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ORIGINAL RESEARCH

Chronic Kidney Disease Is Associated With Increased Cardiac Corin Expression But Decreased Proatrial Natriuretic Peptide Conversion Activity

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BACKGROUND: Chronic kidney disease (CKD) is associated with an increased risk of cardiovascular disease. Corin converts proatrial natriuretic peptide into its active form after being activated by PCSK6 (proprotein convertase subtilisin/kexin type 6) protease. It remains unknown whether the PCSK6/corin/atrial natriuretic peptide pathway plays a role in CKD-induced cardiomyopathy.

METHODS AND RESULTS: Serum corin, left ventricular mass index, and corin–left ventricular mass index correlation were compared between outpatients with versus without CKD. Cardiac corin expression and activity as well as serum corin were compared between 5/6 nephrectomy CKD animal models and sham controls. The effects of indoxyl sulfate, a uremic toxin, on cardiomyocytes were examined in vitro in H9c2 cells. A total of 543 patients were enrolled in this study. Serum corin levels were elevated in patients with CKD compared with levels in patients without CKD. Serum corin levels correlated negatively with left ventricular mass index in participants without CKD, but not in patients with CKD. Compared with sham controls, CKD mice had higher serum corin levels and increased cardiac expression of corin but reduced cardiac corin conversion activity. Indoxyl sulfate stimulated corin expression while suppressing serine protease activity in H9c2 cardiomyoblasts. Lower PCSK6 expression in CKD mouse hearts and indoxyl sulfate–treated H9c2 cardiomyoblasts may explain, at least partly, the observed CKD-associated reduction in corin activity.

CONCLUSIONS: In CKD, cardiac and serum levels of corin are increased, yet corin activity is suppressed. The latter may be attributable to reduced PCSK6 expression. These findings suggest that corin dysfunction may play a significant role in the pathogenesis of CKD-associated cardiomyopathy.

Key Words: atrial natriuretic peptide ■ chronic kidney disease ■ corin ■ PCSK6 ■ uremic cardiomyopathy

There are multiple interactions between cardiac and renal conditions. Decreased renal function is an independent risk factor for cardiovascular outcomes and all-cause mortality.[1,2] Furthermore, cardiomyopathy affects up to 80% of hemodialysis patients and is the main cause of their mortality.[3] Conversely, chronic kidney disease (CKD) and the accumulation of uremic toxins, including indoxyl sulfate (IS), are risk factors for enhanced cardiac remodeling.[4]

Atrial natriuretic peptide (ANP) is a cardioprotective hormone that exerts antihypertrophic and antifibrotic effects upon being activated by the transmembrane serine protease corin.[5] Corin is expressed mainly on cardiomyocytes and is activated by a protease, namely PCSK6 (proprotein convertase subtilisin/kexin type 6).[6,7] A convergence of evidence implies a protective role for the PCSK6/corin/ANP pathway in cardiac remodeling. Notably, corin variants that

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CLINICAL PERSPECTIVE

What Is New?

- Increased serum and cardiac corin expression in chronic kidney disease (CKD) concurrent with a seemingly paradoxical suppression of corin activity may be caused by, at least in part, suppressed PCSK6 (proprotein convertase subtilisin/kexin type 6) expression.
- Reduced proatrial natriuretic peptide activation, attributable to suppressed PCSK6, may underlie CKD-related cardiomyopathy.

What Are the Clinical Implications?

- CKD-related cardiomyopathy is a major cause of death in patients with CKD.
- Restoration of corin activity could represent a potential treatment target for management of CKD-associated cardiovascular abnormalities.

Nonstandard Abbreviations and Acronyms

IS	indoxyl sulfate
LVMI	left ventricular mass index
PCSK6	proprotein convertase subtilisin/kexin type 6

impair ANP processing have been associated with increased risks of hypertension and cardiac hypertrophy.[8,9] In addition, higher baseline serum corin levels have been associated with better prognoses in patients with acute myocardial infarction and chronic heart failure.[10,11]

Despite usually having elevated circulating levels of pro-ANP,[12] patients with CKD have a disproportionately increased risk of cardiac hypertrophy and fibrosis.[13] Although corin hypoactivity has been observed in terminal heart failure tissues,[14,15] corin expression has yet to be evaluated in patients with CKD. The purpose of the present study was to evaluate corin expression and activity in human patients, a mouse model of CKD, and an in vitro model of CKD. We hypothesized that CKD would be associated with inadequate corin signaling, impaired pro-ANP conversion, and accelerated adverse cardiac remodeling.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Patient Enrollment and Study Protocol

Patients were recruited from the outpatient departments of Taipei Veterans General Hospital and Cheng Hsin General Hospital. The study was approved by the institutional review boards of both hospitals (016–08-002AC and [464] 103A-1) and all procedures conformed to the principles outlined in the Declaration of Helsinki. Informed written consent was obtained before the enrollment of participants in this study.

Patients who underwent regular follow-up in cardiovascular and nephrology outpatient departments were eligible for inclusion. Patients with CKD were defined as those with an estimated glomerular filtration rate <60 mL/min per 1.74 m^2 for at least 3 months. Patients were excluded if they were aged <20 years, unwilling or unable to undergo regularly scheduled examinations or follow-ups, or pregnant, or had a recently diagnosed cancer. Power analysis indicated that at a significance level of 0.05, a power level of 0.95, and an effect size d of 0.85, a sample size of ≥78 participants should be included. We included a total of 543 patients in our study.

After enrollment, blood and urinary samples were collected for baseline analyses. Patient characteristics and comorbidities were recorded based on chart reviews and questionnaire responses. Blood pressure (BP) measurements were performed by trained nurses. Serum levels of pro-ANP, ANP, and corin were measured with commercial ELISA kits (RayBiotech, EIA-ANP-1; R&D Systems, DANP00 and DCRN00).

Comprehensive 2-dimensional Doppler echocardiography was performed. Left ventricular dimensions (interventricular septal thickness at diastole, posterior wall thickness, and left ventricular end-diastolic diameter) were measured in M-mode at the end of diastole. Left ventricular mass was determined with the Troy formula, as recommended by the American Society of Echocardiography: $0.8\times1.04x[(LVEDD+IVSd+PW)^3-LVEDD^3]+0.6$, where LVEDD is left ventricular end-diastolic diameter, IVSd is interventricular septal thickness at diastole, and PW is posterior wall thickness. Left ventricular mass (in g). Left ventricular mass was divided by body surface area to obtain a left ventricular mass index (LVMI) value. Left ventricular hypertrophy was defined as LVMI >118 g/m^2 in men and >108 g/m^2 in women.

Animal Model and Study Protocol

Our animal experiments were approved by the Institutional Animal Care and Use Committee of Cheng Hsin General Hospital (CHIACUC 104–22). A total of 60 male C57Bl/6J mice provided from the National Laboratory Animal Center, Taiwan, were used. The mice were randomized into control (sham operation) and CKD model groups (n=20 and n=40, respectively).

We used the 5/6 nephrectomy CKD mouse model wherein two thirds of the left kidney was removed from 8-week-old male C57Bl/6J mice and right nephrectomy was performed 2 weeks later.

After the first operation, body weight was measured weekly. Serum samples were collected, BP was measured, and transthoracic echocardiography was performed every 4 weeks. Serum level of serum urea nitrogen, creatinine, corin, and IS levels were measured. Serum corin concentration was determined with a commercial ELISA kit (MyBioSource, MBS2023262) and IS was measured with high-performance liquid chromatography–tandem mass spectrometry. BP was measured by tail-cuff plethysmography in restrained animals. Mice were habituated for at least 3 consecutive days before baseline BP measurements were taken. Each recording session consisted of 20 measurements, of which the last 10 measurements were used in the analysis. For echocardiography, 2-dimensional B-mode cine loops were recorded in the parasternal long-axis and midpapillary short-axis views. Recordings were analyzed offline with Vevo Lab Version 3.1.1 software (FUJIFILM VisualSonics, Inc, Vevo 3100). All variables of interest were measured for at least 3 heartbeats at end diastole and at the corresponding end systole.

Preparation of Heart Tissues and Cardiomyocyte Morphometry

Animals were euthanized 8 weeks after their 5/6 nephrectomy or sham operation. Hearts were perfused with phosphate-buffered saline in situ, removed carefully, weighed, and then stored at −80 °C or embedded in 4% paraffin. In addition, fresh frozen heart tissues were subjected to reverse transcriptase polymerase chain reaction and Western blot analyses. Paraffin-embedded heart sections (3-μm thickness) were deparaffinized and subjected to hematoxylin and eosin staining, Masson trichrome, and wheat germ agglutinin (Biotium, 29023-1) staining. Three randomly selected fields were inspected, and ≥100 cardiomyocytes/field were measured to obtain a cross-sectional area marked with wheat germ agglutinin. To quantify cardiac fibrosis, micrographs (5 fields per section) were captured under light microscopy (×400), and Masson trichrome–positive areas, including both myocardial and perivascular areas, were analyzed and quantified in CaseViewer software (3DHISTECH). Fibrosis area was expressed by the percentage of the Masson trichrome–positive area within the tissue section.

Corin Immunohistochemistry

Heart tissue sections (3-μm thickness) were cleared in xylene and rehydrated in water solutions with gradually decreasing concentrations of ethanol. Anticorin primary antibodies (Biorbyt, orb100997, 1:50) were applied for 1 hour, and omission of primary antibody served as a negative control. Labeling was enhanced with streptavidin-biotin augmentation and revealed with alkaline phosphatase. At least 3 randomly selected fields per section were inspected under a light microscope (×400 magnification). Corin immunolabeling in these fields was quantified in ImageJ software (National Institutes of Health).

Real-Time Quantitative Polymerase Chain Reaction

Heart samples were homogenized and then subjected to RNA extraction with TRIzol reagent (ThermoFisher Scientific). RNA (1 μg) was reverse transcribed with a cDNA Archival Kit (Life Technologies). Real-time quantitative polymerase chain reaction was performed in a ViiA 7 System (Life Technologies) instrument with SYBR Green Master Mix (ThermoFisher Scientific) and gene-specific primers (sequences in Table S1). Data were normalized and analyzed with the ΔΔCt method. Samples were assayed in triplicate, and β-actin served as an internal control.

Western Blotting

Frozen heart tissue (25 mg) was homogenized in radioimmunoprecipitation assay buffer (Boston BioProducts) with freshly added protease inhibitors (1 mmol/L PMSF, 1 μg/mL aprotinin, 1 μg/mL pepstatin, 1 μg/mL leupeptin). Forty-microgram aliquots of each homogenate were separated by Bis-Tris Midi gel electrophoresis with 10% polyacrylamide in separate gels. Proteins were then transferred to polyvinylidene difluoride membranes. After blocking, membranes were incubated overnight at 4 °C with polyclonal antibodies targeting pro-ANP (Novus Biologicals, NBP1-97752), corin (Genetex, GTX64508), and PCSK6 (Novus Biologicals, H00005046-A01). Detection of GAPDH (Genetex, GTX100118) was performed as a loading control. The blots were imaged by a densitometer (GE Amersham Imager-680) and the optical densities of the bands were measured and normalized relative to an internal control in Multi Gauge V3.0 software (FUJIFILM).

IS Treatment of Rat and Human Atrial Myofibroblasts

To evaluate the effects of uremic toxin accumulation on cardiac corin expression, we employed IS because it has a well-established cardiac remodeling induction effect.[16,17] At concentrations in the range of 3 to 300 μmol/L, IS increases neonatal rat cardiac fibroblast collagen synthesis and myocyte hypertrophy.[18] At concentrations of 0.1, 1, and 300 μmol/L, IS downregulates potassium current channel protein phosphorylation

and potassium current activity in H9c2 cells.[19] At concentrations >1000 μmol/L, IS induces apoptosis in H9C2 cardiomyocytes.[20] Based on the aforementioned findings, we used IS concentrations in the range of 50 to 400 μmol/L to evaluate its effects on corin/PCSK6 expression in H9c2 cells and human atrial myofibroblasts. Rat H9c2 cardiomyoblasts were cultured in DMEM (ATCC 30-2002) supplemented with 10% fetal bovine serum, as previously prescribed.[21] Human atrial myofibroblasts were obtained from outgrowths of auricle biopsies from cardiac surgery patients who underwent cardiopulmonary bypass.[16,22] On reaching 80% confluence, culture media were replaced with serum-free DMEM. After 8 hours, cells were stimulated with IS for 24 hours.

Serine Protease Activity and Pro-ANP Processing Assays

Serine protease activity was measured with a commercial kit (ImmunoChemistry Technologies, FAM-FLISP Kits #950). To quantify pro-ANP processing activity of corin in mouse heart tissue, human embryonic kidney 293 cells were transfected with a plasmid encoding modeled human pro-ANP tagged with V5 at the COOH terminus to facilitate detection. Seventy-two hours after transfection, conditioned medium containing recombinant V5-tagged pro-ANP was collected and mixed with an equal amount of heart tissue cell membrane extracted from mice (control and CKD models). After 8 hours at 37 °C, the mixture was concentrated with centrifugal filter units (Amicon Ultra-0.5 mL 3–100 kDa, Centrifugal Filters). Levels of V5-tagged pro-ANP and V5-tagged ANP present in the mixtures were quantified in Western blots performed with anti-V5 primary antibody (Invitrogen, R960-25). Corin activity was determined based on the ratio of ANP to pro-ANP after standardizing corin concentrations.[23] The corin concentration in each sample's membrane fraction was determined with a commercial ELISA kit (MyBioSource, MBS2023262).

Statistical Analysis

All statistical analyses were performed in SPSS version 26 software (SPSS Inc). Variable normality was evaluated with the Shapiro-Wilks test. All human data are presented as medians (25th–75th percentile) or number (percentage) values. Mann–Whitney *U* test or the Fisher exact test were used to detect differences between group pairs. Comparisons among ≥3 groups were performed with Kruskal-Wallis tests. Quantile (median) regression analysis was used to examine independent predictors of serum corin levels. Otherwise, Student *t* test and linear regression were used in the animal and cell studies. $P<0.05$ were considered statistically significant.

RESULTS

Comparison of Characteristics Between Patients With Versus Without CKD

Among the 543 patients enrolled in this study, 359 (66.1%) had CKD. The baseline characteristics of our patients with CKD and without CKD are reported in Table 1. Compared with the non-CKD group (n=184), patients with CKD were older, more likely to be women, and had a higher prevalence of diabetes, chronic heart failure, and proteinuria. Patients with CKD were also more likely to be taking renin-angiotensin-aldosterone system blockers, β-blockers, and calcium channel blockers. Compared with patients without CKD, our patients with CKD had a greater left ventricular diameter and a higher prevalence of Left ventricular hypertrophy. Compared with individuals with preserved renal function, the patients with CKD exhibited higher levels of uric acid, N-terminal pro-B-type natriuretic peptide, N-terminal pro-ANP, and corin, and had lower hemoglobin levels.

Factors Related to Serum Corin Levels in Human Patients

The results of univariate and multivariate analyses conducted to identify factors that are significantly related to serum corin levels are reported. For patients without CKD (Table 2), age (R=–0.005), body mass index (R=0.009), diastolic BP (R=0.007), history of myocardial infarction (R=–0.714), white blood cell count (R=–0.023), and LVMI (R=–0.001) were found to be independent determinants of serum corin levels. For patients with CKD (Table 3), being a woman (R=–0.146), statin use (R=0.084), serum triglyceride level (R<–0.001), and estimated glomerular filtration rate (R=–0.003) were found to be independent determinants of serum corin levels. More advanced CKD was associated with a more elevated serum corin level (Figure 1A). Serum corin levels correlated negatively with LMVI in the non-CKD group but not in the CKD group (Figure 1B and 1C).

Cardiac Hypertrophy and Fibrosis in CKD Mice

Compared with sham controls, CKD mice had higher serum urea nitrogen and creatinine levels (Figure S1A and S1B). The CKD mice showed body weight loss over the first few postoperative weeks that normalized by week 7 (Figure S1C) and they showed a higher BP in the early stages following surgery (Figure S1D). Echocardiography revealed increased LVMI in the CKD mice with a similar ejection fraction for the 2 groups (Figure S1E and S1F). After euthanization, cardiac hypertrophy and increased heart-weight index values were observed in the CKD mice (Figure 2A and 2B). Compared with the non-CKD group, the CKD group had larger cardiomyocytes and larger areas of

Table 1. Comparison of Baseline Characteristics Between Patients With Versus Without CKD

Characteristic	Total	Non-CKD	CKD*	*P* value
Age, y	70 (59–80)	63 (55–73)	74 (61–82)	<0.001
Men, n (%)	197 (36.3)	102 (55.4)	95 (26.5)	<0.001
BMI, kg/m^2	25.2 (22.9–28.2)	25.8 (23.2–28.6)	24.9 (22.9–28.1)	0.154
Systolic BP, mm Hg	152 (134–175)	150 (125–165)	154 (137–178)	0.065
Diastolic BP, mm Hg	75 (68–85)	76 (70–87)	74 (67–85)	0.081
Medical history				
Diabetes	195 (35.9)	46 (25)	149 (41.5)	<0.001
Hypertension	358 (65.9)	111 (60.3)	247 (68.9)	0.056
Chronic heart failure	61 (11.2)	12 (6.5)	49 (13.6)	0.014
Stroke	34 (6.3)	4 (2.2)	30 (8.4)	0.004
Myocardial infarction	45 (8.3)	2 (1.1)	43 (11.9)	<0.001
Medications				
Antiplatelet	304 (56)	100 (54.3)	204 (56.8)	0.585
RAAS blocker	155 (28.5)	36 (19.6)	119 (33.1)	<0.001
β-Blocker	143 (26.3)	30 (16.3)	113 (31.5)	<0.001
Ca^{2+} channel blocker	168 (30.9)	32 (17.4)	136 (37.9)	<0.001
Statin	174 (32)	45 (24.5)	129 (35.9)	0.07
Laboratory data				
WBC count, ×10^3/μL	6.7 (5.5–7.9)	6.8 (5.7–8.4)	6.6 (5.3–7.8)	0.053
Hemoglobin, g/dL	12.6 (11.1–13.8)	12.9 (11.9–13.9)	12.1 (10.6–13.7)	<0.001
Fasting glucose, mg/dL	104 (93–129)	101 (91–122)	106 (96–131)	0.109
Triglyceride, mg/dL	109 (76–156)	106 (80–152)	111 (75–161)	0.487
Total cholesterol, mg/dL	163 (142–189)	169 (147–194)	160 (138–187)	0.015
eGFR, mL/min per 1.73 m^2	49.2 (30.9–72.1)	84.5 (70.6–91.9)	36.7 (20.4–49.1)	<0.001
Uric acid, mg/dL	6.2 (5–7.4)	5.3 (4.3–6.6)	6.7 (5.4–7.8)	<0.001
Proteinuria	127 (29.9)	22 (13.3)	105 (40.7)	<0.001
NT-proBNP, pg/mL	370.1 (99.4–1483.3)	142.1 (58–542.7)	542.5 (144.7–2258)	<0.001
NT-proANP, ng/mL	10.6 (5.4–24.9)	5.8 (2.9–10.6)	14.8 (7.3–33.5)	<0.001
ANP, pg/mL	70.4 (29.3–176.6)	69.8 (28.5–177.4)	70.4 (29.6–176.6)	0.928
Corin, pg/mL	1153.2 (815.7–1560.2)	935.6 (669.2–1229.2)	1287.5 (945.6–1744.9)	<0.001
Cardiac echo				
LA diameter, mm	40 (35–45)	37 (32–44)	40 (36–45)	0.004
LVMI, g/m^2	112.9 (90.6–140.1)	106.2 (83.2–136.4)	115.9 (92.9–140.6)	0.111
LVH, n (%)	169 (50.9)	33 (40.7)	136 (54.2)	0.041
LVEF. %	58 (52–66)	57 (52–62)	58.7 (52–66)	0.287
RSVP, mm Hg	31 (27–39)	30 (27–35)	32 (27–41)	0.247

Data are presented as median (25th–75th percentile) or number (percentage). ANP indicates atrial natriuretic peptide; BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; LA, left atrial; LVEF, left ventricular ejection fraction; LVH, left ventricular hypertrophy; LVMI, left ventricular mass index; NT-proANP, N-terminal proatrial natriuretic peptide; NT-proBNP, N-terminal pro-B-type natriuretic peptide; RAAS, renin-angiotensin-aldosterone system; RVSP, right ventricular systolic pressure; and WBC, white blood cell.

*Total: 543; without chronic kidney disease (CKD): 184; and with CKD: 359 (including 233 stage 3, 49 stage 4, and 77 stage 5).

cardiac fibrosis (Figure 2C through 2F), greater expression of genes underlying cardiac hypertrophy (*Myh7b*; Figure 2G) and fibrosis (*Col1a1*; Figure 2H), and larger areas of renal fibrosis (Figure S2B and S2C).

Corin Expression and Activity in CKD Mice

Similar to our finding in human patients with CKD, we observed significantly elevated serum corin levels in CKD mice compared with control mice (Figure 3A). They showed elevated corin expression in cardiac tissue at both mRNA (Figure 3B) and protein (Figure 3C and 3D) levels. Corin gene expression was slightly lower 8 weeks postoperatively than it was 4 weeks postoperatively (P=0.01). Immunohistochemistry analysis confirmed that the CKD mice had increased corin expression in the cytoplasm of myocardial tissues (Figure 3E and 3F).

Table 2. Correlation of Corin Concentration (Log Transformed) With Factors in Patients Without CKD

Characteristic	Univariate			Multivariate*		
	R	95% CI	*P* value	*R*	95% CI	*P* value
Age, y	−0.005	−0.008 to −0.002	<0.001	−0.005	−0.008 to −0.002	<0.001
Men, n (%)	0.148	0.073 to 0.222	<0.001	0.033	−0.062 to 0.128	0.495
BMI, kg/m^2	0.13	0.005 to 0.02	<0.001	0.009	0.001 to 0.017	0.026
Systolic BP, mm Hg	0.001	−0.001 to 0.003	0.346			
Diastolic BP, mm Hg	0.009	0.005 to 0.013	<0.001	0.007	0.003 to 0.011	0.001
Medical history						
Diabetes	0.053	−0.029 to 0.134	0.208			
Hypertension	0.091	0.019 to 0.162	0.014	−0.05	−0.126 to 0.026	0.197
Chronic heart failure	−0.049	−0.194 to 0.097	0.51			
Stroke	0.000	−0.246 to 0.246	1.000			
Myocardial infarction	−1.182	−1.527 to −0.837	<0.001	−0.714	−0.956 to −0.473	<0.001
Medications						
Antiplatelet	−0.037	−0.111 to 0.037	0.323			
RAAS blocker	−0.053	−0.15 to 0.044	0.283			
β-Blocker	0.027	−0.069 to 0.122	0.58			
Ca^{2+} channel blocker	0.081	−0.015 to 0.177	0.097			
Statin	0.017	−0.068 to 0.101	0.701			
Laboratory data						
WBC count, ×10^3/μL	−0.015	−0.03 to −0.001	0.039	−0.023	−0.037 to 0.01	0.001
Hemoglobin, g/dL	0.031	0.002 to 0.059	0.035	0.011	−0.015 to 0.037	0.392
Fasting glucose, mg/dL	0.001	0.000 to 0.002	0.092			
Triglyceride, mg/dL	0.000	−0.00002 to 0.001	0.065			
Total cholesterol, mg/dL	0.00005	−0.001 to 0.001	0.921			
eGFR, mL/min per 1.73 m^2	−0.005	−0.007 to −0.003	<0.001	−0.001	−0.004 to 0.002	0.449
Uric acid, mg/dL	0.033	0.01 to 0.056	0.005	0.013	−0.009 to 0.036	0.234
Proteinuria	0.000	−0.111 to 0.111	1.000			
Log NT-proBNP, pg/mL	−0.067	−0.143 to 0.007	0.078			
Log NT-proANP, ng/mL	−0.074	−0.174 to 0.026	0.145			
Log ANP, pg/mL	−0.088	−0.162 to −0.014	0.02	−0.033	−0.1 to 0.034	0.327
Cardiac echo						
LA diameter, mm	−0.004	−0.012 to 0.003	0.26			
LVMI, g/m^2	−0.001	−0.002 to 0.000	0.013	−0.001	−0.001 to −0.00007	0.026
LVH, %	−0.103	−0.24 to 0.033	0.135			
LVEF, %	0.000	−0.006 to 0.006	0.923			
RSVP, mm Hg	−0.002	−0.008 to 0.003	−0.467			

ANP indicates atrial natriuretic peptide; BP, blood pressure; BMI, body mass index; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; LA, left atrial; LVEF, left ventricular ejection fraction; LVH, left ventricular hypertrophy; LVMI, left ventricular mass index; NT-proANP, N-terminal proatrial natriuretic peptide; NT-proBNP, N-terminal pro-B-type natriuretic peptide; RAAS, renin-angiotensin-aldosterone system; RVSP, right ventricular systolic pressure; and WBC, white blood cell.

*Only significant (P<0.05) independent determinants from univariate analysis included in multivariate regression analysis.

Interestingly, the rate of pro-ANP to ANP conversion was found to be reduced in the CKD group, compared with the control group, despite the former having an increase in corin expression (Figure 3G and 3H). Moreover, in contrast to our findings in the heart, corin expression in the kidney was lower in CKD mice than in control mice (Figure S2D and S2E).

Uremic Toxin Effects on Cardiomyoblasts and Heart Tissues

Serum IS levels were greater in CKD mice than in the sham control group (Figure 4A). Serum corin levels in mice correlated with their serum IS concentrations (R=0.806, P<0.001) (Figure 4B).

Table 3. Correlation of Corin Concentration (Log Transformed) With Factors in Patients With CKD

Characteristic	Univariate			Multivariate*		
	R	95% CI	*P* value	*R*	95% CI	*P* value
Age, y	−0.004	−0.006 to −0.002	<0.001	−0.001	−0.003 to 0.002	0.518
Men, n (%)	−0.193	−0.249 to −0.137	<0.001	−0.146	−0.207 to −0.085	<0.001
BMI, kg/m^2	0.006	0.000 to 0.013	0.066			
Systolic BP, mm Hg	0.000	−0.001 to 0.001	0.382			
Diastolic BP, mm Hg	0.002	0.000 to 0.004	0.094			
Medical history						
Diabetes	0.032	−0.019 to 0.083	0.213			
Hypertension	−0.072	−0.128 to −0.16	0.012	−0.037	−0.099 to 0.025	0.238
Chronic heart failure	−0.033	−0.111 to 0.045	0.407			
Stroke	−0.027	−0.123 to 0.068	0.577			
Myocardial infarction	0.051	−0.033 to 0.134	0.234			
Medications						
Antiplatelet	0.041	−0.013 to 0.095	0.134			
RAAS blocker	0.027	−0.03 to 0.084	0.354			
β-Blocker	0.06	0.003 to 0.117	0.038	−0.015	−0.075 to 0.045	0.633
Ca^{2+} channel blocker	0.096	0.042 to 0.15	<0.001	−0.004	−0.011 to 0.02	0.588
Statin	0.101	0.046 to 0.156	<0.001	0.084	0.023 to 0.144	0.007
Laboratory data						
WBC count, ×10^3/μL	−0.009	−0.023 to 0.004	0.186			
Hemoglobin, g/dL	−0.015	−0.028 to −0.002	0.02	0.004	−0.011 to 0.02	0.588
Fasting glucose, mg/dL	−0.00008	−0.001 to 0.001	0.979			
Triglycerides, mg/dL	0.000	0.00002 to 0.001	0.037	0.000	−0.001 to −0.00004	0.029
Total cholesterol, mg/dL	0.001	0.000 to 0.001	0.02	0.000	0.000 to 0.001	0.224
eGFR, mL/min per 1.73 m^2	−0.004	−0.005 to −0.003	<0.001	−0.003	−0.005 to −0.001	0.002
Uric acid, mg/dL	0.019	0.005 to 0.032	0.007	0.003	−0.012 to 0.017	0.724
Proteinuria	0.047	−0.011 to 0.104	0.111			
Log NT-proBNP, pg/mL	−0.042	−0.089 to 0.004	0.073			
Log NT-proANP, ng/mL	0.093	0.027 to 0.159	0.006	−0.1	−0.093 to 0.073	0.807
Log ANP, pg/mL	0.079	0.022 to 0.135	0.006	0.022	−0.044 to 0.088	0.503
Cardiac echo						
LA diameter, mm	−0.001	−0.007 to 0.004	0.657			
LVMI, g/m^2	0.001	0.000 to 0.001	0.173			
LVH, n (%)	0.02	−0.05 to 0.91	0.575			
LVEF. %	0.005	0.003 to 0.008	<0.001	0.001	−0.002 to 0.003	0.661
RVSP, mm Hg	0.001	−0.002 to 0.004	0.411			

ANP indicates atrial natriuretic peptide; BMI, body mass index; BP, blood pressure; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; LA, left atrial; LVEF, left ventricular ejection fraction; LVH, left ventricular hypertrophy; LVMI, left ventricular mass index; NT-proANP, N-terminal proatrial natriuretic peptide; NT-proBNP, N-terminal pro-B-type natriuretic peptide; RAAS, renin-angiotensin-aldosterone system; RVSP, right ventricular systolic pressure; and WBC, white blood cell.

*Only significant (P<0.05) independent determinants from univariate analysis included in multivariate regression analysis.

In H9c2 cardiomyoblasts, IS exposure induced significantly increased expression of hypertrophy (*Myh7b*) and fibrosis (*Co1a1*) genes (Figure 5A and 5B). IS treatment also stimulated corin gene (Figure 5C) and protein expression (Figure 5D and 5E) in H9c2 cardiomyoblasts. Notably, all 4 IS doses tested (50, 100, 200, and 400 μg) had significant stimulatory effects on the expression of *Co1a1* and corin genes, without evidence of a dose-dependent effect. Similar results were obtained in IS-treated primary cultures of human atrial myofibroblasts (Figure S2). Our serine protease activity assay showed that although net corin activity was increased at a low IS dose (50 μmol/L), it was suppressed at IS concentrations ≥100 μmol/L (Figure 5F).

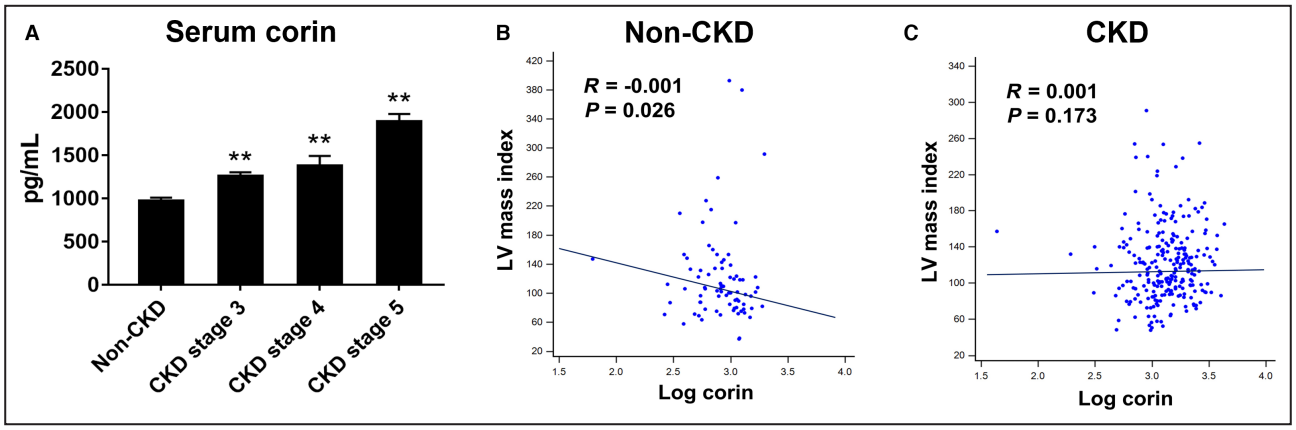

Figure 1. Mean serum corin levels by chronic kidney disease (CKD) stage and associations of corin levels with left ventricular (LV) mass index.
A, Serum corin levels were higher in patients with CKD than in patients without CKD, and, among patients with CKD, a trend of increasing levels with stage progression was observed (*P* for trend <0.001). **B** and **C**, Serum corin levels (log) correlated inversely with LV mass index in participants with preserved renal function (*R*=−0.355, *P*=0.001) but not in the patients with CKD. Non-CKD, n=184; CKD stage 3, n=233; CKD stage 4, n=49; CKD stage 5, n=775. **P*<0.05, ***P*<0.001 vs non-CKD.

In H9c2 cardiomyoblasts, *PCSK6* gene expression was downregulated in response to treatment with IS ≥50 μmol/L (Figure 6A), while PCSK6 protein expression was suppressed by IS doses ≥100 μmol/L (Figure 6B). Compared with levels observed in control mice, PCSK6 gene expression was downregulated at 8 weeks and PCSK6 protein expression was suppressed at 4 weeks and 8 weeks after the final 5/6 nephrectomy operation in CKD mice (Figure 6C and 6D).

DISCUSSION

In this study, our patients with CKD were found to have higher serum levels of corin and pro-ANP than our patients without CKD. Furthermore, we observed that cardiac corin was upregulated, while net corin activity was suppressed, in the CKD model mice. Corin gene expression in CKD mice was slightly lower at 8 weeks postoperatively than at 4 weeks, possibly because of increased cardiac fibrosis reducing the number of

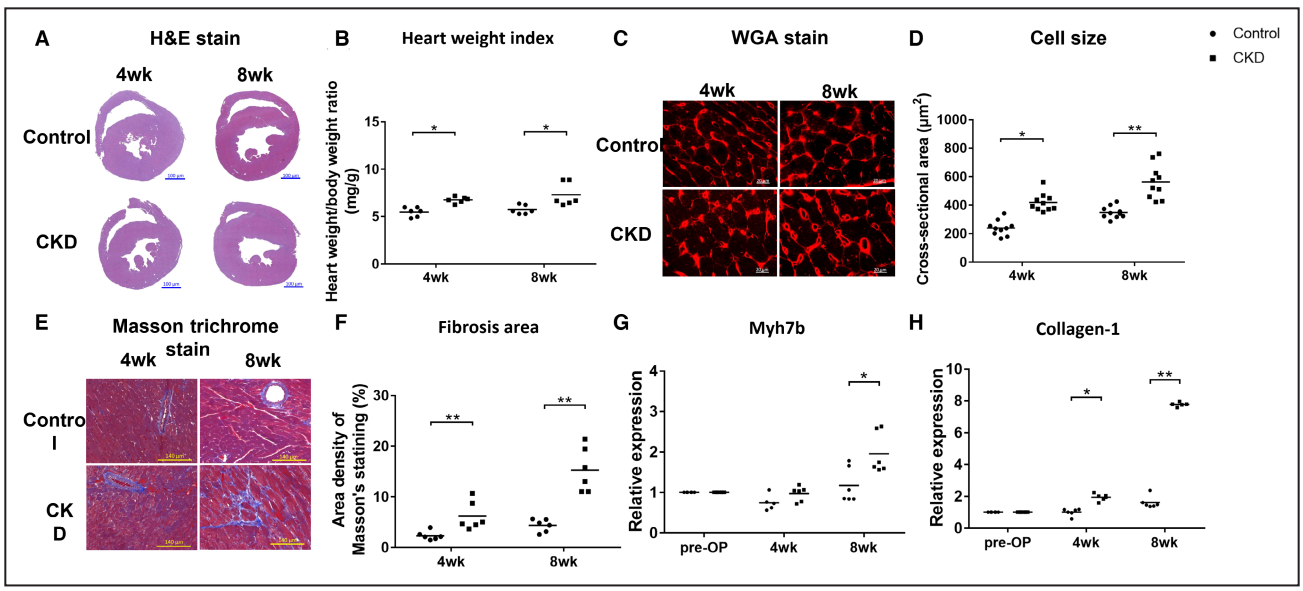

Figure 2. Chronic kidney disease (CKD) mice develop cardiac hypertrophy and fibrosis.
A, Representative cross-sections of the left ventricle in CKD and control mice. **B**, Heart weight ratios by group. **C** and **D**, Size analysis of wheat germ agglutinin (WGA)–stained cardiomyocytes revealed that CKD hearts had larger cardiomyocytes than controls. **E** and **F**, Analyses of Masson trichrome–stained left ventricles showed that CKD mice showed greater fibrosis development than did controls. **G** and **H**, The expression of hypertrophy (*Myh7b*) and fibrosis (*Col1a1*) associated genes were upregulated in CKD mice. **P*<0.05, ***P*<0.001 between groups. H&E indicates hematoxylin and eosin.

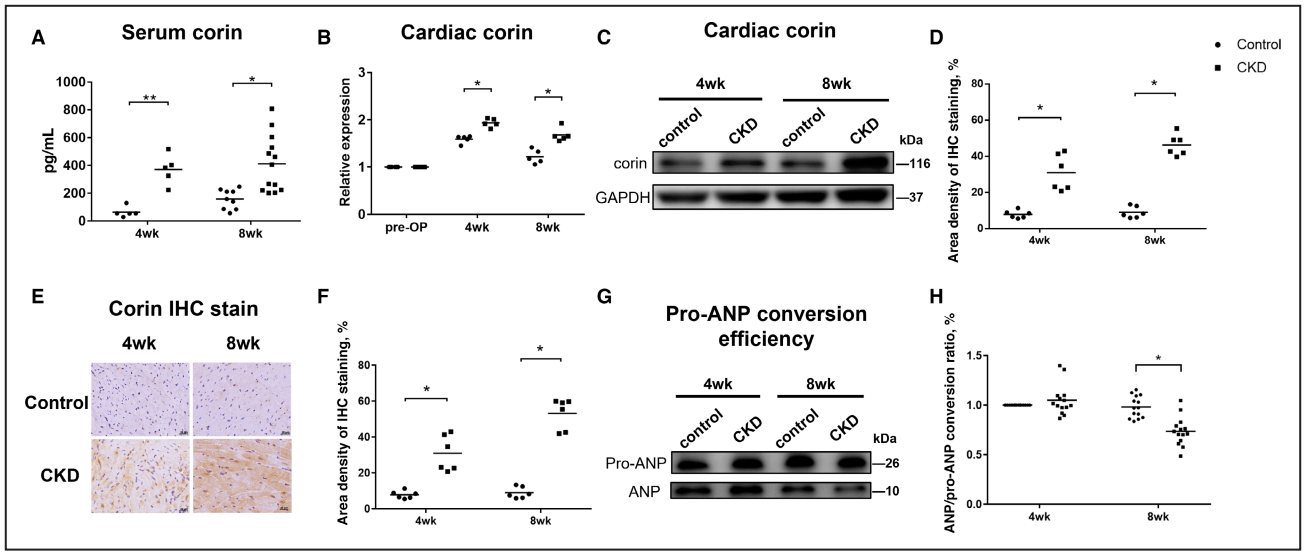

Figure 3. Chronic kidney disease (CKD) model mice exhibit elevated serum corin levels and cardiac corin expression.
A, Serum corin levels before, 4 weeks after, and 8 weeks after 5/6 nephrectomy or sham surgery. **B**, Relative mRNA expression of the corin gene. **C**, Western blot for corin protein quantitation in cardiac tissues. **D**, Corin protein expression levels in sham control mouse and CKD mouse heart tissues 8 weeks postsurgery. **E** and **F**, Immunohistochemistry (IHC) staining for corin shows enhanced cytoplasmic corin labeling in CKD mouse heart tissues 8 weeks postsurgery. **G** and **H**, Proatrial natriuretic peptide conversion assay and associated calculated conversion ratio data (8 weeks postsurgery). *P<0.05, **P<0.001 between groups. ANP indicates atrial natriuretic peptide.

functioning cardiomyocytes.[18,24] IS stimulated corin expression while also suppressing the corin activator PCSK6, resulting in net suppression of corin activity. Taken together, these results indicate that suppression of cardiac corin activity may represent an underappreciated factor in CKD-induced cardiovascular pathology despite the presence of upregulated corin expression.

Previous studies have shown that circulating corin levels are increased in patients with hypertension,[25] hyperglycemia,[26] hyperlipidemia,[27] obesity,[28] and metabolic syndrome,[29] and are decreased in patients with acute myocardial infarction[30] and decreased systolic function.[31] Our study provides evidence showing a link between impaired renal function altered serum corin levels. In addition to having higher serum corin levels than patients without CKD, the determinants of serum corin levels in patients with CKD were also distinct. Notably, a lower estimated glomerular filtration rate was associated with higher serum corin levels in the CKD group. Our cell experiments suggest that

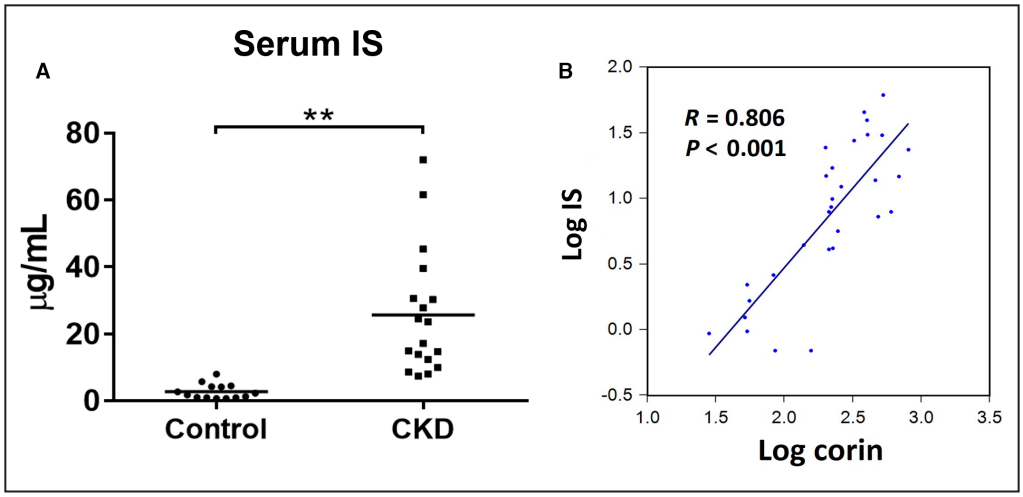

Figure 4. Elevated serum in chronic kidney disease (CKD) mice and a strong association of serum indoxyl sulfate (IS) with serum corin levels.
A, Serum IS levels. **B**, Positive correlation between serum corin and serum IS levels. *P<0.05, **P<0.001 between groups.

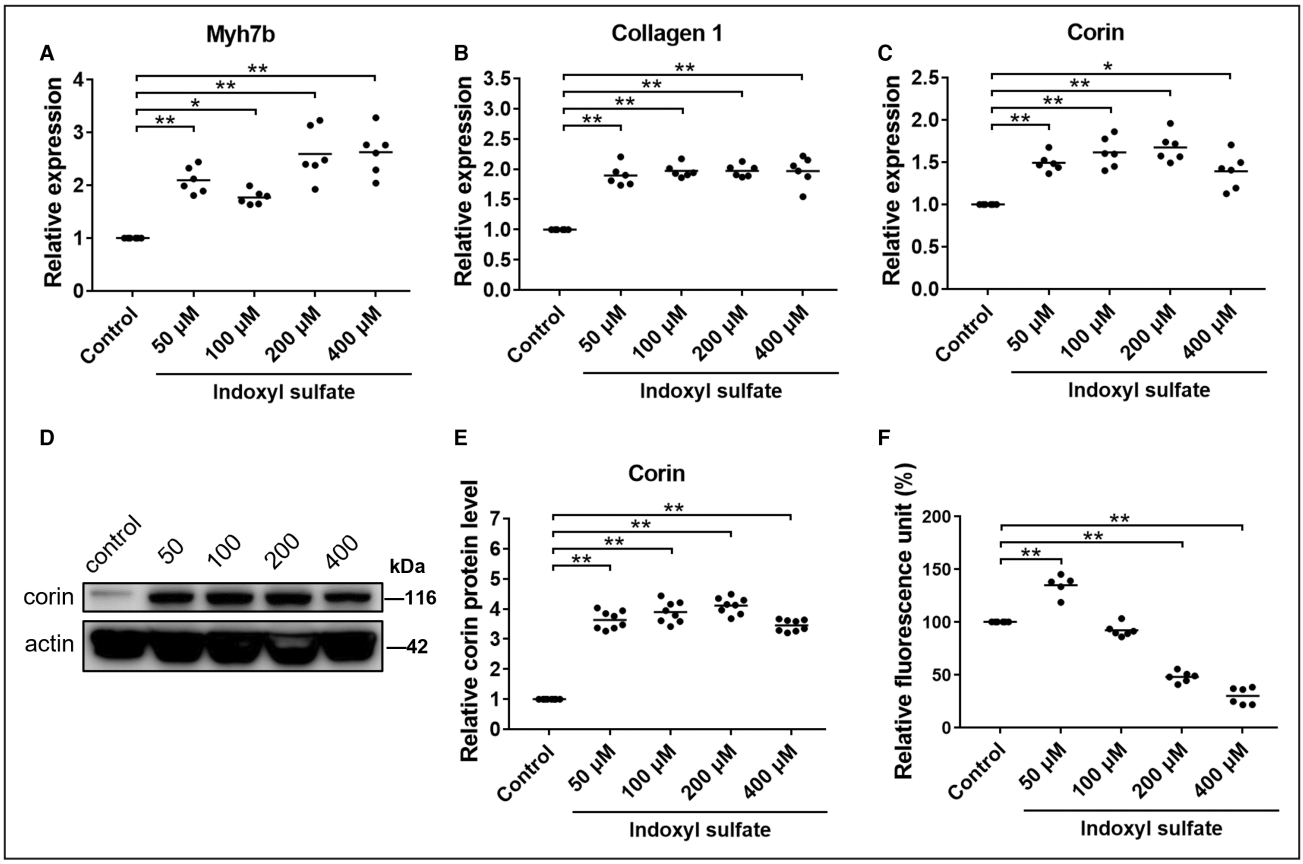

Figure 5. Indoxyl sulfate (IS) stimulates hypertrophy, stimulates fibrosis, and modulates corin expression in H9c2 cardiomyoblasts.
A and **B**, IS upregulates *Myh7b* and *Col1a1* in cultured H9C2 cardiomyoblasts. **C** through **E**, Stimulatory effect of IS on relative corin mRNA (**C**) and protein expression (**D** and **E**). **F**, In contrast with our corin protein expression data in (**E**), corin activity was elevated at a low IS concentration but suppressed at high IS concentrations. **P*<0.05, ***P*<0.001 vs control; all experiments were performed in triplicate.

uremic toxin accumulation, which is a sequela of CKD, may play a role in stimulating cardiac corin expression. Furthermore, we found that statin use was associated with higher serum corin levels in patients with CKD, and this relationship remained significant after adjustment for hyperlipidemia and underlying cardiovascular disease, suggesting that serum corin may be a potential prognostic marker in patients with cardiovascular disease.[10,32–34] The interaction between statin and corin expression has not been studied before and more data are needed to confirm this finding.

The heart is the major source of circulating corin, which is released into circulation via corin autodigestion or a disintegrin and metalloproteinase 10–mediated shedding.[35] Corin is also expressed by epithelial cells of the renal tubule and it has been previously reported that patients with CKD have lower levels of renal corin expression and urinary corin than individuals without CKD.[36] Our data support the conclusion that CKD is associated with decreased renal expression of corin. Knockdown of corin in high salt-treated cortical collecting duct cells has been shown to increase expression of aquaporin 2 channels and β-epithelial Na^+ channels,[37] suggesting that renal corin insufficiency may play a role in CKD-related sodium retention. However, we observed the opposite trend for serum corin and corin expression within cardiac tissues and the reasons for these contrary effects remain to be elucidated.

Patients with CKD have a high prevalence of cardiovascular disease,[38,39] and uremic cardiomyopathy is characterized by both diastolic dysfunction and cardiac hypertrophy.[40] Corin/ANP signaling in heart tissues has been shown to exert antihypertrophy and antifibrosis effects during pathological cardiac remodeling.[5] However, before the present work, cardiac corin expression and activity had not been studied in patients with CKD. We analyzed potential correlations of serum and cardiac corin with remodeling markers (Myh7b and collagen-1), but no significant correlations were seen in either group. However, we did obtain evidence indicating that cardiac corin expression, in association with enhanced cardiac remodeling, was increased in a well-established animal model of CKD. Although cardiac corin overexpression has been

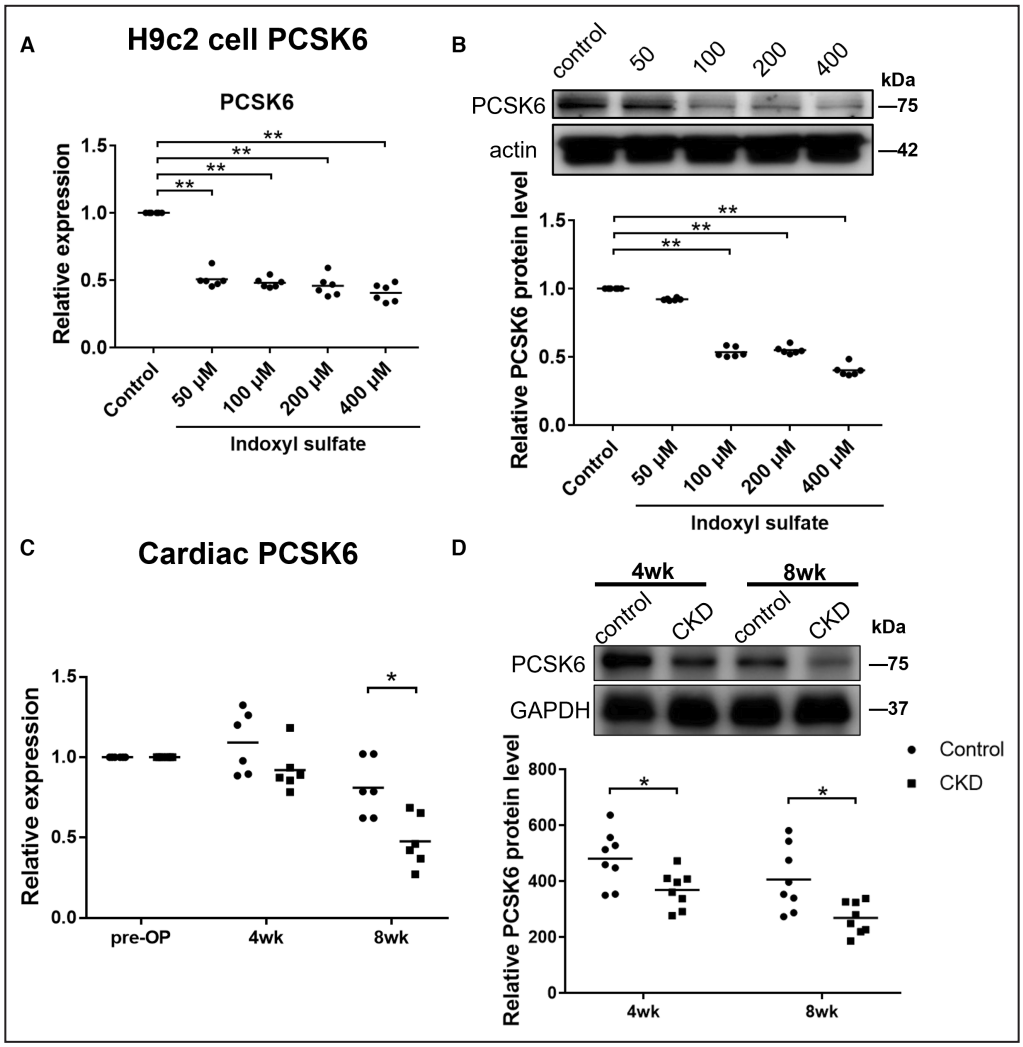

Figure 6. PCSK6 (proprotein convertase subtilisin/kexin type 6) expression is suppressed in H9c2 cardiomyoblasts and chronic kidney disease (CKD) hearts treated with indoxyl sulfate (IS). **A** and **B**, In H9c2 cardiomyoblasts, both PCSK6 mRNA (**A**) and protein (**B**) levels decreased after IS treatment. **C** and **D**, Cardiac expression of PCSK6 mRNA (**C**) and protein (**D**) was suppressed in CKD mice. *P<0.05, **P<0.001 vs control; all experiments were performed in triplicate.

shown to improve cardiac fibrosis, cardiac function, and survival in a mouse model of dilated cardiomyopathy,[41] our data suggest that insufficient cardiac corin activation in CKD mice may be at least a contributing mechanism of CKD-related cardiomyopathy. In our study, we observed increased cardiac corin expression, along with profound cardiac hypertrophy and fibrosis in the CKD mice. Additionally, we found that the corin gene expression level was also upregulated in IS-treated H9c2 cardiomyoblasts. We demonstrated that corin expression in human cardiac myofibroblasts was also increased at an IS dose of 100 μmol/L, but was decreased when the cells were exposed to 400 μmol/L of IS. Because high-dose IS had been shown to induce apoptosis in cardiomyocytes and in human kidney proximal tubular cells,[20,42] we speculate that higher IS levels may suppress metabolic activity in human cardiac myofibroblasts. Further studies are needed to address this finding.

Cardiac corin overexpression has been shown to improve cardiac fibrosis, cardiac function, and survival in a mice model of dilated cardiomyopathy.[41] However, some studies have shown elevated protein levels in failing hearts, without a concomitant increase in corin activity.[14,43] Therefore, insufficient corin activity may contribute to impaired cardiac function during pathological cardiac remodeling. In our human study, serum corin correlated with LVMI in patients with preserved renal function but not in our CKD patient sample, suggesting that corin/ANP pathway activity may be altered in patients with CKD. We further found that corin activity (assessed by measuring pro-ANP conversion rate) and serine protease activity were suppressed in CKD mice and in IS-treated H9c2 cardiomyoblasts,

respectively. These results support the hypothesis that reduced cardiac corin activity may be involved in the pathogenesis of CKD-related cardiomyopathy.

We found that expression of the corin activator[7] PCSK6 was attenuated in the heart tissues of CKD mice and in IS-treated cardiomyocytes. These results suggest that impaired corin/ANP signaling in CKD may be at least partly attributable to decreased PCSK6 expression. Accumulation of IS could have an impairing effect on the PCSK6/corin/ANP pathway. Further studies are needed to evaluate the mechanism mediating IS-induced PCSK6/corin/ANP pathway suppression and whether this pathway should be investigated as a possible treatment target for CKD-related cardiomyopathy.

Although our data show an augmenting effect of 50 μmol/L of IS on the expression of *Co1a1* and corin, we did not observe evidence of an IS dose-dependent response. Analogous apparent plateau stimulation effects on collagen synthesis and cardiac myocyte hypertrophy have been previously reported,[17] suggesting that IS may not have a dose-dependent influence on cardiac fibrosis and hypertrophy. Interestingly, the results from our in vivo and in vitro experiments show that IS stimulates corin expression at low doses, while upregulating corin expression, thus increasing net activity of corin. However, at IS concentrations beyond 100 μmol/L, which are representative of levels observed in patients with CKD,[44,45] cardiac PCSK6 is suppressed and corin activation is decreased. Given that PCSK6 levels did not further decrease with IS concentrations >100 μmol/L, other factors may be involved in corin activation at higher IS concentrations.

This study has several limitations. First, because all of our patients were of Asian ethnicity, it is not known how well our serum corin data would generalize to patients with CKD of other ethnicities. Second, we did not measure serum IS in our human participants and, therefore, do not know whether the serum corin-IS correlation seen in animals would be present in humans. Third, we analyzed corin expression and activity in hearts from our CKD animal models (which undergo cardiac remodeling similar to that seen in patients with CKD), but not in human heart samples.[46,47] Fourth, the accuracy of tail cuff plethysmography (utilized in our animal study) may be reduced by stress-induced hypertension, operator-dependent measurements, or other environmental factors, potentially masking BP differences. Finally, H9c2 cardiomyoblast responses in vitro may not represent cardiomyocyte responses in vivo. To minimize this limitation, experiments were performed in primary cultured human atrial myofibroblasts, and consistent results were obtained (Figure S3).

In conclusion, we found that patients with CKD had elevated serum corin levels and that CKD mouse hearts and in IS-treated cardiomyocytes had suppressed pro-ANP processing rates. These findings suggest that impaired corin activation may be a previously unrecognized pathological mechanism of uremic cardiomyopathy. Downregulation of PCSK6, the main activator of corin, is a potential cause of cardiac corin/ANP pathway impairment in CKD. Thus, restoration of corin activity could represent a potential treatment target for management of CKD-associated cardiovascular abnormalities.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Table S1
Figures S1-S3

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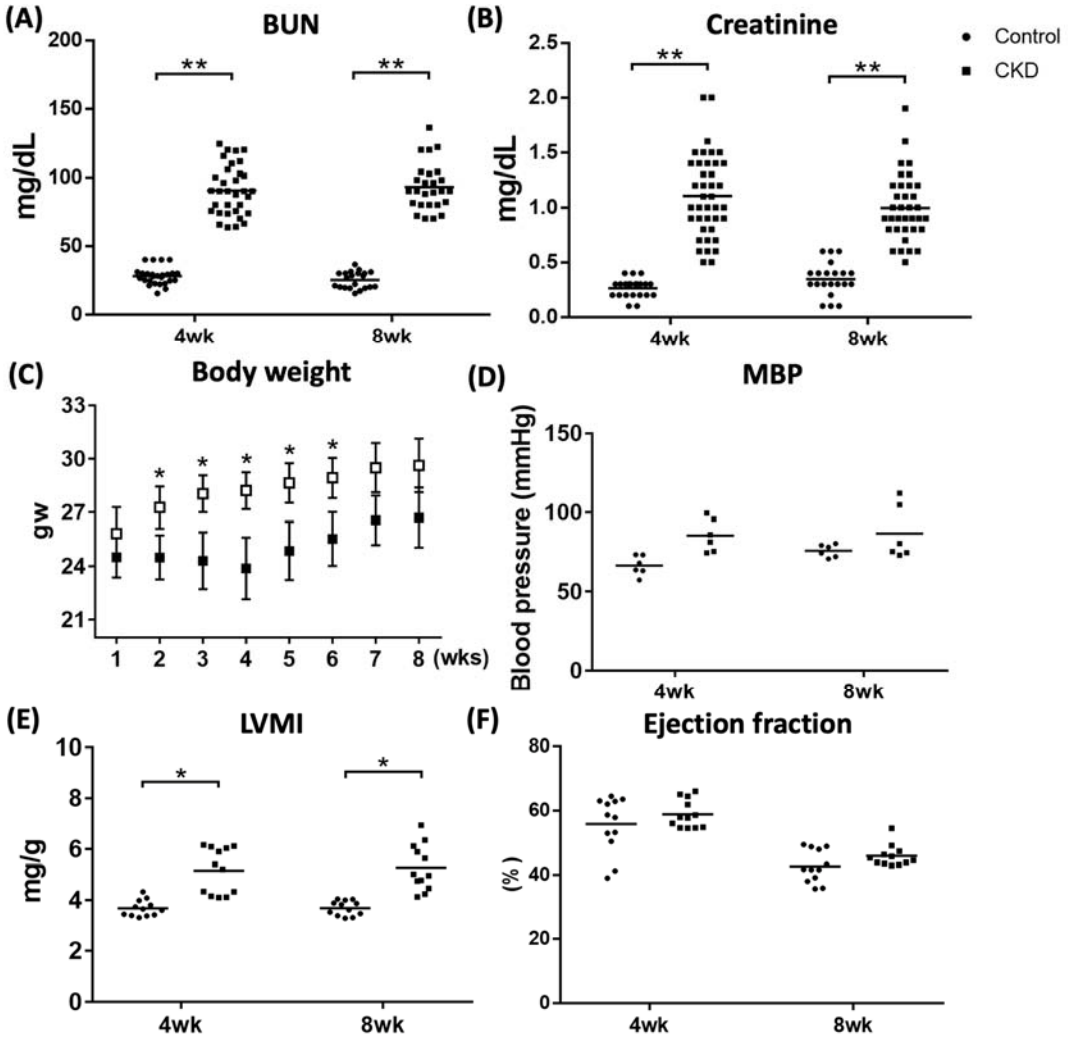
SUPPLEMENTAL MATERIAL

Table S1. Primer sequences used by target gene.

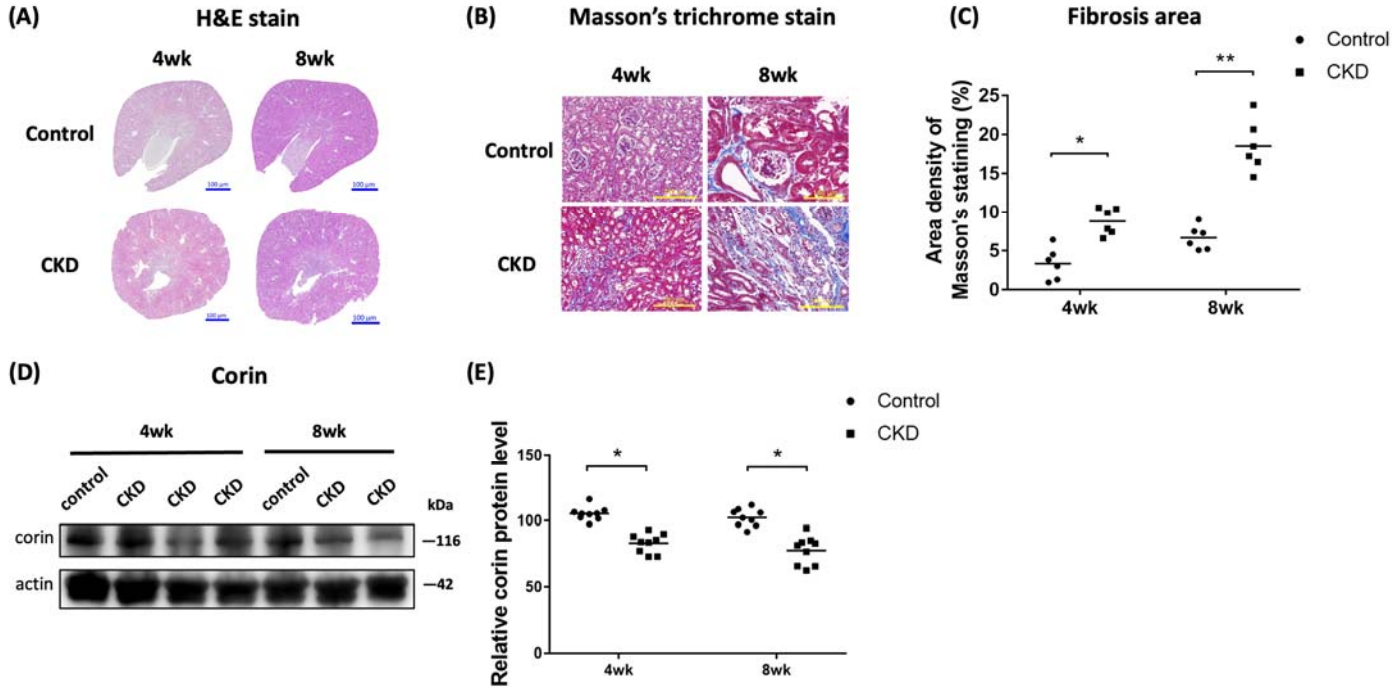
Target gene	Forward (5' – 3')	Reverse (5' – 3')
Mouse		
Corin	TGG AGG TGC CTA TCA GAG AGA	GTG AGA TCC AGT AAC GCA TTC A
PCSK6	CAG GCG CGA AGT GAC TCT C	GAC CGA CAG CGA CTG TTC TT
Myh7b	GCT CCC TCG ACA GTT CTT TAT C	GCT TTC TTG CGC TTC TCT TTC
Nppa	CCA GCA TGG GCT CCT TCT CCA	CCG GAA GCT GTT GCA GCC TAG T
CTGF	CTA CCG ACT GGA AGA CAC ATT T	GTC CCT TAC TTC CTG GCT TTA C
Collagen 1	GCT CCT CTT AGG GGC CAC T	CCA CGT CTC ACC ATT GGG G
TGFβ	CGA AGC GGA CTA CTA TGC TAA A	GTG TGT CCA GGC TCC AAA TA
IL-6	TAG TCC TTC CTA CCC CAA TTT CC	TTG GTC CTT AGC CAC TCC TTC
IL-1β	GTG TGT GAC GTT CCC ATT AGA	TTA GAA ACA GTC CAG CCC ATA C
β-actin	GGC TGT ATT CCC CTC CAT CG	CCA GTT GGT AAC AAT GCC ATG T
Rat		
Corin	CTC CTC ATT CCT GAC TGT TCA C	GGA CTC ATA GCC AGC ACA TAT C
PCSK6	GCT AGC CGA AAG ACC TCT AAT G	TGA GTG TGG AGG CCA AAT G
Myh7b	GTG TGG AGC AGG TGG TAT TT	GGT GAC TTC CCA GAG TGA TTG
Collagen 1	GAC ATC CCT GAA GTC AGC TGC	TCC CTT GGG TCC CTC GAC
IL-6	GAG TTG TGC AAT GGC AAT TC	ACT CCA GAA GAC CAG AGC AG
β-actin	AAG TCC CTC ACC CTC CCA AAA G	AAG CAA TGC TGT CAC CTT CCC
Human		
Corin	AAT GGG AGT GAA CCT TTG GTC A	GTC GGG ATG TGC AGT AGA CA
β-actin	CAT GTA CGT TGC TAT CCA GGC	CTC CTT AAT GTC ACG CAC GAT

Figure S1. Renal function was significantly impaired in the chronic kidney disease (CKD) group. (**A**) BUN levels. (**B**) Creatine levels. (**C**) Following surgery, CKD mice initially lost body weight, but returned to a normal weight range by 7 weeks after 5/6 nephrectomy. (**D**) Mean blood pressure (MBP) appeared to be trending higher in CKD mice 4 weeks postsurgery, but then appeared to be trending toward normalizaton by 8 weeks. (**E, F**) Echocardiography revealed that the CKD group had an increased LVMI but an unchanged ejection fraction compared with control mice.



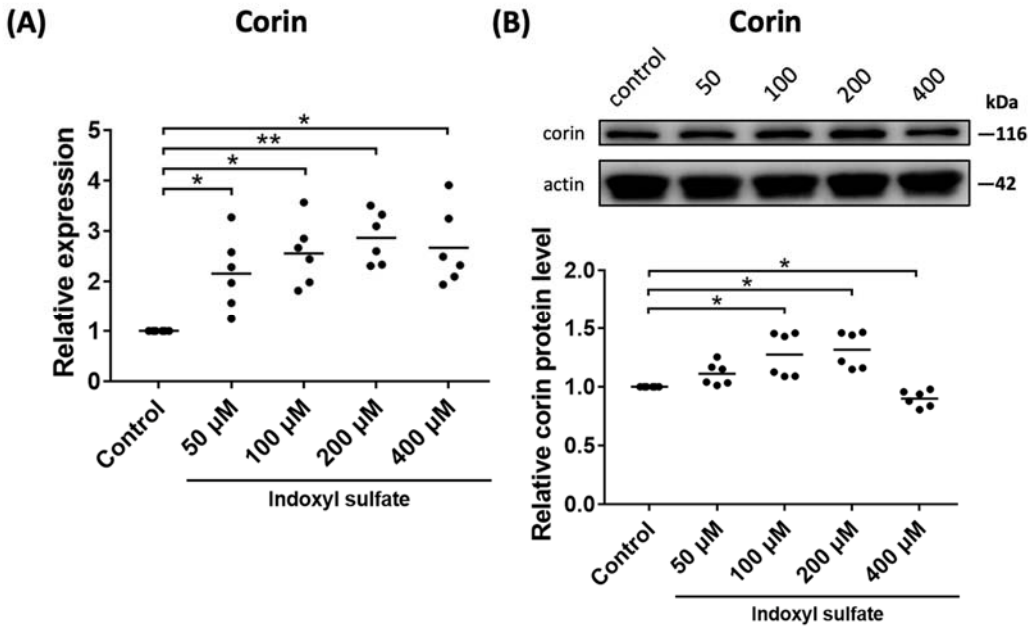
N ≥ 5 per group; *$P < 0.05$, **$P < 0.001$ between groups.

Figure S2. Chronic kidney disease (CKD) mice develop renal fibrosis and corin up-regulation. (**A**) Representative H&E-stained kidney cross-sections from control and CKD mice. (**B**) Representative MT-stained kidney sections from control and CKD mice. (**D, E**) Corin protein expression levels were upregulated in the kidney of CKD mouse kidneys compared to control mouse kidneys.



N ≥ 5 per group; *P < 0.05, **P < 0.001 between groups.

Figure S3. Indoxyl sulfate (IS) effects on corin expression. (**A**) Relative corin mRNA expression determined by real-time qRT-PCR. (**B**) Western blot and associated relative corin protein expression.



*P < 0.05, **P < 0.001 vs. control group.