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Abstract—Blacks represent a high-risk population for salt-sensitive hypertension and heart disease, but the underlying mechanism remains unclear. Corin is a cardiac protease that regulates blood pressure by activating natriuretic peptides. A corin gene variant (T555I/Q568P) was identified in blacks with hypertension and cardiac hypertrophy. In this study, we tested the hypothesis that the corin variant contributes to the hypertensive and cardiac hypertrophic phenotype in vivo. Transgenic mice were generated to express wild-type (WT) or T555I/Q568P variant corin in the heart under the control of α-myosin heavy chain promoter. The mice were crossed into a corin knockout (KO) background to create KO/TgWT and KO/TgV mice that expressed WT or variant corin, respectively, in the heart. Functional studies showed that KO/TgV mice had significantly higher levels of proatrial natriuretic peptide in the heart compared with that in control KO/TgWT mice, indicating that the corin variant was defective in processing natriuretic peptides in vivo. By radiotelemetry, corin KO/TgV mice were found to have hypertension that was sensitive to dietary salt loading. The mice also developed cardiac hypertrophy at 12 to 14 months of age when fed a normal salt diet or at a younger age when fed a high-salt diet. The phenotype of salt-sensitive hypertension and cardiac hypertrophy in KO/TgV mice closely resembles the pathological findings in blacks who carry the corin variant. The results indicate that corin defects may represent an important mechanism in salt-sensitive hypertension and cardiac hypertrophy in blacks. **(*Hypertension*. 2012;60:1352-1358.)** ● **Online Data Supplement**

Key Words: cardiac hypertrophy ■ corin ■ natriuretic peptide ■ mouse models ■ salt-sensitive hypertension

Hypertension is a major risk factor for cardiovascular disease, such as stroke and myocardial infarction. The prevalence of hypertension is particularly high in blacks, but the underlying mechanism is unclear.[1,2] Environmental, socioeconomic, and genetic factors may all contribute to the disease.[3–6] Genome-wide linkage analyses indicate several chromosomal loci that may influence blood pressure in blacks.[7,8] Genetic variants in enzymes in epinephrine synthesis and the renin-angiotensin-aldosterone system also are associated with hypertension in this population.[9–11]

Natriuretic peptides are important for maintaining salt–water balance and normal blood pressure.[12] Corin is a serine protease highly expressed in cardiac myocytes.[13,14] It activates natriuretic peptides, thereby regulating blood pressure and cardiac function.[15,16] In mice, corin deficiency prevented atrial natriuretic peptide (ANP) activation and caused salt-sensitive hypertension.[17,18] Corin-deficient mice had cardiac hypertrophy and poor cardiac function.[17,19,20]

Single nucleotide polymorphisms (T555I/Q568P) in the *CORIN* gene were identified in blacks with hypertension and cardiac hypertrophy.[21] These single nucleotide polymorphisms are located in exon 12 of a minor *CORIN* allele that is more common in blacks than whites (≈12.0% versus <0.2% with 1 or 2 copies of the allele).[21,22] In patients with heart failure, individuals with this minor *CORIN* allele had impaired natriuretic peptide processing and worse clinical outcomes compared with those without this allele.[23] Biochemical studies showed that recombinant corin variant T555I/Q568P had a reduced biological activity, indicating that the single nucleotide polymorphisms may alter corin protein structure and function.[24] The results suggested that corin variant T555I/Q568P may contribute to hypertension and cardiac hypertrophy in blacks.

To test this hypothesis, we generated transgenic (Tg) mice expressing the corin variant in a corin null background and examined corin variant function in vivo and its effect on blood pressure and cardiac morphology. Here we report that the Tg mice had impaired ANP processing in the heart and developed hypertension and cardiac hypertrophy, a phenotype similar to that in blacks with the *CORIN* variant allele. Our results

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indicate that defects in the corin-ANP pathway may be an important contributing factor in hypertension and heart disease in humans, especially in blacks.

Methods

Generation of Tg Mice

Plasmid encoding mouse corin variant T623I/Q636P, corresponding with human corin variant T555I/Q568P, was made by mutagenesis. To generate Tg mice with heart-specific corin expression, corin wild-type (WT) and variant cDNAs were inserted into a plasmid driven by the mouse α-myosin heavy chain promoter (Figure S1A in the online-only Data Supplement).[25] The plasmids were used for pronuclear microinjection to produce Tg mice, which were crossed with corin knockout (KO) mice to generate KO/Tg mice expressing WT or variant corin in the heart in a null background. Heterozygous mice with 1 null allele and 1 WT or variant transgene allele were studied. The animal procedures were approved by the institutional animal care and use committee of the Cleveland Clinic. Detailed methods for making Tg mice are described in the online-only Data Supplement.

Western Blotting and ELISA

To analyze corin protein in hearts, tissues were homogenized in a buffer containing 50 mmol/L of Tris–HCl, pH 8.0, 150 mmol/L of NaCl, 1% Triton X-100 (vol/vol), and a protease inhibitor mixture (1:100 dilution, Sigma). Proteins were analyzed by SDS–PAGE and Western blotting with a polyclonal antibody (Berlex Biosciences).[26] Cardiac pro-ANP expression was analyzed by Western blotting with a polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA). Plasma levels of N-terminal (NT)-pro-ANP were measured by ELISA (Alpco Diagnostics Salem, NH).

Heart Membrane Fractions and Pro-ANP Processing Assay

Cell membrane fractions from hearts were prepared by ultracentrifugation.[26] Cell membrane pellets were resuspended in an NP-40 buffer, and protein concentrations were determined by a Bradford method (Bio-Rad). Recombinant pro-ANP from transfected HEK293 cells was added to the heart membranes and incubated at 37°C over time. Pro-ANP conversion to ANP was analyzed by immunoprecipitation and Western blotting.[27,28] Detailed methods are described in the online-only Data Supplement.

Blood Pressure Measurement

Blood pressure was monitored continuously by radiotelemetry in conscious and unrestrained mice.[25] Detailed methods for radiotelemetry are described in the online-only Data Supplement.

Effects of Dietary Salt on Blood Pressure

Mice were fed normal (0.3% NaCl), or high (4.0% and 8.0% NaCl) salt diets (Harlan Teklad) for 3 weeks. Blood pressure was monitored by radiotelemetry before, during, and after different salt diets.

Histological Analysis of Hearts

Hearts were isolated, weighed, and fixed with 10% formalin. Longitudinal and transversal sections (5 μm in thickness) were stained with hematoxylin and eosin. Computer-assisted measurement (Measure IT, Olympus) at a high magnification (×400) was used to determine the diameter of ≈100 individual cardiac myocytes in 5 randomly selected fields in left ventricular (LV) sections. The analysis was done in a blind fashion.

Statistical Analysis

Data were analyzed using the Prism software (Graph Pad) and presented as mean±SD. Comparisons between 2 groups were made using the Student *t* test. Three or more groups were compared using ANOVA followed by post hoc least significant difference. A *P* value of <0.05 was considered to be statistically significant.

Results

Generation of Corin Tg Mice

To test corin variant function in vivo, we generated Tg mice with cardiac-specific expression of the corin variant and WT control. Transgene copy numbers in founder lines were determined by Southern blotting (Figure S1B). WT and corin variant founders with similar transgene copy numbers were selected to cross with corin KO mice to create KO/Tg mice expressing WT or corin variant (V) in the heart in a null background (Figure S1C). The tissue specificity of Tg corin expression was verified by RT-PCR (Figure S1D). Similar levels of heart-specific corin protein expression were confirmed by Western analysis (Figure S1E and S1F).

Impaired Pro-ANP Processing in Corin KO/TgV Mice

Previously, corin variant T555I/Q568P was found to have a reduced pro-ANP processing activity in cell-based assays.[24] To determine whether the variant had an impaired activity in vivo, pro-ANP levels in hearts from KO/TgWT and KO/TgV mice were analyzed by Western blotting. An ≈20-kDa band, representing pro-ANP, was found to be stronger in intensity in corin KO and KO/TgV mice compared with that in WT and KO/TgWT mice (Figure 1A). Quantitatively, pro-ANP levels in WT and KO/TgWT mice were comparable ($P>0.05$, n=6), whereas the levels in corin KO and KO/TgV mice were ≈3-fold higher than that of WT or KO/TgWT mice ($P<0.01$, n=6) (Figure 1B). On Western blots, ANP was not detected, indicating that it was secreted from the heart once activated from pro-ANP.

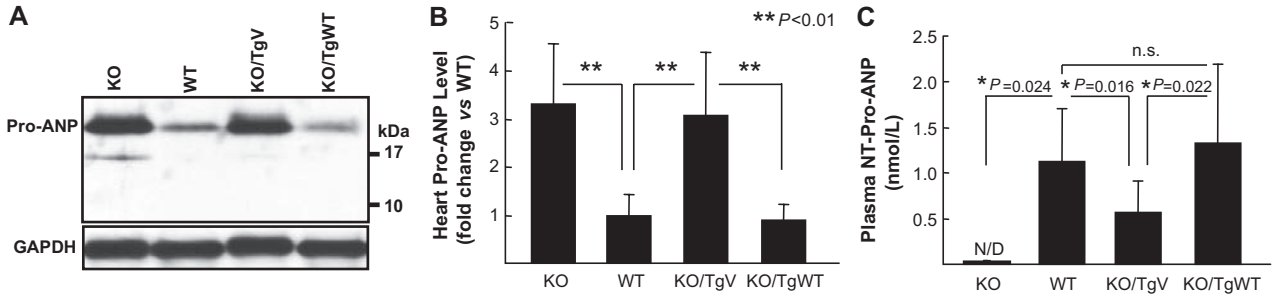

Figure 1. Pro-atrial natriuretic peptide (ANP) expression and processing. **A**, Western analysis of pro-ANP in hearts from corin KO, WT, KO/TgWT, and KO/TgV mice. **B**, Quantitative data of Western blots from 6 independent experiments. **C**, Plasma levels of NT-pro-ANP in corin KO, WT, KO/TgWT, and KO/TgV mice. n≥8 per group. N/D indicates not detectable; n.s., not significant; ANP, atrial natriuretic peptide; KO, knockout; WT, wild type.

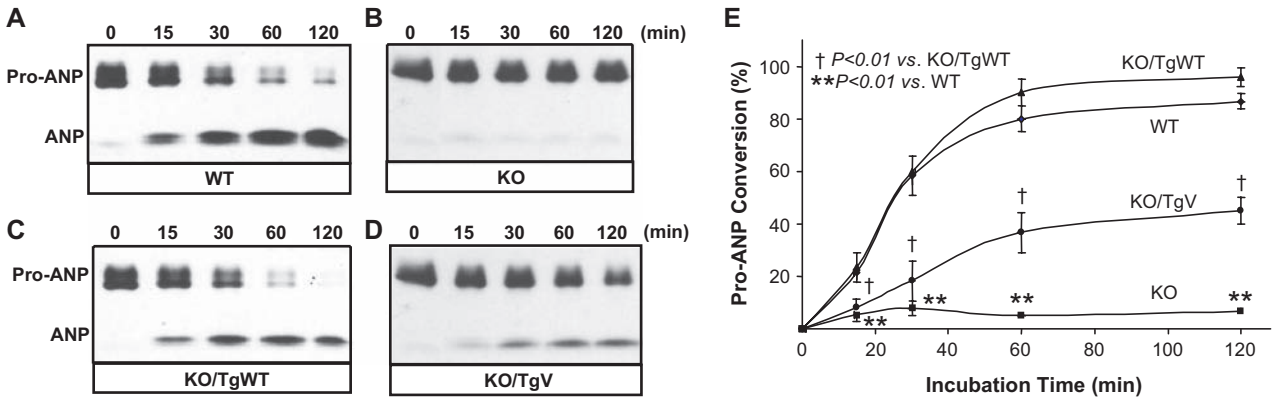

Figure 2. Pro-ANP processing activity in Tg mouse hearts. **A–D**, Pro-ANP processing activity was assayed using membrane fractions from corin WT, KO, KO/TgWT, and KO/TgV mouse hearts. Conversion of pro-ANP to ANP was analyzed by immunoprecipitation and Western blotting. **E**, Quantitative data of Western blots from 3 independent experiments. ANP indicates atrial natriuretic peptide; WT, wild type; KO, knockout.

By ELISA, plasma NT-pro-ANP was measured. The levels in WT mice were 1.13±0.58 nmol/L (n=11) but undetectable in KO mice (n=8) (Figure 1C). In corin KO/TgV mice, the levels were significantly lower than that of WT and KO/TgWT mice (0.57±0.35 versus 1.13±0.58 and 1.33±0.87 nmol/L, respectively; both *P* values <0.05, n=8–10). There was no significant difference between WT and KO/TgWT mice (Figure 1C). The results indicated that detected plasma NT-pro-ANP represented cleaved NT-pro-ANP fragments and that pro-ANP processing was impaired in corin KO/TgV mice.

Pro-ANP Processing by Heart Membranes

Corin is a membrane protein.[28,29] To determine corin activity in hearts, we prepared heart membrane fractions from Tg mice and measured pro-ANP processing activity. The activity was detected in a time-dependent manner in WT but not KO mice (Figure 2A and 2B). A similar activity was observed in KO/TgWT mice (Figure 2C). The activity was significantly reduced in KO/TgV mice (Figure 2D and 2E).

Hypertension in Corin KO/TgV Mice

Corin variant T555I/Q568P was associated with hypertension and cardiac hypertrophy in blacks.[21,30] We measured blood pressure in corin KO/TgWT and KO/TgV mice. On a normal salt (0.3% NaCl) diet, corin KO mice had higher blood pressures than WT mice [systolic (SBP) 124±4 versus 112±3 mm Hg, *P*<0.01; diastolic (DBP) 91±4 versus 83±3 mm Hg, *P*<0.01] (Figure 3). In KO/TgWT mice, both SBP and DBP were restored to normal levels (SBP 113±4 versus 112±3 mm Hg in WT; DBP 83±3 versus 83±3 mm Hg in WT, both *P* values >0.05) (Figure 3). In KO/TgV mice, blood pressures remained high (SBP 121±4 mm Hg; DBP 88±2 mm Hg, *P*<0.01 versus WT or KO/TgWT) (Figure 3).

Salt-Sensitive Hypertension in Corin KO/TgV Mice

We next tested the effects of high-salt diets on blood pressure. On a 4% NaCl diet, SBP in KO/TgV mice increased within a week from 121±2 to 129±1 mm Hg (*P*<0.01) (Figure 4A). Similarly, DBP also increased in these mice (data not shown). Similar salt-sensitive hypertension occurred in corin KO mice (Figure 4A). In contrast, blood pressure did not increase significantly in WT or KO/TgWT mice (Figure 4A). When the mice were switched to the normal salt diet, blood pressure in KO and KO/TgV mice remained high for 2 more weeks (Figure 4A).

When the mice were fed with an 8% NaCl diet, blood pressure in KO/TgV mice increased further (SBP from 120±2 to 137±10 mm Hg, *P*<0.01), which was similar to that in KO mice (Figure 4B). On this high-salt diet, blood pressure in WT and KO/TgWT mice also increased (Figure 4B). When the mice were switched to the normal salt diet, blood pressure in WT and KO/TgWT mice quickly returned to normal levels, whereas that in KO and KO/TgV mice remained high for 3 to 4 weeks (Figure 4B).

Cardiac Hypertrophy in Corin KO/TgV Mice

Previous studies showed that corin KO mice developed cardiac hypertrophy at ≈12 months of age.[17] Consistently, no apparent cardiac hypertrophy was observed in 4-month-old KO/TgV mice (Figure 5A). LV wall thickness increased significantly by 12 to 14 months in these mice (Figure 5B). Such a change was not observed in KO/TgWT mice

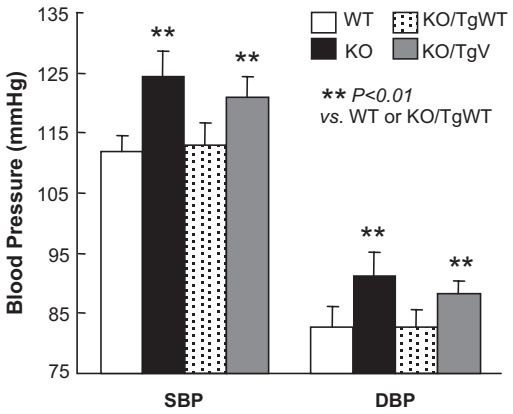

Figure 3. Hypertension in corin KO/TgV mice. SBP and DBP in Tg mice on a normal salt (0.3% NaCl) diet were measured by radiotelemetry. Data are mean±SD from ≥6 mice per group. **P<0.01 vs WT or KO/TgWT mice by 2-way ANOVA. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; WT, wild type; KO, knockout.

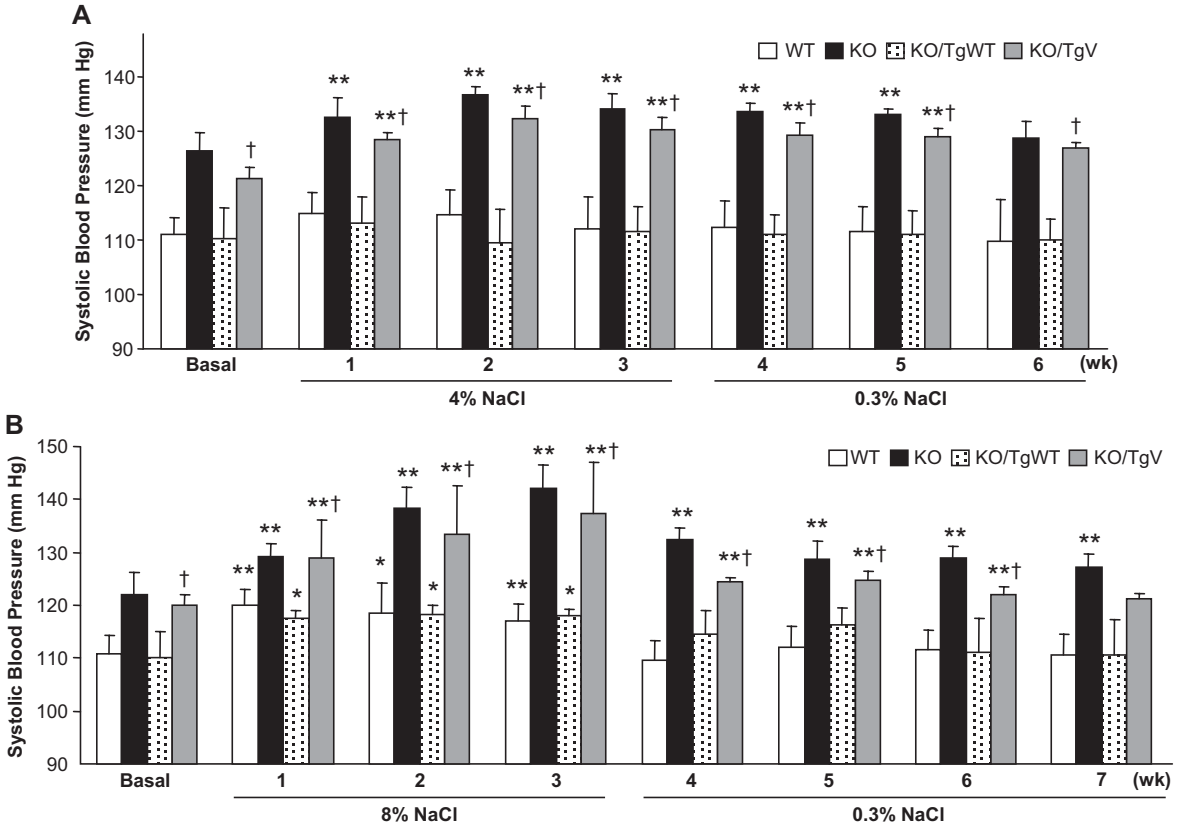

Figure 4. Salt-sensitive hypertension in corin KO/TgV mice. **A**, Tg mice on a 0.3% NaCl diet (basal) were switched to a 4% NaCl diet for 3 weeks (wk) and then back to the 0.3% NaCl diet. SBP data from 6 to 10 mice per group are shown. Corin WT and KO mice were included as controls. **B**, Similar studies were conducted in the Tg mice on an 8% NaCl diet. SBP data from 4 to 6 mice per group are shown. **P*<0.05 or ***P*<0.01 vs. basal of the same genotype; †*P*<0.01 vs. KO/TgWT of the same group by 2-way ANOVA. SBP indicates systolic blood pressure; WT, wild type; KO, knockout.

(Figure 5B). In 12- to 14-month-old KO/TgV mice, LV muscle fibers were much thicker with an average diameter of 20.1±1.5 μm, significantly greater than that in KO/TgWT mice (14.6±1.3 μm, *P*<0.01) (Figure 5B). The ratio of heart weight to body weight or tibia length was significantly greater in 12- to 14-month-old KO/TgV mice compared with that in KO/TgWT mice of similar age (Figure S2A and S2B).

We next tested the effect of high-salt diet on cardiac hypertrophy. When 4-month-old WT, KO, KO/TgWT, and KO/TgV mice, which did not have LV hypertrophy, were fed with an 8% NaCl diet for 3 weeks, LV wall thickness and ratio of heart weight to body weight or tibia length all increased in KO and KO/TgV mice (Figure 6A–D and Figure S3A and S3B). In contrast, these changes were not observed in WT and KO/TgWT mice (Figure 6A–D and Figure S3A and S3B).

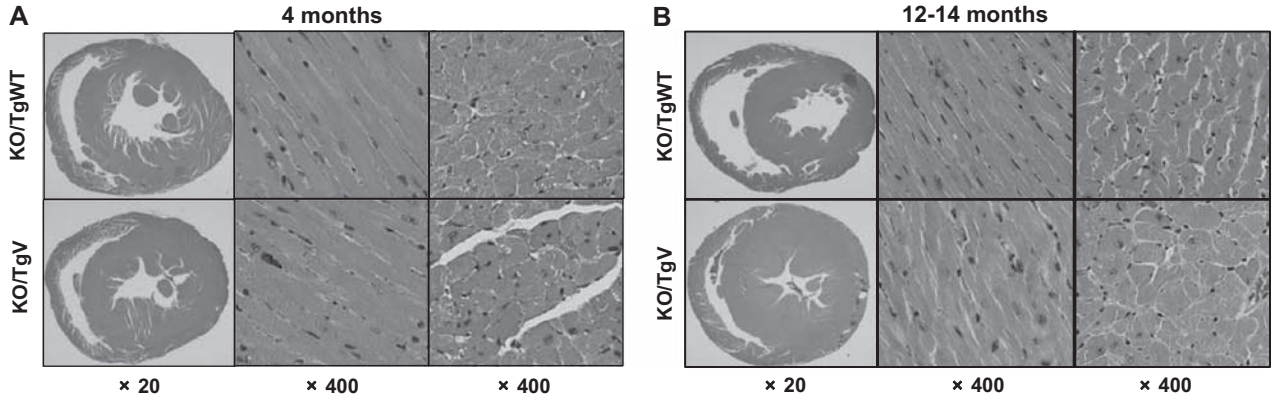

Figure 5. Cardiac hypertrophy in corin KO/TgV mice on normal salt diet. Hematoxylin-eosin-stained heart sections from corin KO/TgWT and KO/TgV mice at 4 months (**A**) or 12 to 14 months (**B**) of age on a 0.3% NaCl diet were shown at low (×20) and high (×400) magnifications. WT indicates wild type; KO, knockout.

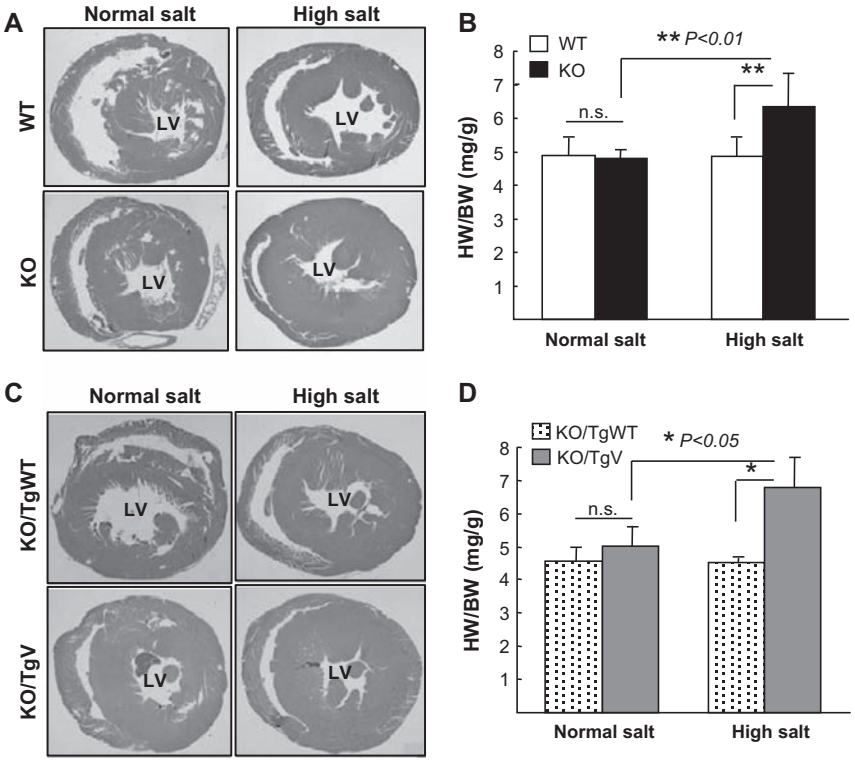

Figure 6. Cardiac hypertrophy in corin KO/TgV mice on high-salt diet. Hematoxylin-eosin-stained heart sections from corin WT and KO (**A**) or KO/TgWT and KO/TgV (**C**) mice at 4 months of age on normal and 8% NaCl diets were shown at a lower (×20) magnification. Ratios of heart weight (HW) to body weight (BW) (**B** and **D**) were calculated from 8 to 10 mice per group. n.s. indicates not significant.

Discussion

Hypertension occurs in all ethnic groups but its high prevalence in blacks is striking.[1,2] Corin is a cardiac protease that regulates blood pressure. Genetic studies have identified corin variant T555I/Q568P in blacks who had hypertension and cardiac hypertrophy.[21,30] In biochemical studies, recombinant corin variant T555I/Q568P had an impaired natriuretic peptide processing activity,[24] suggesting that genetic variations in the *CORIN* gene may reduce corin activity in vivo, thereby contributing to hypertension in blacks.

Mouse models are useful tools to study human genetic variants.[31,32] To test our hypothesis, we generated KO/TgWT and KO/TgV mice that had comparable cardiac corin levels in a corin null background. We found similarly low levels of pro-ANP in hearts from WT and KO/TgWT mice, whereas the levels were much higher in corin KO and KO/TgV mice (Figure 1A and 1B). Consistently, comparable plasma levels of NT-pro-ANP fragments were detected in WT and KO/TgWT mice, whereas the levels were low in KO/TgV mice and undetectable in KO mice (Figure 1C). The results show that pro-ANP processing was restored in the heart in KO/TgWT but not KO/TgV mice, supporting that the corin variant was defective in vivo.

In our previous in vitro studies,[24] the corin variant exhibited impaired zymogen activation and hence reduced activity. Because of the lack of a suitable antibody that recognizes the activated corin protease fragment, we were unable to directly determine corin zymogen activation in mouse hearts. To circumvent this problem, we developed an assay measuring corin activity in heart membrane fractions. The results showed that corin activity was significantly lower in KO/TgV mice than that in KO/TgWT mice (Figure 2). Because corin protein levels were similar in KO/TgWT and KO/TgV mouse hearts (Figure S1E and S1F), reduced corin activity in KO/TgV mouse hearts was probably attributed to impaired corin zymogen activation, consistent with our previous in vitro findings. Recent studies indicated that impaired corin zymogen activation and reduced corin activity may be important in the pathogenesis of heart failure in patients.[26,33,34]

Corin is essential for maintaining normal blood pressure.[35] We found elevated SBP and DBP in corin KO/TgV mice (Figure 3). Moreover, blood pressure in KO/TgV mice was highly sensitive to dietary salt loading, a phenotype similar to that of corin KO mice (Figure 4). Recent studies in mice showed that corin deficiency caused sodium retention in an ENaC-dependent mechanism, which may underlie salt-sensitive hypertension.[17,18,36,37] Blacks are known for high prevalence of salt-sensitive hypertension.[3–5] Population studies show that corin variant T555I/Q568P allele was more common in blacks than in whites.[21] It is possible, therefore, that the corin variant may contribute to high prevalence of salt-sensitive hypertension in blacks.

Natriuretic peptides are shown to have a direct antihypertrophic function in the heart.[38–40] Mice lacking either corin or ANP developed cardiac hypertrophy.[17,19,20,41] In blacks, corin variant T555I/Q568P was associated with severe cardiac hypertrophy.[30] In this study, we showed that mice carrying the corin variant developed significant cardiac hypertrophy either at an older age when on a normal salt diet or at a younger age when on a high-salt diet (Figures 5 and 6). Thus, the results from our mouse model studies helped to establish a link between the corin variant and the cardiac phenotype in vivo.

Perspectives

The results from this study showed that Tg mice expressing the corin variant identified in blacks developed hypertension and cardiac hypertrophy. The phenotype mimics the clinical features in blacks who carry the *CORIN* variant allele. The results provide direct experimental evidence that this *CORIN* allele is defective in vivo, suggesting that the corin variant may contribute to hypertension and heart disease in blacks. Previously, single nucleotide polymorphisms in the genes coding for ANP or its receptor also were reported in patients with hypertension and cardiac hypertrophy.[42,43] Together, these data suggest that defects in the corin-ANP pathway may be an important mechanism in hypertension and cardiac hypertrophy in patients. Most recently, corin and ANP have been found to act locally in the pregnant uterus to regulate spiral artery remodeling, which is critical for preventing pregnancy-induced hypertension.[25] Our findings should encourage more genetic studies to determine whether additional corin gene variants or mutations may play a role in hypertensive disease in patients.

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Disclosures

None.

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Novelty and Significance

What Is New?

- This study shows that Tg mice expressing the corin variant identified in blacks developed salt-sensitive hypertension and cardiac hypertrophy.
- The data provide direct experimental evidence that this CORIN variant allele is defective in vivo.

What Is Relevant?

- Blacks are a high-risk population for salt-sensitive hypertension and heart disease.
- The CORIN variant gene allele is associated with blacks with hypertension and cardiac hypertrophy but its contribution to the disease was unknown.

Summary

These data indicate that corin gene defects may be an important mechanism in salt-sensitive hypertension and cardiac hypertrophy in patients, especially in blacks.

Online Supplemental Data

Salt-Sensitive Hypertension and Cardiac Hypertrophy in Transgenic Mice Expressing a Corin Variant Identified in African Americans

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Running Title: Transgenic Mice Expressing Corin Variant

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Supplemental Methods

Generation of Tg mice

Plasmid expressing mouse corin variant T623I/Q636P was made by mutagenesis using mouse wild-type (WT) corin plasmid as a template. Corin WT and variant cDNAs were inserted into a plasmid with the mouse α-myosin heavy chain (MHC) promoter and a 3' human growth hormone poly(A) site (Figure S1A).[1, 2] The plasmids were used for pronuclear microinjection to produce Tg mice, which were crossed with corin knockout (KO) mice to generate KO/Tg mice expressing WT or variant corin in the heart in a null background.

Tg founder mice were analyzed by Southern blotting. Genomic DNA was extracted from tissues using the DNeasy kit (Qiagen), digested with *Hin*dIII endonuclease, separated in agarose gels and transferred onto nylon membranes, which were hybridized with a digoxigenin-dUTP-labeled probe. The transgene copy number was estimated by comparing with copy number standards.

To examine tissue specific transgene expression, total RNAs were isolated from tissues using TRIzol reagents (Invitrogen) to synthesize first strand cDNAs by SuperScript III reverse transcriptase (Invitrogen). RT-PCR was done using oligonucleotide primers specific for the corin transgene: sense 5'-AAG CCT ATC CCT AAC CCT CTC-3' and antisense 5'-ACA GGA ATA ACA CCA GGC ACT C-3'. Primers for the mouse β-actin gene were used as controls. PCR products were analyzed on 1% agarose gels.

Pro-ANP Processing Assay

Plasmid expressing human pro-ANP was transfected in HEK cells using Lipofectamine 2000 (Invitrogen), as described previously.[3] Cells were cultured at 37°C for 24-48 h. Conditioned medium containing recombinant human pro-ANP was collected, added to the heart membranes, and incubated at 37°C over time. Pro-ANP and ANP in the medium were analyzed by immunoprecipitation and Western blotting. Western blots were developed using enhanced chemiluminescent (ECL) reagents (Denville Scientific) and exposed to X-ray films. The optical density of bands representing pro-ANP and ANP was measured by densitometry, and the

percentage of pro-ANP to ANP conversion was calculated using computer software (Bio-Rad), as described previously.[4]

Blood Pressure Measurement

Blood pressure was monitored by radiotelemetry in conscious and unrestrained mice.[1] Mice were anesthetized with ketamine and xylazine on a 37°C warming pad. A TA11PA-C10 telemetry device (Data Science International) was inserted into the left common carotid artery under a microscope. After the surgery, mice were singly caged and fed with standard diet and water *ad libitum* for ~7 days. Blood pressure was recorded by telemetry receivers (model RPC-1) and the Dataquest System (Data Science International).

Supplemental References

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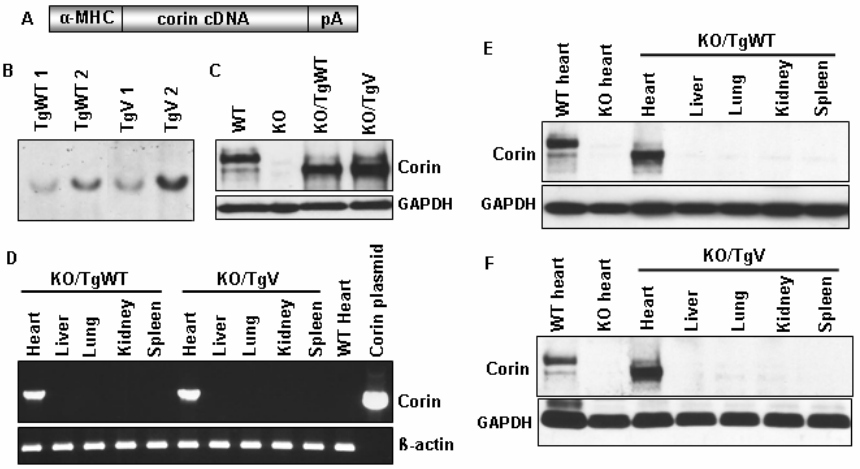
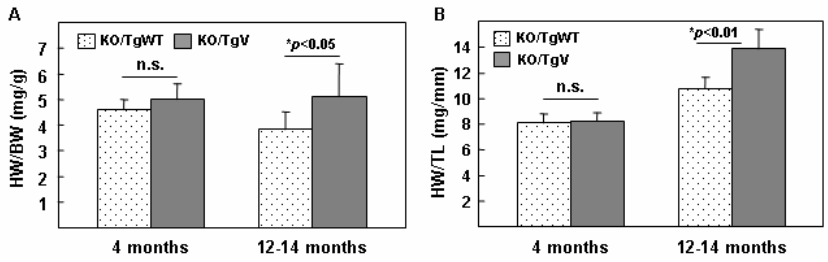
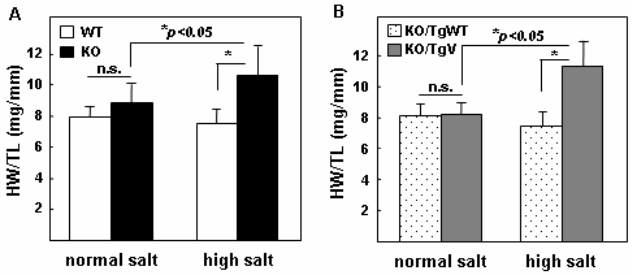
Supplemental Figures and Legends



Supplemental Figure S1. Generation of corin Tg mice. (**A**) Plasmid expressing mouse corin transgene contained a 5' α-MHC promoter and a 3' human growth hormone poly (A) (pA) site. (**B**) Southern analysis of corin transgene in founder mice. WT and corin variant founders with similar transgene copy numbers (TgWT1 and TgV1) were selected to cross with corin KO mice. (**C**) Western analysis of corin protein in hearts from corin WT, KO, KO/TgWT and KO/TgV mice. (**D**) RT-PCR analysis of specific corin transgene expression in the heart. Negative (WT heart) and positive (corin plasmid) controls and β-actin control were included. (**E**, **F**) Western analysis of WT and variant corin proteins in hearts from KO/TgWT and KO/TgV mice. GAPDH control was included. On Western blots, recombinant WT and corin variant migrated slightly faster than endogenous corin (**E**, **F**) due to differences in protein glycosylation (data not shown).



Supplemental Figure S2. **Cardiac hypertrophy in corin KO/TgV mice.** Ratios of heart weight (HW) to body weight (BW) (**A**) or tibia length (TL) (**B**) were calculated in KO/TgWT and KO/TgV mice on a normal salt diet at 4 and 12-14 months of age. Data were from 8-10 mice per group. n.s., not significant.



Supplemental Figure S3. Cardiac hypertrophy in corin KO/TgV mice on high salt diet. Corin WT, KO, KO/TgWT and KO/TgV mice at 4-months of age were on normal or 8% NaCl diets. Ratios of heart weight (HW) to tibia length (TL) (**A, B**) were calculated from 8-10 mice per group. n.s., not significant.