Corin Variant Associated With Hypertension and Cardiac Hypertrophy Exhibits Impaired Zymogen Activation and Natriuretic Peptide Processing Activity

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Abstract—Corin is a cardiac serine protease that acts as the pro–atrial natriuretic peptide (ANP) convertase. Recently, 2 single-nucleotide polymorphisms (SNPs) (T555I and Q568P) in the human corin gene have been identified in genetic epidemiological studies. The minor I555/P568 allele, which is more common in African Americans, is associated with hypertension and cardiac hypertrophy. In this study, we examined the effect of T555I and Q568P amino acid substitutions on corin function. We found that corin frizzled-like domain 2, where T555I/Q568P substitutions occur, was required for efficient pro-ANP processing in functional assays. Mutant corin lacking this domain had 30% (P<0.01) activity compared to that of wild type. Similarly, corin variant T555I/Q568P had a reduced (38%, P<0.01) pro-ANP processing activity compared to that of wild type. The variant also exhibited a low activity (44%, P<0.05) in processing pro–brain natriuretic peptide (BNP). We next examined the biochemical basis for the loss of activity in T555I/Q568P variant and found that the zymogen activation of the corin variant was impaired significantly, as indicated by the absence of the activated protease domain fragment. This finding was confirmed in human embryonic kidney (HEK)293 cells and murine HL-1 cardiomyocytes. Thus, our results show that the corin gene SNPs associated with hypertension and cardiac hypertrophy impair corin zymogen activation and natriuretic peptide processing activity. Our data suggest that corin deficiency may be an important mechanism in hypertensive and heart diseases. (Circ Res. 2008;103:502-508.)

Key Words: natriuretic peptides ■ genetic variants ■ protease

Atrial natriuretic peptide (ANP) is a cardiac hormone that regulates blood pressure and salt-water balance.1-3 The ANP pathway also has an antihypertrophic function in the heart, which is independent of its systemic action on blood pressure.4-7 Corin is a cardiac enzyme of the type II transmembrane serine protease family.8-10 It has a cytoplasmic tail and a transmembrane domain near the N terminus. In its extracellular region, there are 2 frizzled-like domains, 8 LDL receptor (LDLR) repeats, a scavenger receptor–like domain, and a C-terminal trypsin-like protease domain. Corin converts pro-ANP into active ANP,11,12 In mice, corin deficiency prevented pro-ANP activation,13 demonstrating that corin is the physiological pro-ANP convertase. Corin-null mice developed hypertension and cardiac hypertrophy.13 Corin also cleaved pro–brain natriuretic peptide (BNP) in vitro, although the cleavage was less efficient than that for pro-ANP.12

Hypertension is the most common cardiovascular disease, and its prevalence is even higher in African Americans, but the underlying mechanism is unclear.14,15 Recently, 2 nonsynonymous and nonconservative single-nucleotide polymorphisms (SNPs) (T555I and Q568P) are found in the human corin gene. These 2 SNPs are in complete linkage disequilibrium in the population and, as a result, are colocalized in a minor allele (I555/P568).16 Epidemiological studies of large population-based cohorts, including the Dallas Heart Study, the Multi-Ethnic Study of Atherosclerosis (MESA), and the Chicago Genetics of Hypertension Study, have shown that the minor corin 1555/P568 allele is more common in African Americans than in whites (>12% versus <0.2% carrying 1 or more copies of the allele) and associated with an increased risk for hypertension.16 Moreover, this corin minor allele is associated with an enhanced concentric cardiac hypertrophic response to high systolic blood pressure in African Americans from the Dallas Heart and MESA cohorts.17,18 The results suggest that the SNPs may impair corin function, thereby attenuating the antihypertensive and antihypertrophic actions of the natriuretic peptide system.

T555I and Q568P amino acid changes caused by the SNPs occur in the second frizzled-like domain in the corin propeptide.16,19 Both T555 and Q568 residues are conserved in corin...
molecules of most vertebrates. To date, the functional significance of T555I and Q568P changes has not been determined. Here, we examined the effect of T555I and Q568P, either individually or together, on corin biological activity. We show that corin variant T555I/Q568P, but not variants T555I or Q568P, had a markedly reduced natriuretic peptide processing activity. We further identified impaired zymogen activation as a molecular basis for the defect of T555I/Q568P variant.

Materials and Methods

Cell Culture
Human embryonic kidney (HEK)293 cells were cultured in DMEM with 10% FBS. The murine HL-1 cells,20 a generous gift from Dr William Claycomb (Louisiana State University Medical Center, New Orleans), were cultured in Claycomb medium (JRH Biosciences) with 10% FBS, 100 μmol/L norepinephrine, and 4 mmol/L L-glutamine in gelatin/fibronectin-coated flasks.

Expression Plasmid Constructs
Plasmids expressing human wild-type corin and active site mutant S985A were described previously.21 Recombinant corin proteins contained a C-terminal V5 tag to facilitate protein detection. Plasmids expressing human corin variants T555I, Q568P, and T555I/Q568P were made by site-directed mutagenesis using plasmid pcDNAhumanCorin21 as a template with the following oligonucleotide primers: sense 5′-GTG GCC TGA AGA CAT AGA TTG-3′ and antisense 5′-CAA TCT ATG TCT TCA GGC CAC-3′ for mutant T555I; sense 5′-CAG ACA ATC CAA CCT GCC TG-3′ and antisense 5′-CAG ACA ATC CAA CCT GCC TG-3′ for mutant Q568P. To express corin proteins (WT-EK and I/P-EK) with an enteropeptidase (EK) cleavage site, plasmids were made by mutagenesis to replace the original amino acid sequence RMNKR at the zymogen activation cleavage site with an EK recognition sequence DDSDK.

Proteins were analyzed by Western blotting using an anti-V5 antibody. 

Transfection, Immunoprecipitation, and Western Blotting
HEK 293 and HL-1 cells were transfected with plasmids using FuGENE reagent (Roche Diagnostics). Conditioned medium was collected overnight. Cells were lysed in a buffer containing 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% Triton X-100 (vol/vol), and a protease inhibitor cocktail (1:100 dilution, Sigma). Immunoprecipitation and Western blotting were done as described previously.21

Natriuretic Peptide Processing
Conditioned medium with pro-ANP was prepared in transfected cells. The medium was then added to HEK 293 cells expressing corin proteins and incubated at 37°C for 1 hour. Pro-ANP and ANP in the conditioned medium were analyzed by immunoprecipitation and Western blotting as described previously.22 To quantify pro-ANP processing, Western blots were scanned by a densitometer (The PharosFx System, Bio-Rad). The percentage of pro-ANP to ANP processing, Western blots were scanned by a densitometer (The PharosFx System, Bio-Rad). The percentage of pro-ANP to ANP processing was analyzed similarly as for pro-ANP processing.

Statistical Analysis
All data are presented as means±SD. Multigroup comparisons were done by ANOVA followed by post hoc least significant difference and Turkey honestly significant difference tests. A probability value of <0.05 was considered to be statistically significant.

Results

Importance of Corin Frizzled-Like Domains for Pro-ANP Processing
The 2 SNPs found in patients with hypertension are located in corin gene exon 12,16,19 causing T555I and Q568P changes in frizzled-like domain 2. Previously, we found that propeptide domains such as frizzled-like domain 1 and LDLR repeats 1 to 4 were required for corin-mediated pro-ANP processing,22 but the functional importance of corin frizzled-like domain 2 was not examined. In this study, we made a mutant corin without frizzled-like domain 2 (ΔFz2) and tested its activity. We used wild-type corin and mutants lacking either frizzled-like domain 1 (ΔFz1) or LDLR repeats 1 to 4 (ΔR1-4) as positive and negative controls, respectively (Figure 1A). Recombinant pro-ANP was incubated with HEK 293 cells expressing wild-type and corin mutants. Pro-ANP processing was analyzed by immunoprecipitation and Western blotting. As shown in Figure 1B (top) and 1C, ΔFz2 mutant had 30±5% of activity compared to that of wild-type. Consistent with our previous results,22 ΔFz1 and ΔR1-4 mutants had 44±7 and 4±3% of activities, respectively (Figure 1B and 1C). In controls, comparable levels of total recombinant corin proteins were confirmed in the transfected cells (Figure 1B, middle). By biotin-labeling of cell surface proteins followed by immunoprecipitation and Western blotting, we also confirmed that these corin proteins were present on the cell surface (Figure 1B, bottom). The results show that corin frizzled-like domain 2 is important for pro-ANP processing.

Pro-ANP Processing Activity of T555I/Q568P Variant
To examine if T555I/Q568P changes after corin function, we transfected HEK 293 cells with plasmids expressing human pro-ANP or corin variants in separate plates. Conditioned medium containing recombinant pro-ANP then was collected and added to the cells expressing corin proteins. After 1-hour at 37°C, the conditioned medium was analyzed for pro-ANP processing. As shown in Figure 2A (top), pro-ANP was processed similarly by wild-type corin and variants T555I and Q568P. In contrast, the activity of variant T555I/Q568P was greatly reduced. In these experiments, a negative control, corin S985A, in which the active site Ser was mutated to Ala, was used. This inactive mutant produced little ANP (Figure 2A, top). The Western blots were quantified by densitometry.
Values from S985A negative control were used as background and subtracted in calculating values for wild-type and variants. As shown in Figure 2B, T555I/Q568P variant had 38±7% of activity compared to that of wild-type (n=6, P<0.01). Variants T555I (84±12%) and Q568P (80±8%) had lower activities than that of wild-type, but the differences were not statistically significant (n=6, P>0.05). We verified corin proteins in the transfected cells by Western blotting using an anti-C-terminal V5 tag antibody and found comparable levels of corin proteins, which appeared as 2 band of ~150 and ~170 kDa (Figure 2A, bottom), representing differentially N-glycosylated molecules.21 These results indicate that T555I/Q568P variant had a markedly reduced pro-ANP processing activity.

Pro-BNP Processing by Corin Variants

Previously, we showed that corin also cleaved pro-BNP, although the reaction was less efficient than that for pro-ANP.12 To test pro-BNP processing by corin variants, we transfected plasmids for corin variants in HEK 293 cells stably expressing human pro-BNP. The conditioned medium was collected and pro-BNP processing was analyzed. As shown in Figure 3A, a small fraction of pro-BNP was processed in control 293 cells (control), possibly by an unknown protease. The processing was enhanced in cells expressing wild-type corin and variants T555I and Q568P, but was barely increased in cells expressing variant T555I/Q568P. By densitometric analysis of Western blots (Figure 3B), variant T555I/Q568P had 44±15% of activity (n=3, P<0.05) compared to that of wild-type, whereas variants T555I and Q568P had 44±15% and 81±6% of the activity, respectively (n=3, probability values >0.05). The results support that variant T555I/Q568P had a reduced biological activity.

Cell Surface Expression of Corin Variants

Corin is a transmembrane protein. We next examined if the reduced activity of variant T555I/Q568P was caused by low...
cell surface expression. Total surface proteins in transfected cells were labeled by biotinylation and precipitated using Streptavidin-Sepharose beads followed by Western blotting. Figure 4 (top left) shows similar levels of biotin-labeled corin proteins in HEK 293 cells expressing wild-type corin and variants. Densitometric analysis of four independent experiments showed that the expression levels for variants T555I, Q568P, and T555I/Q568P were 120±13, 114±8, and 96±8% of the wild-type (probability values >0.05). In controls, similar levels of corin proteins in cell lysates were found by Western analysis (Figure 4, top right). As another control for the specificity of cell surface labeling, an antibody against intracellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detected the protein in cell lysates but not in Streptavidin precipitates (Figure 4, bottom gels). The results show that T555I/Q568P changes did not prevent corin expression on the cell surface.

**Impaired Zymogen Activation for Variant T555I/Q568P**

Like most trypsin-like proteases, corin is made as a zymogen, which is activated proteolytically at the conserved site, Arg801↓Ile802 (Figure 1A).24,25 A disulfide bond links the activated protease fragment to the propeptide. On Western blots under reducing conditions, this fragment is expected to migrate as a band of ≈35 to 40 kDa, whereas it should attach to the propeptide under nonreducing conditions. In transfected cells, corin had activity, but the activated protease fragment was not readily detectable (Figure 2A, bottom), indicating that only a small fraction of the total corin molecules was activated, making it difficult to assess the status of corin activation. To circumvent this problem, we used immunoprecipitation to isolate all recombinant corin proteins in cell lysates instead of analyzing only a fraction of samples. The rationale was to enrich corin proteins and hence detect the activated protease fragment.

We transfected HEK 293 cells with plasmids expressing wild-type corin and variants. As a control, we included a plasmid encoding corin mutant R801A, in which the activation site Arg-801 was replaced by Ala (Figure 1A). This mutation prevents corin activation cleavage and, as a result, mutant R801A had no detectable pro-ANP processing activity.21,24 Lysates from the transfected cells were immunoprecipitated by an anti-V5 antibody to pull down recombinant corin proteins and then analyzed by Western blotting under reducing and nonreducing conditions. As shown in Figure 5A (top), an ≈40-kDa band was detected in samples containing wild-type corin and variants T555I and Q568P but not variant T555I/Q568P. This band also was absent in mutant R801A sample, indicating that it was the activated protease fragment. Consistent with the predicted corin structure, the protease fragment was absent under nonreducing conditions (Figure 5A, bottom). Analysis of 5 independent experiments by densitometry showed that the activated protease fragment represented 20±3%, 22±3%, and 20±4%, respectively, of total corin protein for the wild-type and variants T555I and Q568P. This fragment was undetectable in variant T555I/Q568P and mutant R801A samples, suggesting that T555I/Q568P amino acid changes impaired corin zymogen activation.

We verified these results in physiologically relevant murine HL-1 cardiomyocytes, which retain phenotypic characteristics of adult cardiomyocytes.20,26 A recent study indicates that corin activation in HL-1 cells is similar to that in the heart.27 HL-1 cells were transfected with corin-expressing plasmids. Recombinant corin proteins in cell lysates were analyzed by immunoprecipitation and Western blotting. As shown in Figure 5B (top), the activated corin protease fragment, represented by the ≈40-kDa band, was detected under reducing conditions in samples of wild-type and variants T555I and Q568P but not variant T555I/Q568P or mutant R801A. A nonspecific band of ≈50 kDa was present...
in all samples including parental HL-1 cells under reducing conditions (Figure 5B, top). Similar to the results from HEK 293 cells, the activated corin protease fragment was not detected under nonreducing conditions (Figure 5B, bottom). Analysis of three independent experiments by densitometry showed that the activated protease fragment represented 16±1%, 19±1%, and 17±1%, respectively, of total wild-type and variants T555I and Q568P proteins. The fragment was undetectable for variant T555I/Q568P and mutant R801A. The results show that impaired zymogen activation of variant T555I/Q568P occurred not only in HEK 293 cells but also in cardiomyocytes.

Restoration of Variant T555I/Q568P Activity by An EK Cleavage Site
To confirm that T555I/Q568P changes alter only corin zymogen activation but not substrate recognition, we designed recombinant corin proteins that can be activated in a controlled manner. Mutagenesis was made to replace the corin activation sequence RMNKR at residues 797 to 801 with a specific EK cleavage sequence DDDDK (Figure 6A).24 Wild-type and variant T555I/Q568P versions of corin with the EK site were expressed in HEK 293 cells. Cell lysates were prepared and incubated with increasing concentrations of recombinant human EK. Western blotting showed that WT-EK and variant T555I/Q568P-EK proteins were activated similarly by EK in a dose-dependent manner (Figure 6B). More importantly, the EK-activated corin variant T555I/Q568P converted pro-ANP to ANP as efficiently as WT-EK in functional assays (Figure 6C). In controls, recombinant EK did not convert pro-ANP to ANP (data not shown). These results show that T555I/Q568P variant, once activated, functioned normally, indicating that the impaired zymogen activation is responsible primarily for the reduced activity of the variant.

Discussion
The ANP pathway is important in regulating blood pressure.1–3 Knockout mice lacking either ANP or its receptor are hypertensive.28,29 Corin is an essential enzyme for ANP production.13 Recently, human corin gene SNPs have been found to be associated with an increased risk for hypertension and cardiac hypertrophy,16,18 suggesting that gene variants involved in the natriuretic peptide system may contribute to hypertensive disease. Further studies are needed to determine whether the gene variants alter corin function.

Figure 6. Activation and activity of WT-EK and T555I/Q568P-EK corin proteins. A, Schematic illustrations of wild-type (WT-EK) and T555I/Q568P (I/P-EK) versions of corin that contain an EK recognition sequence (DDDDK) at the conserved activation site. B, Recombinant WT-EK and I/P-EK were activated by increasing concentrations of recombinant EK (rEK), as shown by Western blot analysis under reducing conditions. C, The activity of EK-activated WT-EK and I/P-EK was tested in pro-ANP processing assays by Western blotting. The data shown were representative of 3 independent experiments.
In this report, we characterized human corin variants in functional studies. We showed that frizzled-like domain 2, where the naturally occurring variants occur, was required for corin to process pro-ANP efficiently (Figure 1). Previously, we showed that corin frizzled-like domain 1 and LDLR 1 to 4 repeats are important for pro-ANP processing. The new data indicate that other domains such as frizzled-like domain 2 are also important for corin function. Consistently, in other transmembrane serine proteases such as EK and matriptase, structural elements in the propeptide region are critical for their interactions with substrates and inhibitors. We also showed that corin variant T555I/Q568P had significantly lower activities in processing pro-ANP and pro-BNP (Figure 2 and 3) than that of wild-type, indicating that the amino acid substitutions caused by the SNPs altered corin protein structure, thereby lowering its biological activity.

To understand the molecular basis for the reduced activity in variant T555I/Q568P, we examined the cell surface expression of corin variants and found similar expression levels for wild-type and the variants (Figure 4), indicating that the cell surface expression is not altered by the amino acid changes. We next examined the zymogen activation of corin variants. As a trypsin-like protease, corin is made as a zymogen that is activated by cleavage at a conserved site. For many proteolytic enzymes, zymogen activation represents a critical step in regulating their activities. Our recent data indicated that a protease present in HEK 293 cells and cardiomyocytes was responsible for initial activation of corin, although the identity of this enzyme remains unknown.

In transfected HEK 293 cells and HL-1 cardiomyocytes, we showed that corin variant T555I/Q568P activation was impaired, as indicated by the absence of the activated protease fragment (Figure 5). Because the T555I/Q568P variant still exhibited some activities, it is likely that some variant molecules were activated but not enough to be detected by the methods used in our studies. To confirm that impaired zymogen activation is the principal mechanism responsible for the reduced activity of the corin variant, we made wild-type and T555I/Q568P versions of corin that can be activated by EK. We showed that, once activated, the variant and wild-type versions of corin had similar activities toward pro-ANP (Figure 6). Together, the results indicate that the naturally occurring variants reduce corin function by impairing zymogen activation. Our data are consistent with findings from other trypsin-like proteases in which mutations in the propeptide cause disease. For example, mutations in pro-thrombin propeptide are shown to prevent the zymogen activation, resulting in bleeding in patients. In TMPRSS3, another membrane protease involved in hearing, mutations in its propeptide prevent zymogen activation and cause autosomal recessive deafness. Because frizzled-like domains are present in many biologically important proteins, including Wnt signaling molecules, mutations in different frizzled-like domains may also cause other human diseases.

Interestingly, the activity and zymogen activation of corin variants with single amino acid change, either T555I or Q568P, did not appear to be reduced significantly. In functional assays, T555I and Q568P variants had slightly lower activities than that of wild-type, but the difference was not statistically significant. At this time, the corin crystal structure is not available. Based on a computer model using mouse Frizzled 8 as a template, single amino acid change, either T555I or Q568P, could alter the corin frizzled-like domain structure (data not shown). It appears, however, that conformational changes induced by single amino acid change are not sufficient to disrupt corin function, suggesting that more profound structural alterations occur when both T555 and Q568 residues are substituted. In mouse Frizzled 8, the frizzled domain is involved in protein dimerization and amino acid changes in this domain abolish its function. At this time, we do not have experimental evidence that corin protein dimerizes or its frizzled domains interact, but such possibilities cannot be excluded. The population-based genetic studies have shown that T555I and Q568P SNPs are in complete linkage disequilibrium. The minor allele, which is associated with hypertension and cardiac hypertrophy, encodes both I555 and P568. As a result, the corin protein encoded by this allele is expected to have a lower activity. It will be interesting to know whether corin alleles with 1 SNP, either T555I or Q568P, exist in the general population and whether such alleles are associated with a hypertensive phenotype.

Hypertension is a major cardiovascular disease, but its pathogenesis remains unknown in most patients with the disease. Genome-wide searches for SNPs associated with hypertension yielded few candidate genes, which may reflect the fact that blood pressure is regulated by many biological pathways. Our studies have established corin as the long-sought pro-ANP convertase. The identification of the naturally occurring corin variants in patients with hypertension links the corin gene to human disease. Interestingly, I555/P568 corin allele is found mostly in African Americans, a population known for its high prevalence of salt-sensitive hypertension. In mice, corin deficiency causes salt-sensitive hypertension and cardiac hypertrophy. Here, we show that the naturally occurring corin variant had a markedly reduced activity in vitro, which was most likely caused by impaired zymogen activation. At this time, we do not know whether corin T555I/Q568P variant is associated with impaired pro-ANP/pro-BNP processing in vivo, nor do we know whether this variant directly causes hypertension and cardiac hypertrophy in patients. Further studies also will be important to examine if corin expression, activity, and regulation in the myocardium are affected in individuals carrying this polymorphism. Our findings should encourage more genetic studies to screen additional corin mutations in patients with hypertension and heart disease. The results will help to understand the role of this newly discovered enzyme in cardiovascular disease.

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Disclosures
None.

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