SK-4105) was from Vector Laboratories (Burlingame, CA). The nitrocellulose transfer membrane was from Amersham Bioscience (Piscataway, NJ). The SuperScript III First Strand and Lipofectamine® 3000 Transfection Kits were purchased from Invitrogen Life Technologies (Carlsbad, CA). CatK-specific siRNAs (#F5129442005, #F5129442006) and nontargeting control siRNA (#F5129292-921) were purchased from Sigma-Aldrich (St Louis, MO). The Amersham ECL Prime Western Blotting Detection kit was from GE Healthcare (Freiburg, Germany). Azan-Mallory solution and periodic acid-Schiff solution purchased from Muto Pure Chemicals (Tokyo). The 0.5w/v% sterilized methyl cellulose 400 solution was purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan). The ImmPACT™ DAB peroxidase substrate kit (cat. no. SK-4105) was from Vector Laboratories (Burlingame, CA). Silamin A/C (D0010500105) used as a positive control was from Dharmaco (Brébières, France). CatK plasmid plasmid was purchased from Invitrogen Life Technologies. CatK inhibitor II
[1-(NBenzyloxycarbonyl-leucyl)-5-(N-Boc-phenylalanyl-leucyl) carbohydrazide] was purchased from Sigma-Aldrich.

**Mice**

The male CatK$^{-/-}$ mice (C57BL/6J background; a gift from Harvard University, Boston, MA$^1$) and CatK$^{+/+}$ mice (C57BL/6J background) used in this study were 7 weeks old and weighed 21–25 g. All animals were provided with a normal diet and tap water ad libitum and housed two per cage under standard conditions (50 ± 5% humidity, 23 ± 1°C), with a 12-hr light/dark cycle (dark beginning at 7:00 pm) in a viral pathogen-free facility at the Laboratory Animal Research Division of the Nagoya University Graduate School of Medicine. The animal protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University Graduate School of Medicine (protocol no. 27304^2) and performed according to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

**5/6Nx surgery, stress procedures, and evaluations**

The 5/6Nx surgery was performed as described. In brief, we cut a small wound from the posterior peritoneum and exposed the left kidney. Following ligation at Up and down one-third place, 2/3 of the kidney was removed with a cut and performed compression hemostasis with a cotton swab both cutting sides for 1 minute. One week later, the same method was applied to remove the entire right kidney. Three days later, the mice were subjected to a stress protocol; the mice were exposed to chronic variable stress for 8 weeks as described. To prevent the mice from becoming accustomed to the restraint stress, we administered several different combinations of stressors over each week from Mondays to Sundays and changed the stress order randomly as follows. (1) Cage horizontal and damp: We removed the sawdust and placed some water in the stress cage, then suspended the stress cage horizontally so that the mouse's tail was in the water for 4 hr once every 2 days. (2) Cage tilt: We put the mouse into a stress cage and suspended the cage at a 45° angle for 4 hr once every 2 days. (3) Overnight illumination: The mouse was placed separately in a cage in a room with all-night lighting, 3x/week (from 21:00 to 9:00).

First, to explore the impact of chronic stressors on kidney injury, CatK$^{+/+}$ mice
(n=8) were divided into non-stress/sham operation, 5/6Nx surgery, and 5/6Nx+stress groups for 8 weeks. Non-stressed control mice were left undisturbed and allowed contact with each other.

For the mechanistic studies, four independent experiments were performed as follows. (1) In a separate 5/6Nx+stress experiment (Exp. 1), CatK−/− and CatK+/+ mice that had undergone sham surgery (CatK+/++sham; CatK−/−+sham) and CatK−/− and CatK+/+ mice that had undergone 5/6Nx surgery (S-CatK+/+5/6Nx; S-CatK−/−5/6Nx) were subjected to chronic stress for 8 weeks and then subjected to the evaluation. (2) In a separate CatKII to 5/6Nx+stress experiment (Exp. 2), CatK+/+ mice that had undergone 5/6Nx surgery were signed to one of two groups and given either the vehicle (0.5% carboxymethylcellulose, S-5/6Nx+veh) or CatKII (5 mg/kg per day, S-5/6Nx+CatKII, dosed as above) every day for 8 weeks with continued daily immobilized stress and then subjected to the evaluation. (3) In a separate 5/6Nx experiment (Exp. 3), CatK−/− and CatK+/+ mice that had undergone the sham surgery (CatK+/++sham; CatK−/−+sham) or 5/6Nx surgery (CatK+/+5/6Nx; CatK−/−5/6Nx) were subjected to the evaluation 8 weeks later. (4) In a separate CatKII to 5/6Nx experiment (Exp. 4), CatK+/+ mice that had undergone 5/6Nx surgery were signed to one of two groups and given by oral gavage either the vehicle (5/6Nx+veh) or CatKII (5/6Nx+CatKII, dosed as above) every day for 8 weeks and then subjected to the evaluation.

At the end of the 2-month stress protocol, all mice (all stressed animals were left undisturbed for 2 h before sacrifice) were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Dainippon Pharmaceutical, Osaka, Japan), and the kidney and left ventricular blood samples were collected for biological analyses (including creatinine, blood urea nitrogen [BUN], an enzyme-linked immunosorbent assay [ELISA], and gene and protein assays) and histological analyses.

Urinalysis and blood pressure measurement

Twenty-four-hour urine was collected from mice with the use of a metabolic cage at the end of treatments, and the urine albumin levels were determined by a pyrogallol assay (MicroTP-AR, Wako, Osaka, Japan). Blood pressure (BP) values were measured by the tail-cuff device (MK-2000; Muromachi, Tokyo) and/or the Powerlab blood pressure
system (ADInstruments, Castle Hill, NSW, Australia) at baseline and every week thereafter (day 0, 14, 28, 42, and 56). We measured three times and averaged for each mouse.

**ELISA and biochemical analyses**

Plasma and serum samples from experimental mice (n=5–6 per group) were assayed for the levels of tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-17 using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions. The plasma creatinine and BUN levels were measured at a commercial laboratory (SRL, Tokyo, Japan).

**Gene expression assay**

Whole RNA was harvested from the tissues with an RNAeasy Mini Kit according to the recommended protocol. A SuperScript III Cells Direct cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA) was used to generate complementary deoxyribonucleic acid (cDNA). Quantitative real-time gene expression was studied by using the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with SYBR Green Master Mix using the following conditions: 50°C (2 min) for uracil-N-glycosylase incubation, 94°C (10 min) for AmpliTaq Gold activation, 95°C (15 s), and 59°C (1 min) for 40 cycles. All experiments were performed in triplicate.

The sequences of the primers for the targeted genes are shown in Supplementary Table S1: collagen type I, collagen type III, matrix metalloproteinase-2/-9 (MMP-2/-9), CatK, CatL, CatS, IL-1β, TLR-2/-4, TNF-α, intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), and angiotensin II receptor type 1α (AT1Rα). The transcription of targeted genes was normalized to that of the gene for the internal housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene.

**Western blot analysis**

Proteins were lysed from the cells and the tissues using lysis buffer containing 20 mM Tris-Cl, (pH 8.0), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.05% SDS, 1% Na-deoxycholate, and fresh 1× protease inhibitors. The concentration of each protein
was measured by the DC Protein Assay kit (Bio-Rad Laboratories, Marnes-la-Coquette, France) before the proteins were equally loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to FluoroTrans-W® membranes and incubated overnight with primary antibodies against gp91phox, PPAR-γ, Bcl-2, C-Cas-8, p-p38MAPK, p38MAPK, Erk1/2, p-Erk1/2, Sirt1, NLRP3, p21, p16INK4A, and GAPDH (1:1,000 for each). The membranes were then treated with the horseradish peroxidase (HRP)-conjugated secondary antibody at a 1:10,000–15,000 dilution. The Amersham ECL Prime Western Blotting Detection kit was used for the determination of targeted proteins. Protein levels quantitated from western blots were normalized by loading the internal control GAPDH.

**Histology and immunohistochemistry**

Kidneys were fixed in ice-cold 4% paraformaldehyde solution for 24 h, embedded in paraffin, and processed for histology and immunohistochemistry as described. Renal transverse tissue sections (3 μm) were stained with hematoxylin-eosin solution for routine histological examination; Masson solution was used to evaluate glomerular and perivascular fibrosis, and periodic acid-Schiff (PAS) solution was used to evaluate glomerulosclerosis and tubulointerstitial injury. The glomerulosclerosis index was semiquantitatively calculated through the examination of 20 glomeruli from four independent sections of each animal (n=5–7 per group) and graded from 0 to 4+. The glomerulosclerosis index was scored as 0 (0%), 1+ (1%–25%), 2+ (26%–50%), 3+ (51%–75%), or 4+ (76%–100%) based on the described scoring system. Glomerular fibrosis was analyzed in 20 glomeruli from each section and four independent sections of each animal (n=5–7 per group) and is expressed as the area percentage stained per glomerulus.

For immunohistochemistry, sections were stained with a mouse monoclonal antibody (mAb) against CD68, a rabbit anti-ssDNA, or a mouse mAb against human desmin (1:100 for each antibody). After being washed with phosphate-buffered saline (PBS) five times, the sections were sequentially treated with appropriate secondary antibodies (1:200) for 2 h at 4°C, and then visualized with a corresponding substrate kit. For negative controls, primary antibodies were replaced with nonimmune IgG. Macrophages that infiltrated the glomeruli were counted in four random microscopic
fields from three independent sections (of each animal \(n=5–7\)), and the infiltration is expressed as the number of macrophages per glomerulus (mm\(^2\)) (400×).

Apoptotic cells in glomerular regions were counted in 20 glomeruli from four independent sections of each animal \((n=5–7)\), and the apoptosis is expressed as the number of ssDNA\(^+\) cells per glomerulus. Glomerular desmin staining was analyzed in 20 glomeruli with ImageJ software from four independent sections of each animal \((n=5–7)\) and is expressed as the area percentage stained per glomerulus. All morphometric measurements were performed with WinROOF ver. 5.0 image-processing software (Mitani, Tokyo) by two observers.

**Transmission electron microscopy (TEM)**

Kidney cortical tissue was thinly sliced, placed immediately in primary electron microscopy fixative, and prepared as described.\(^4\) A JEM-1400EX transmission electron microscope (JEOL, Tokyo) was used to view all renal samples (at magnifications of 3,000× and 30,000×). A total of 50–70 podocyte cross-sections from 5–7 sections were calculated and averaged for each mouse, and distribution diagrams were obtained separately for each group.

**Cell culture**

Mesangial cells (American Type Culture Collection, VA, UAS) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics at 37°C with 5% CO\(_2\). At 60%–80% confluence, the cells were subjected to the biological analysis, transfection experiments, and apoptosis assay. To explore the molecular mechanisms of chronic stress-mediated kidney injury, the serum used all of in vitro experiments were from the mice with and without stress for 4 weeks.

**Overexpression and silencing of CatK**

The overexpression of CatK was performed as described.\(^5\) In brief, mesangial cells were typically seeded in six-well plates at 5×10\(^5\) cells/well. The cells were then transfected with a CatK plasmid (pICatK) at 80% confluence for 24 h with the use of the Lipofectamine\(^\circledast\) 3000 Transfection Kit (Thermo Fisher Scientific). An empty vector alone was transfected as a mock transfection control (CONT).
For the silencing of CatK, the cells were grown on 60-mm dishes until they reached 80% confluence and then transiently transfected with siCatK and non-targeting control siRNA (siRNA-C) with the use of Lipofectamine® Transfection reagent according to the manufacturer's instructions. Arranged cells were then subjected to western blotting and cellular function assays.

Cell apoptosis assay
For the cell apoptosis assay, the mesangial cells were seeded onto coverslips (2×10³ cells/ml). After being cultured in H₂O₂ (100 μM) or 5% stress-serum overnight, the cells were fixed with 4% paraformaldehyde and washed three times with PBS containing 1% glycerol. The cells were then treated with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining reagents for the apoptosis assay.

Statistical analysis
All results are presented as the mean ± standard error of the mean (SEM). Before applying statistical methods, we evaluated whether the data fit a normal distribution by conducting a Pearson normality test. Student's t-tests (for comparisons of two groups) or a one-way analysis of variance (ANOVA) (for comparisons of three or more groups) followed by Tukey post hoc tests were used for the statistical analyses. The blood pressure data were subjected to a two-way repeated-measures ANOVA and Bonferroni post hoc tests. A probability (p)-value <0.05 was considered significant. All of the morphometric measurements were performed by two observers in a blind manner, and the values they obtained were averaged. The authors had full access to and take full responsibility for the data. All authors have read and agree to the manuscript as written.

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Supplementary Figure Legends

Figure S1. CatK deletion decreased the targeted oxidative stress-, inflammation-, and proteolysis-related gene expressions in 5/6Nx mice in response to stress. A though F: Quantitative PCR data show the levels of collagen type I (A) and type III (B), MMP-2 (C) and MMP-9 (D), gp91phox (E) and p22phox (F), and TLR-2 (G) and TLR-4 (H) mRNAs in the kidneys of four experimental groups. Data are mean±SEM (n=5–8). *P<0.05, **P<0.01 vs. corresponding S-CatK+/++5/6Nx by ANOVA and Tukey's post hoc tests; NS, no significant.

Figure S2. CatK knockout (KO) decreased systolic blood pressure (SBP) and proteinuria in response to 5/6Nx injury. A: SBP recordings in two experimental groups measured at each indicated time point (n=6-8). B: Urine protein test results of mice in four groups at 8 weeks after stress. C and D: ELISA results showing the levels of plasma IL-17 and TNF-α at 8 weeks after stress. E and F Laboratory test showing the levels of blood creatinine and BUN at 8 weeks after stress. G though I: Representative immunoblot images (G) and quantitative data (H and I) show the target protein levels (gp91phox, NLRP3, PPAR-γ, Bcl-2, C-Cas-8, p-p38MAPK, p16, Sirt1, and p-Erk1/2) in the kidneys of four experimental groups. Data are mean±SEM (n=3–8). *P<0.05, **P<0.01 vs. day 0; #P<0.05, ##P<0.01 vs. corresponding CatK+/++5/6Nx ANOVA and Tukey's post hoc tests (B through F, H and I); NS, no significant.

Figure S3. CatK KO alleviated the podocyte and glomerular injuries in mice after 5/6 nephrectomy. A though E: Representative images (A) combined with quantitative data obtained by electron microscopy (B), periodic acid-Schiff (PAS, C), Masson's trichrome (D) and desmin (E), ssDNA (single strain DNA, F) and macrophage (Mac, G) staining show the levels of podocyte foot process, glomerulosclerosis, glomerular fibrosis, ssDNA+ apoptosis, and macrophage infiltration in the kidneys of four experimental groups. TEM scale bar, 500 nm. Scale bar for others, 75 μm. Data are mean±SEM (n=5–8). *P<0.05, **P<0.01 vs. corresponding CatK+/++5/6Nx mice by ANOVA and Tukey's post hoc tests (B though G); NS, no significant.

Figure S4. CatKII ameliorated the blood pressure elevation and proteinuria in mice that had undergone a 5/6 nephrectomy. A: SBP recordings in two experimental groups measured at each indicated time point (n=6-8). B: Urine protein test results of mice in four groups at 8 weeks after stress. Quantitative PCR data show the levels of TLR-2, TNFα, MCP-1, collagen type I, collagen type III, MMP-2, and MMP-9 mRNAs. Data
are mean±SEM (n=5–8). *P<0.05, **P<0.01 vs. day 0; #P<0.05, ##P<0.01 vs. corresponding 5/6Nx+veh by two-way ANOVA and Tukey's post hoc tests (A) or unpaired t tests (B though D).

Figure S5. CatKII alleviated the podocyte injuries and glomerular fibrosis in mice that had undergone a 5/6 nephrectomy. A though G: Representative images (A) and quantitative data of electron microscopy (B), PAS (C), Masson (D), desmin (E), ssDNA (F) and macrophage (G) staining. TEM scale bar, 500 nm. Scale bar, 75 μm. Data are mean±SEM (n=5–8). *P<0.05, **P<0.01 vs. corresponding 5/6Nx+veh by ANOVA and Tukey's post hoc tests (B though G); NS, no significant.

Figure S6. CatKII mitigated the blood pressure elevation and proteinuria in 5/6Nx mice under chronic stress conditions. A: SBP recordings in four experimental groups measured at each indicated time point (n=7-8). B: Urine protein test results of mice in four groups at 8 weeks after stress. C and D: Representative western blotting images (C) and quantitative data (D) show the targeted molecular protein levels (PPAR-γ, C-Cas-8, and Bcl-2) in the kidney of both experimental groups. Data are mean±SEM (n=3–8). *P<0.05, **P<0.01 vs. day 0; #P<0.05, ##P<0.01 vs. corresponding S-5/6Nx+veh by two-way ANOVA and Tukey's post hoc tests (A) or unpaired t tests (B though D); NS, no significant.

Figure S7. Effect of oxidative stress on the expression of CatK and apoptosis-related protein levels in mesangial cells. A: The mRNA levels of CatK in mesangial cells in response to various H2O2 concentrations. B and C: Representative western blotting images (A) and quantitative data (C) show the targeted molecular protein levels (PPAR-γ, C-Cas-8, and Bcl-2) in mesangial cells in response to various concentrations of H2O2. Data are mean±SEM (n=3-8). *P<0.05, **P<0.01 vs. corresponding H2O2 0 or 50 μM by one-way ANOVA and Tukey's post hoc tests; NS, no significant.

Figure S8. CatK silencing exerted a beneficial effect on the levels of apoptosis-related proteins in mesangial cells in response to H2O2 at 100 μM. A: Real-time PCR data show the H2O2-induced CaK mRNA expression in mesangial cells transfected with and without siCatK. B and C: Representative western blotting images (B) and quantitative data (C) show H2O2-induced target molecular protein levels (PPAR-γ, C-Cas-8, and Bcl-2) in mesangial cells transfected with and without siCatK. D and E: Representative TUNEL staining images (E) and quantitative data (D) show the number of apoptotic
cells in mesangial cells transfected with and without siCatK. Scale bar, 75 μm. Data are mean±SEM (n=3–7). *p<0.05, **p<0.01 vs. corresponding Con+100μM H₂O₂ by one-way ANOVA and Tukey's post hoc tests; NS, no significant.

**Figure S9.** plCatK-mediated overexpression accelerated the apoptosis-related protein expression and apoptosis in mesangial cells. **A:** Real-time PCR data show the CaK mRNA expression in mesangial cells transfected with and without plCatK. **B** and **C:** Representative western blotting images (B) and quantitative data (C) show H₂O₂-induced target molecular protein levels (PPAR-γ, C-Cas-8, and Bcl-2) in mesangial cells transfected with and without plCatK. **D** and **E:** Representative TUNEL staining images (E) and quantitative data (D) show the number of apoptotic cells in mesangial cells transfected with and without plCatK. Scale bar, 75 μm. Data are mean±SEM (n=3–7). *p<0.05, **p<0.01, *p<0.05, ###p<0.01 vs. the corresponding controls by one-way ANOVA and Tukey's post hoc tests.
### Table S1. Primer sequences for the quantitative real-time PCR

| Genes     | Forward Primers          | Reverse Primers          |
|-----------|--------------------------|--------------------------|
| p22phox   | AACTACCTGGGAGCCAGTTGAG   | AATTAGGAGGTGGTGAATATCGG  |
| gp91phox  | ACTTTCCATAAGATGGTAGCTTG  | GCATTACACACACACTCAACG    |
| IL-1β     | TGCCACCTTTTGACAGTGATG    | ATGTGCTGCTGGCAGATTTTG    |
| MCP-1     | GCCCACTCCTCTGCTCTACT     | CCTGCTGCTGGTGATCCTCTTG   |
| ICAM-1    | CCCGCAGGTCCTAATCC        | CCAGAGCGGCAGAGCAA        |
| TNF-α     | AGGCTGCCGCCGACTAGCT      | GAACCTTCCTCAGTGATGAGAAA  |
| MMP-2     | CCCCATGAAGCCTTTTACC      | TTGTAGGAGGTGGCCCTGGAA    |
| MMP-9     | CCAGACGCTCTCTCGA GAACC   | GTTATAGAAGTGCGGTTGTTG    |
| CatK      | AGCAGGCTGGGAAGCTAAAGT    | TTTGTGCATCTCAGTGGAAGCT   |
| CatL      | GGCAACCCGATGCCGC         | TGTGTGACTCCTGTGAAGAACCA  |
| CatS      | GTGGCCACTA AAGGGCCTG     | ACCGCTTTTGTAGAAGAGAAGGAG |
| AT1Rα     | TTTCAGATCAAGTGCATTGTA    | AGAGTTAAGGGCCATTGCTTT    |
| TLR2      | AAGAAGCTGGCATCCCGAGGC    | CGTCTGACCTCAGGGGTTG      |
| TLR-4     | AGTGGGTCAGAAGAACAGAAAGCA| CTTTACCAGCTATTTCTCACC    |
| Coll-I    | TGACTGGAGAGCGGGAGAGT     | GACGGC TGAGTA GGAAACAC   |
| Coll-III  | AGGTTTCTCCTGTTGCTGTCT    | GGATGCCACCTTGTCATCAT     |
| GAPDH     | ATGTGTCGGTCGTGGATCTGA    | ATGCGCTTCCACACCTTCTCT    |

AT1Rα: angiotensin receptor 1α, CatK: cathepsin K, CatL: cathepsin L, CatS: cathepsin S, ICAM-1: intercellular adhesion molecule-1, IL-1β: interleukin-1beta, MCP-1: monocyte chemoattractant protein-1, MMP-2: matrix metalloproteinase-2, PCR: polymerase chain reaction TLR-2: toll-like receptor-2, TNF-α: tumor-necrosis factor-alpha; Coll-I: collagen type I; Coll-III: collagen type III; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.
Table S2. CatK deletion reduced targeted gene expression in the kidneys of 5/6 Nx mice

|                  | CatK^{+/+}5/6Nx | CatK^{-/-}5/6Nx |
|------------------|-----------------|-----------------|
| Coll-I           | 27.89±1.61      | 14.77±1.91**    |
| Coll-III         | 784.03±52.70    | 288.15±15.48**  |
| MMP-2            | 2312.19±279.70  | 1401.06±67.35** |
| MMP-9            | 40.03±2.61      | 10.42±0.76**    |
| CatK             | 8.75±0.78       | 0.01±0.00**     |
| CatS             | 120.02±5.23     | 92.59±3.85**    |
| CatL             | 9.73±0.58       | 3.53±0.44**     |
| IL-1β            | 86.52±3.50      | 41.17±2.94**    |
| gp91^{phox}      | 62.61±2.48      | 23.30±1.37**    |
| p22^{phox}       | 9.35±0.67       | 3.77±0.62**     |
| TLR2             | 52.17±4.46      | 22.44±1.99**    |
| TLR4             | 500.73±38.08    | 259.92±4.08**   |
| ICAM-1           | 932.71±49.81    | 524.26±15.89**  |
| TNF-α            | 4.45±0.29       | 2.66±0.18**     |
| MCP-1            | 11.92±1.01      | 5.14±0.30**     |
| AT1Rα            | 13.73±0.98      | 6.92±0.78**     |

Coll-I: collagen type I; Coll-III: collagen type III; ATR1α: angiotensin receptor 1α; CatK: cathepsin K; CatL: cathepsin L; CatS: cathepsin S; ICAM-1: intracellular cell adhesion molecule-1; IL-1β: interleukin-1beta; MCP-1: monocyte chemoattractant protein-1; MMP: matrix metalloproteinase; TLR: toll-like receptor; TNF-α: tumor-necrosis factor-alpha. Data are mean±SEM (n=6). *p<0.05, **p<0.01 by unpaired t test.
Figure S1.

A. Collagen type I

B. Collagen type III

C. MMP2

D. MMP9

E. gp91phox

F. p22phox

G. TLR2

H. TLR4
Figure S2.
Figure S3.
Figure S4.
Figure S5.
Figure S6.
Figure S7.
Figure S8.
Figure S9.