HHS Public Access

Author manuscript

Nat Med. Author manuscript; available in PMC 2016 September 01.

Published in final edited form as:

Nat Med. 2015 September ; 21(9): 1048–1053. doi:10.1038/nm.3920.

PCSK6-mediated corin activation is essential for normal blood pressure

Shenghan Chen[1,7], **Pengxiu Cao**[1,7], **Ningzheng Dong**[2,3], **Jianhao Peng**[1], **Chunyi Zhang**[1,7], **Hao Wang**[1], **Tiantian Zhou**[2], **Junhua Yang**[4], **Yue Zhang**[2], **Elizabeth E Martelli**[1], **Sathyamangla V Naga Prasad**[1], **Rachel E Miller**[5,6], **Anne-Marie Malfait**[5,6], **Yiqing Zhou**[2], and **Qingyu Wu**[1,2]

[1]Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, USA

[2]Cyrus Tang Hematology Center, Collaborative Innovation Center of Hematology, Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases, Soochow University, Suzhou, China

[3]Key Laboratory of Thrombosis and Hemostasis, Jiangsu Institute of Hematology, the First Affiliated Hospital of Soochow University, Suzhou, China

[4]Department of Cardiology, the First Affiliated Hospital of Soochow University, Suzhou, China

[5]Department of Internal Medicine (Rheumatology), Rush University Medical Center, Chicago, Illinois, USA

[6]Department of Biochemistry, Rush University Medical Center, Chicago, Illinois, USA

Abstract

Hypertension is the most common cardiovascular disease, afflicting >30% of adults[1]. The cause of hypertension in most individuals remains unknown[2,3], suggesting that additional contributing factors have yet to be discovered. Corin is a serine protease that activates the natriuretic peptides, thereby regulating blood pressure[4]. It is synthesized as a zymogen that is activated by proteolytic cleavage. *CORIN* variants and mutations impairing corin activation have been identified in people with hypertension and pre-eclampsia[5–9]. To date, however, the identity of the protease that activates corin remains elusive. Here we show that proprotein convertase subtilisin/kexin-6

Correspondence should be addressed to Q.W. (wuq@ccf.org).

[7]Present addresses: Department of Gynecology and Obstetrics, Jiangxi Provincial People's Hospital, Nanchang, China (S.C.); Department of Internal Medicine, University of Michigan Health System, Ann Arbor, Michigan, USA (P.C.); Biochemistry and Molecular Biology Department, Shanghai Medical School, Fudan University, Shanghai, China (C.Z.).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS

S.C., P.C., N.D. and Q.W. designed the study. S.C., P.C., J.P., C.Z. and H.W. performed molecular biology, biochemistry, cell biology and mouse model studies. N.D., T.Z., J.Y. and Y. Zhang studied hypertensive patients, collected blood samples, sequenced *PCSK6* exons, and made plasmids expressing corin variants. E.E.M. and S.V.N.P. did echocardiographic analysis in mice. R.E.M. and A.-M.M. provided *Pcsk6*-KO mice. S.C., Y. Zhou and Q.W. wrote the manuscript. All authors critically read and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

(PCSK6, also named PACE4; ref. 10) cleaves and activates corin. In cultured cells, we found that corin activation was inhibited by inhibitors of PCSK family proteases and by small interfering RNAs blocking PCSK6 expression. Conversely, PCSK6 overexpression enhanced corin activation. In addition, purified PCSK6 cleaved wild-type corin but not the R801A variant that lacks the conserved activation site. *Pcsk6*-knockout mice developed salt-sensitive hypertension, and corin activation and pro-atrial natriuretic peptide processing activity were undetectable in these mice. Moreover, we found that *CORIN* variants in individuals with hypertension and pre-eclampsia were defective in PCSK6-mediated activation. We also identified a *PCSK6* mutation that impaired corin activation activity in a hypertensive patient. Our results indicate that PCSK6 is the long-sought corin activator and is important for sodium homeostasis and normal blood pressure.

Maintenance of normal blood pressure is of fundamental importance in health. Hypertension is a major risk factor for leading cardiovascular diseases such as myocardial infarction, heart failure and stroke. Mechanistically, hypertension reflects inadequate control of salt and body fluid balance[11,12]. Consistent with this idea, genetic mutations that alter sodium homeostasis have been identified in subgroups of hypertensive patients[2,3,13]. Given the high prevalence of hypertension in general populations[1], it is probable that more genes involved in sodium homeostasis remain unidentified.

Atrial natriuretic peptide (ANP) is a hormone essential for sodium homeostasis[14]. Common genetic variants in the *NPPA* gene locus (encoding ANP) are associated with circulating natriuretic peptide and blood pressure levels in general populations[15]. ANP variants have been reported in hypertensive patients[16–19]. In cardiomyocytes, the precursor form of ANP, pro-ANP, is cleaved by corin, a transmembrane serine protease, to produce ANP[4]. A trypsin-like enzyme, corin is synthesized as an inactive zymogen that is activated by cleavage at a conserved site between Arg801 and Ile802 (ref. 20) (Fig. 1a). Activated corin may undergo auto-cleavage and a disintegrin and metalloprotease (ADAM)-mediated shedding[21] (Fig. 1a), which reduces corin activity on the cell surface. The enzyme responsible for activation of the corin zymogen has not been identified.

To understand how corin is activated, we expressed wild-type (WT) corin and either of two inactive corin variants in HEK293 human embryonic kidney cells (Fig. 1b) or mouse HL-1 cardiomyocytes (Supplementary Fig. 1). The inactivated variants were R801A, in which the activation site is abolished, and S985A, in which the catalytic serine is mutated (Fig. 1a). On western blots, corin zymogen migrated at ~170–200 kDa. An ~40-kDa band, representing the activated corin protease domain fragment (Corin-p), was detected in cells expressing WT corin. This band was present under reducing but not non-reducing conditions owing to the presence of a disulfide bond that connects the cleaved fragment to the propeptide (Fig. 1a). The ~40-kDa band was absent in cells expressing the R801A variant but was present in cells expressing the S985A variant, indicating that, in both HEK293 and HL-1 cells, corin is activated specifically at Arg801 by an unknown enzyme or enzymes.

We next tested different protease inhibitors for their effects on corin activation in transfected HEK293 cells. Western blot analysis showed that benzamidine (serine protease inhibitor) and dec-RVKR-cmk (PCSK protease inhibitor), but not GM6001 (metalloproteinase

inhibitor) or ALLM (*N*-acetyl-leucyl-leucyl-methionine; cysteine protease inhibitor), inhibited corin activation, as indicated by reduced levels of the ~40-kDa band and increased levels of an ~190-kDa zymogen band (Fig. 1c and Supplementary Fig. 2). Trypsin digestion studies showed that the ~190-kDa band represents corin zymogen on the cell surface (Supplementary Fig. 3). These results suggest that one or more PCSK family proteases activate corin in these cells. Consistent with this hypothesis, the corin activation cleavage sequence is RMNKR↓, and PCSKs are known to cleave following two basic residues[10,22].

There are nine PCSK family members[10,22]. We transfected plasmids encoding each of the PCSKs (PCSK1–PCSK9) into HEK293 cells expressing human corin. Western blot analysis showed that PCSK6 expression enhanced corin activation, as indicated by loss of the corin zymogen band (Fig. 1d and Supplementary Figs. 4 and 5), suggesting that PCSK6 is a corin activator. By RT-PCR, we detected PCSK6 mRNA in HEK293 and HL-1 cells as well as in mouse and human hearts, where corin normally is produced[20] (Supplementary Fig. 6). These results are consistent with a previous report of PCSK6 expression in cardiomyocytes[23]. Using small interfering RNAs (siRNAs) targeting either human or mouse *PCSK6* genes, we showed that blocking PCSK6 expression inhibited corin activation cleavage in transfected HEK293 and HL-1 cells, whereas scrambled siRNAs or siRNAs targeting *PCSK1*, *PCSK3* or *PCSK5* had no such effects under similar conditions (Fig. 1e–j and Supplementary Fig. 7).

We next examined the cellular mechanism by which PCSK6 activates corin. In corin-expressing cells treated with brefeldin A (BFA), which blocks protein endoplasmic reticulum (ER) trafficking, corin cell surface expression and zymogen activation were inhibited (Fig. 2a,b), indicating that corin is not activated in the ER. We then tested monensin, a Golgi-perturbing agent that blocks the secretory pathway. Monensin did not prevent corin cell surface expression, but it did inhibit corin activation, as indicated by the lack of the ~40-kDa band (Fig. 2c,d). As expected, monensin reduced the levels of PCSK6, which is secreted via the secretory pathway[10], in the conditioned medium but not in the cell lysate (Fig. 2e). These data suggest a model in which PCSK6 and corin traffic via separate intracellular pathways to the cell surface, where PCSK6 activates corin (Fig. 2f). This model is consistent with the results from a trypsin digestion experiment (Supplementary Fig. 3), in which activated corin was detected on the cell surface but not intracellularly.

To further test this hypothesis, we added conditioned medium containing PCSK6 to HEK293 cells expressing corin. Increased corin activation was detected with increasing amounts of PCSK6 in the conditioned medium (Fig. 2g,h). Such activation was not observed if the conditioned medium was first immunodepleted using a PCSK6-specific antibody (Fig. 2i). We also showed that purified recombinant PCSK6 (Supplementary Fig. 8) activated WT corin but not the R801A variant on HEK293 cells (Fig. 2j). These results support a cellular mechanism in which corin is activated only after reaching the cell surface, thereby preventing proteolytic activity inside the cell.

On the basis of these *in vitro* studies indicating that PCSK6 acts as a corin activator, we tested whether PCSK6 can activate corin *in vivo* using WT and *Pcsk6*-knockout (KO) mouse hearts. On western blots, three corin bands of ~190, ~160 and ~110 kDa were detected in

Pcsk6-KO mouse hearts, whereas only the ~160- and ~110-kDa bands, at lower levels than in the KO hearts, were detected in WT hearts (Fig. 3a,b). As a negative control, no bands were detected in *Corin*-KO hearts (Fig. 3a,b). In *Pcsk6*-KO hearts, high levels of the ~190-kDa band—which, as shown in trypsin and glycosidase digestion experiments, represents the cell surface corin zymogen (Supplementary Figs. 3 and 9)—indicated impaired corin activation. In agreement with this result, we found little pro-ANP processing activity in cell membranes from *Pcsk6*- or *Corin*-KO hearts as compared with those from WT mouse hearts (Fig. 3c,d). As a control, we detected corin activity in cell membranes from corin-expressing, but not parental, HEK293 cells (Fig. 3c). Pro-ANP levels were high in *Pcsk6*- and *Corin*-KO hearts compared with WT control hearts (Fig. 3e–g). In contrast, pro-BNP levels were similar in WT, *Pcsk6*-KO and *Corin*-KO mouse hearts (Supplementary Fig. 10). As assessed by ELISA, plasma pro-ANP and/or ANP levels in WT, *Pcsk6*- and *Corin*-KO mice were below the linear detection limit. To circumvent this problem, we measured plasma natriuretic peptide activities in a cell-based cGMP stimulation assay. We detected reduced natriuretic peptide activities in plasma samples from *Pcsk6*- and *Corin*-KO mice as compared with WT mice (Fig. 3h). These results indicate that PCSK6 is a primary corin activator *in vivo* and that lack of PCSK6 prevents corin activation and pro-ANP processing.

In mice, corin deficiency impairs sodium homeostasis, causing salt-sensitive hypertension[24]. If PCSK6 deficiency prevented corin activation *in vivo*, we would expect to observe a similar hypertensive phenotype in *Pcsk6*-KO mice. Indeed, *Pcsk6*-KO mice were hypertensive on a normal-salt (0.3% NaCl) diet (Fig. 3i). The hypertensive phenotype was exacerbated when the mice were placed on high-salt (4% and 8% NaCl) diets (Fig. 3i), indicating that PCSK6-mediated corin activation is essential for sodium homeostasis and normal blood pressure in mice and that PCSK6 deficiency may cause salt-sensitive hypertension in humans. In histological analyses, cardiac hypertrophy was detected in *Corin*-KO but, surprisingly, not in *Pcsk6*-KO mice (Supplementary Fig. 11). Moreover, echocardiography showed normal cardiac function in *Pcsk6*- and *Corin*-KO mice (Supplementary Figs. 12 and 13). By electron microscopy, sarcomeric structures were similar in WT and *Pcsk6*-KO mice, whereas they had a thicker appearance in *Corin*-KO mice (Supplementary Fig. 14). These results suggest that PCSK6 may have other substrates (for example, transforming growth factor beta (TGF-β; ref. 25)) and that the action of PCSK6 on these substrates may promote tissue remodeling and cardiac hypertrophy.

In humans, a *CORIN* variant allele encoding a protein with two amino acid substitutions, T555I and Q568P, has been identified in African-Americans with hypertension and heart disease[7,8]. The variant protein encoded by this allele is defective in pro-ANP processing *in vitro* and *in vivo*[9,26]. Activation of the corin variant containing both the T555I and Q568P amino acid substitutions, but not corin variants containing either T555I or Q568P, is markedly reduced[9]. We found that PCSK6 was able to cleave WT corin and variants containing either T555I or Q568P, but not the variant containing both T555I and Q568P (Fig. 4a,b and Supplementary Fig. 15a).

Recently, corin was found to act in the uterus to prevent pregnancy-induced hypertension[5]. *CORIN* mutations encoding the K317E, S472G and R539C corin variants have been identified in patients with pre-eclampsia and hypertension, and these variants have impaired

activation and reduced pro-ANP processing activity[5,6]. Biochemical studies showed that the S472G variant is retained in the ER, whereas the K317E and R539C variants are present on the cell surface[6,27]. By western blot analysis, we found that the K317E, S472G and R539C variants showed 61%, 1% and 38%, respectively, of the PCSK6-mediated activation seen with WT corin (Fig. 4a,c and Supplementary Fig. 15b), indicating that these natural corin variants are defective in PCSK6-mediated activation. Retention of the S472G variant in the ER would be expected to prevent its trafficking to the cell surface and hence its zymogen activation. In trypsin-like proteases, domain structures in the propeptide are known to interact with their activators[28]. In the case of the protease TMPRSS3, for example, mutations in its propeptide prevent zymogen activation, causing deafness[29]. Thus, our results may provide insights into the biochemical basis of defective corin function in people with hypertension.

We next sequenced the *PCSK6* gene in 100 hypertensive individuals and identified a mutation causing an Asp282Asn (D282N) change in the PCSK6 catalytic domain in one such person (Fig. 4d,e and Supplementary Table 1). This mutation was not found in any of 118 normal control individuals. In transfected HEK293 cells, the D282N variant was associated with reduced corin-activating activity and a dominant-negative effect on endogenous PCSK6 activity (Fig. 4f,g). Compared with that of WT PCSK6, secretion of the D282N variant was impaired (Fig. 4h). When HEK293 cells expressing WT corin were treated with conditioned medium containing increasing amounts of D282N protein, corin activation was dose-dependently increased (Fig. 4i,j). When the D282N variant was co-transfected with WT PCSK6, the D282N variant exhibited a dominant-negative effect, inhibiting WT PCSK6 secretion into the culture medium and reducing corin activation in the transfected cells (Supplementary Fig. 16). These results are consistent with the cellular model in which PCSK6 activates corin on the cell surface but not intracellularly (Fig. 2f). It remains to be determined whether the *PCSK6* mutation we identified impairs corin activation and contributes to hypertension in humans.

Corin-mediated natriuretic peptide activation is essential for normal blood pressure and cardiac function[24,30]. In individuals with heart failure, levels of unprocessed natriuretic peptides are high, indicating that corin activity is a rate-limiting factor in failing hearts[31–33]. A recent study showed that corin overexpression improves cardiac function in a mouse model of heart failure[34]. PCSK6 has been reported to process growth factors and metalloproteinases[10,25]. Here we show that PCSK6 also acts as a primary corin activator in the heart, and that PCSK6 deficiency causes salt-sensitive hypertension in mice. Consistent with these findings, the *PCSK6* gene was previously linked to hypertension in humans[35]. Among PCSK family members, PCSK9 has been identified as a key regulator that binds to the low-density lipoprotein (LDL) receptor and promotes the receptor's degradation[36–38]. PCSK9 inhibitors are being developed as therapeutic agents to lower LDL cholesterol levels[38–40]. Our findings from this study provide new evidence that PCSK family members may be exploited therapeutically to treat hypertension and heart disease.

METHODS

Methods and any associated references are available in the online version of the paper.

ONLINE METHODS

Cell culture

Human embryonic kidney 293 (HEK293) and baby hamster kidney (BHK) cells were cultured in DMEM with 10% fetal bovine serum (FBS). HL-1 cardiomyocytes were provided by William Claycomb (Louisiana State University Medical Center) and cultured in Claycomb medium (Sigma) with 10% FBS, and 4 mM L-glutamine[41]. All cells were cultured at 37 °C in humidified incubators with 5% CO_2.

Plasmids

Plasmids expressing human WT corin and the variants R801A, S985A, T555I, Q568P, T555I/Q568P, K317E, S472G and R539C were described previously[9,27,42,43]. Plasmids expressing human PCSK1-9 were made by cloning their respective cDNAs into the pcDNA3.1 vector (Invitrogen). All corin and PCSK proteins contained a C-terminal V5 or FLAG tag for protein detection by western blotting.

Transfection, immunoprecipitation, and western blotting

HEK293 and HL-1 cells were transfected with corin or PCSK6 expression plasmids using FuGENE HD (Promega) or Lipofectamine 2000 (Invitrogen) reagents. After 48–72 h, cells were lysed in a buffer containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (vol/vol) NP-40 and 1% (vol/vol) protease inhibitor cocktail (Sigma)[32]. Proteins were immunoprecipitated with an anti-V5 (Invitrogen, R96025) or anti-FLAG antibody (Agilent Technologies, 200471). Western blotting was performed to analyze corin expression and activation or PCSK6 expression under reducing or non-reducing conditions, as described previously[41,44]. The percentage of corin activation (corin-p versus corin fragments) was quantified by densitometric analysis of western blots[44]. An anti-GAPDH antibody (Millipore, MAB374) was used on western blots as a control for protein loading.

Cell surface protein labeling

HEK293 and HL-1 cells expressing corin in six-well plates were incubated with 1.5 ml (200 μM) of membrane impermeable Sulfo-NHS-SS-biotin (Thermo Scientific) for 5 min (refs. 21,44). A quenching solution (100 mM glycine in PBS) was added to the wells and incubated on ice for 10 min. Cells were lysed in lysis buffer and labeled proteins were isolated with avidin-coupled agarose beads and analyzed by western blotting.

Trypsin digestion

To verify corin expression on the cell surface, a trypsin digestion experiment was conducted. HEK293 cells expressing corin were cultured with or without the PCSK inhibitor, dec-RVKR-cmk (20 μM) (Enzo Life Sciences, ALX-260-022). Cell surface proteins were labeled with Sulfo-NHS-SS-biotin, as described above. A solution containing 0.05% trypsin and 0.53 mM EDTA (Mediatech) was added to the cells on ice. After 20 min, DMEM with 10% FBS was added to stop the reaction. After washing with PBS, the cells were lysed. Corin protein in the cell lysate and surface-labeled protein fractions was analyzed by western blotting[44].

Effects of BFA and monensin

To examine the cellular mechanism of PCSK6-mediated corin activation, we tested cell organelle-disturbing agents BFA and monensin, which block protein trafficking in the ER and the secretory pathway, respectively[45]. HEK293 cells expressing corin were treated with BFA (0.5 and 1 μM) (Sigma, B7651) or monensin (0.2 and 2 μM) (Sigma, M5273) at 37 °C for 24–48 h. The cells were lysed in the lysis buffer and corin protein on the cell surface and in cell lysate was analyzed by western blotting.

Effect of protease inhibitors on corin activation cleavage

To identify the corin activator, we examined corin activation cleavage in HEK293 and HL-1 cells expressing corin in the presence of protease inhibitors including benzamidine (5 mM) (Sigma, 434760), GM6001 (50 μM) (Enzo Life Sciences, BML-EI300), dec-RVKR-cmk (20 μM) (Enzo Life Sciences, ALX-260-022) and ALLM (*N*-acetyl-leucyl-leucyl-methionine, 50 μM) (Santa Cruz, sc-201268) (ref. 41). The inhibitors were added to the cells in separate wells. After 24 h, cell lysates were prepared and corin protein was analyzed by western blotting, as described above.

PCSK6 mRNA expression

Total RNAs were isolated from HEK293 and HL-1 cells and human and mouse hearts using TRIzol reagents (Invitrogen). First-strand cDNAs were synthesized using the SuperScript III kit (Invitrogen). Human and mouse *PCSK6* mRNAs were amplified by RT-PCR with specific oligonucleotide primers. Negative controls included reactions without cDNA templates. PCR products were analyzed on 1.3% (wt/vol) agarose gels followed by ethidium bromide staining.

PCSK6 gene knockdown

On-TARGET plus SMARTpool siRNAs against human *PCSK6* (LU-005983-00-0002) or mouse *Pcsk6* (LU-059121-01-0002) were obtained from Thermo Scientific. Each siRNA pool contained siRNAs targeting four different sites in the gene. The siRNAs were transfected into HEK293 or HL-1 cells expressing recombinant corin. Non-targeting scrambled siRNAs were used as negative controls. After 48–68 h at 37 °C, corin expression on the cell surface and in cell lysate was analyzed by western blotting. PCSK6 expression in siRNA-treated cells was verified by western blotting. In parallel, siRNAs targeting human *PCSK1*, *PCSK3* and *PCSK5* genes also were transfected into HEK293-corin cells. Corin activation in the transfected cells was examined by western analysis.

Corin activation in the presence of PCSK6 conditioned medium

Conditioned medium (CM) from HEK293 cells stably expressing PCSK6 was collected and centrifuged to remove cellular debris. Increasing volumes of CM were added to corin-expressing HEK293 cells that were cultured in the presence of monensin (2 μM). After 2 h at 37 °C, the cells were lysed. Corin protein in the cell lysate was analyzed by western blotting. To deplete PCSK6 from the CM, an anti-PCSK6 antibody (Sigma, SAB2101751, 5 μg) or control normal IgG (Sigma, I5006, 5 μg) was incubated with 2 ml of CM from PCSK6-transfected cells for 2 h. After immunoprecipitation, the supernatant was collected

and added to the HEK293-corin cells. Corin activation was examined by western blot analysis, as described above.

Mice

Corin-KO mice have previously been described[24,30]. *Pcsk6*-KO mice were generated as previously[25] and were backcrossed onto C57BL/6 background for at least ten generations[46]. No apparent cardiac defects were observed in adult *Pcsk6*-KO mice, as indicated in Supplementary Figures 11–14. Male WT, *Corin*-KO and *Pcsk6*-KO mice (4–6 months old) were used for tissue collection and blood pressure measurements. All procedures were carried out in accordance with US National Institutes of Health (NIH) guidelines for the ethical treatment and handling of animals in research, and approved by the Cleveland Clinic Institutional Animal Care and Use Committee. The sample size for mouse studies was determined on the basis of our previous experience with these mouse models. No randomization was used, and the investigators who performed the experiments were not blinded to mouse genotypes.

Corin, PCSK6, pro-ANP and pro-BNP protein expression in mouse hearts

Mouse heart tissue homogenates were prepared in a buffer with or without a protease inhibitor cocktail[32]. Corin, PCSK6, pro-ANP and pro-BNP protein levels were analyzed by western blotting using antibodies against corin made in our laboratory[5], PCSK6 (Abcam, ab151562), pro-ANP (Abcam, ab126149) and pro-BNP (Santa Cruz, sc-67455), respectively.

Glycosidase digestion

Transfected HEK293 cells expressing human or mouse corin were lysed in the lysis buffer described above. Mouse heart membrane fractions were prepared using hearts from WT and *Pcsk6*-KO mice, as described previously[32]. The cell lysate (25 μg) and membrane proteins (40 μg) were incubated in a deglycosylation denaturing buffer (New England BioLabs) at 100 °C for 10 min. Peptide N-glycosidase F (PNGase F) (1 unit) or endoglycosidase H (Endo H) (1 unit) (New England BioLabs) was added to the protein mixture and incubated at 37 °C for 2 h[47]. Corin proteins were analyzed by SDS-PAGE and western blotting[44].

Pro-ANP processing assay

Membrane fractions from hearts were extracted by ultracentrifugation, as described previously[32]. The membrane pellets were resuspended in an NP-40 buffer[32]. Conditioned medium from stable HEK293 cells expressing human pro-ANP was incubated with increasing amounts of heart membrane proteins. The conversion of pro-ANP to ANP was analyzed by western blotting[32]. The percentage of pro-ANP to ANP conversion was quantified by densitometric analysis of western blots, as described previously[44].

Expression and purification of recombinant PCSK6

Stable HEK293 cells were established to express human PCSK6 containing a C-terminal FLAG tag. The cells were cultured in DMEM with 10% FBS and 600 μg/ml G418. At ~80% confluency, the cells were washed with PBS and cultured with OPTI-MEM I reduced-serum

medium (Life Technologies, 31985-070). After 72 h, the conditioned medium was collected, centrifuged (10 min at 17,000*g*) to remove cell debris, and concentrated to one-fifth of the original volume using an Amicon Ultra-15 Centrifugal filter unit with Ultracel-50 membrane (Millipore) at 4 °C. The concentrated medium was incubated with anti-FLAG M2 affinity bead slurry (Sigma, A2220) in a volume ratio of 25:1 on a rotator at 4 °C for 2 h. After centrifugation, the beads were washed five times with a buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). PCSK6 protein was eluted using a solution (53 mM Tris-HCl, pH 7.4, and 156 mM NaCl) containing 160 ng/μl of FLAG peptide (Sigma, F4799) on a rotator at 4 °C for 1 h. After centrifugation, the supernatant containing purified PCSK6 was collected and analyzed by SDS-PAGE, followed by silver staining and western blotting.

Corin activation by purified PCSK6

HEK293 cells stably expressing human corin were cultured in six-well plates in DMEM with 10% FBS and 2 μM monensin. After the cells reached confluency, they were washed and a mixture of OPTI-MEM I reduced-serum medium and TBS solution in a ratio of 1:4 was added. Increasing concentrations of purified PCSK6 were added to the cell culture and incubated at 37 °C. After 2 h, cell surface proteins were labeled and corin protein was analyzed by western blotting, as described above.

Blood pressure measurements

Systolic blood pressure (SBP) in mice was measured by a non-invasive tail-cuff method using the RTBP1001 system (Harvard Apparatus). Mice went through daily sessions of unrecorded measurements for 4–5 d to become accustomed to the tail-cuff experimental procedure. SBP was then measured for 3 consecutive days. For each mouse, 10 consecutive pulse readings were recorded. The values are presented as mean ± s.d.

Effects of dietary salt on blood pressure and cardiac morphology

Six-month-old male mice were fed with normal-salt (0.3% NaCl) or high-salt (4% and 8% NaCl) diets for 3 weeks (refs. 24,26). Blood pressure was measured, as described above. Hearts from WT, *Pcsk6*-KO and *Corin*-KO mice were isolated, weighed and fixed with 4% paraformaldehyde. Tissue sections (5 μm in thickness) were made and stained with H&E. Computer-assisted analysis (Measure IT, Olympus) of cardiomyocyte diameter was done at a high magnification (×400), as described previously[26]. At least 350 cardiomyocytes in ten randomly selected fields were examined for each mouse group.

Electron microscopy

Hearts were collected from WT, *Pcsk6*-KO and *Corin*-KO male mice (8–9 months old) on a high-salt diet (8% NaCl) for three weeks. Heart tissues were fixed in 3% glutaraldehyde, treated with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in an Araldite-Epon mixture. Ultrathin sections (87 nm in thickness) were cut and stained with uranyl acetate and lead citrate. The sections were examined using a transmission electron microscope (JEOL JEM-1210) at the Lerner Image Core of the Cleveland Clinic.

Echocardiography

Echocardiography was performed using a Vevo770 machine (VisualSonics) on anesthetized WT, *Pcsk6*-KO and *Corin*-KO mice (8-month-old males) on a normal-salt diet (0.3% NaCl) or a high-salt diet (8% NaCl) for three weeks. M-mode views were recorded including left ventricular systolic and diastolic dimensions, as described previously[48].

Plasma natriuretic peptide activity assay

A cell-based cGMP stimulation assay[43] was used to examine natriuretic peptide activity in WT, *Pcsk6*-KO and *Corin*-KO mouse plasma. BHK cells were cultured in DMEM medium with 10% FBS in 96-well plates. Confluent cells were washed once with serum-free DMEM medium. Plasma samples (25 μl) were added to each well with serum-free DMEM medium (75 μl) and incubated at 37 °C for 30 min. The cells were lysed with 0.1 M HCl. Intracellular cGMP concentrations in the cell lysate were measured using an ELISA kit (Enzo Life Sciences, ADI-900-014). In this experiment, human ANP (Calbiochem, 05-23-0300) was used as a positive control. Each experimental condition was assayed in duplicate in at least three independent experiments.

Natriuretic peptide concentrations in mouse plasma

The following commercial kits were used to measure natriuretic peptides in plasma samples from WT, *Pcsk6*-KO and *Corin*-KO mice. An ELISA kit from Alpco Diagnostics (04-BI-20892) was used for pro-ANP; the antibodies in the kit are against human pro-ANP 1-98. An EIA (enzyme immunoassay) kit from Sigma-Aldrich (RAB00385) was used for ANP; the antibodies in the kit are against human ANP sequences that are conserved in other species including mouse. ELISA kits from Biotang were used for pro-BNP (M7594) and BNP (M1437); the antibodies in these kits are against mouse N-terminal (NT) pro-BNP and BNP sequences, respectively. The assays were performed according to the manufacturers' instructions.

PCSK6 gene mutation in individuals with hypertension

Blood samples were obtained from 100 individuals with hypertension, as defined by having diastolic pressure >90 mm Hg and/or systolic pressure >140 mm Hg on at least two occasions, or having histories of hypertension and taking anti-hypertensive medications. Age-, gender- and ethnic background (Han Chinese)-matched normal individuals (n = 118) were included as controls. The clinical characteristics of the hypertensive patients and the normal controls are shown in Supplementary Table 1. The study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University and all participants gave written informed consent. Venous blood was collected into tubes containing EDTA as an anticoagulant, as described previously[49]. Genomic DNA, extracted from white blood cells using the QIAamp DNA Blood mini kit (Qiagen), was used in PCR experiments to amplify each of the 24 exons of the *PCSK6* gene. PCR products were used directly for DNA sequencing. The D282N mutation (NM_002570.4: c.1158G>A) found in one hypertensive person was confirmed by a separate round of PCR and direct DNA sequencing.

Functional analysis of the D282N PCSK6 variant

Using a plasmid encoding PCSK6 WT as a template, site-directed mutagenesis was carried out to generate a pcDNA3.1-based (Agilent Technologies) plasmid expressing D282N variant. WT or D282N PCSK6 proteins expressed by these plasmids contained a C-terminal FLAG tag, allowing detection by an anti-FLAG antibody (Agilent Technologies, 200471). HEK293 cells were co-transfected with plasmids expressing corin and WT or D282N PCSK6 using FuGENE HD (Promega) reagents. After 48 h, the cells were lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40 (vol/vol) and a protease inhibitor cocktail (Sigma, 1:100). Corin and PCSK6 proteins in cell lysate or conditioned medium were analyzed by immunoprecipitation and western blotting, as described above. In separate experiments, conditioned medium containing increasing amounts of WT PCSK6 or the D282N variant was incubated with HEK293-corin cells at 37 °C. After 2 h, the cells were lysed and corin activation was examined by western blot analysis, as described above.

Statistical analysis

All values are presented as means ± s.d. Statistical analysis was done with Student's *t* test using the GraphPad Prism software. Comparisons for three or more groups were done using one-way ANOVA followed by Tukey's *post hoc* test or two-way ANOVA followed by Bonferroni *post hoc* test, as specified in figure legends. $P < 0.05$ was considered as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. Yin of the Lerner Imaging Core at the Cleveland Clinic for electron microscopic analysis and W. Claycomb of Louisiana State University Medical Center for HL-1 cells. This work was supported in part by grants from the US National Institutes of Health (R01HL089298, R01HD064634, R01HL126697) (Q.W.), a Rush University Pilot Grant (A.-M.M.), the National Natural Science Foundation of China (81170247, 81370718) (N.D.), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

1. Go AS, et al. Heart disease and stroke statistics–2013 update: a report from the American Heart Association. Circulation. 2013; 127:e6–e245. [PubMed: 23239837]
2. Lifton RP, Gharavi AG, Geller DS. Molecular mechanisms of human hypertension. Cell. 2001; 104:545–556. [PubMed: 11239411]
3. Oparil S, Zaman MA, Calhoun DA. Pathogenesis of hypertension. Ann Intern Med. 2003; 139:761–776. [PubMed: 14597461]
4. Yan W, Wu F, Morser J, Wu Q. Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme. Proc Natl Acad Sci USA. 2000; 97:8525–8529. [PubMed: 10880574]
5. Cui Y, et al. Role of corin in trophoblast invasion and uterine spiral artery remodelling in pregnancy. Nature. 2012; 484:246–250. [PubMed: 22437503]
6. Dong N, et al. Corin mutation R539C from hypertensive patients impairs zymogen activation and generates an inactive alternative ectodomain fragment. J Biol Chem. 2013; 288:7867–7874. [PubMed: 23372161]

7. Dries DL, et al. Corin gene minor allele defined by 2 missense mutations is common in blacks and associated with high blood pressure and hypertension. Circulation. 2005; 112:2403–2410. [PubMed: 16216958]
8. Rame JE, et al. Corin I555(P568) allele is associated with enhanced cardiac hypertrophic response to increased systemic afterload. Hypertension. 2007; 49:857–864. [PubMed: 17296875]
9. Wang W, et al. Corin variant associated with hypertension and cardiac hypertrophy exhibits impaired zymogen activation and natriuretic peptide processing activity. Circ Res. 2008; 103:502–508. [PubMed: 18669922]
10. Seidah NG, Sadr MS, Chretien M, Mbikay M. The multifaceted proprotein convertases: their unique, redundant, complementary, and opposite functions. J Biol Chem. 2013; 288:21473–21481. [PubMed: 23775089]
11. Guyton AC. Blood pressure control–special role of the kidneys and body fluids. Science. 1991; 252:1813–1816. [PubMed: 2063193]
12. Mullins LJ, Bailey MA, Mullins JJ. Hypertension, kidney, and transgenics: a fresh perspective. Physiol Rev. 2006; 86:709–746. [PubMed: 16601272]
13. Cowley AW Jr. The genetic dissection of essential hypertension. Nat Rev Genet. 2006; 7:829–840. [PubMed: 17033627]
14. McGrath MF, de Bold ML, de Bold AJ. The endocrine function of the heart. Trends Endocrinol Metab. 2005; 16:469–477. [PubMed: 16269246]
15. Newton-Cheh C, et al. Association of common variants in *NPPA* and *NPPB* with circulating natriuretic peptides and blood pressure. Nat Genet. 2009; 41:348–353. [PubMed: 19219041]
16. Barbato E, et al. Influence of rs5065 atrial natriuretic peptide gene variant on coronary artery disease. J Am Coll Cardiol. 2012; 59:1763–1770. [PubMed: 22575314]
17. Cannone V, et al. Atrial natriuretic peptide genetic variant rs5065 and risk for cardiovascular disease in the general community: a 9-year follow-up study. Hypertension. 2013; 62:860–865. [PubMed: 24041948]
18. Hodgson-Zingman DM, et al. Atrial natriuretic peptide frameshift mutation in familial atrial fibrillation. N Engl J Med. 2008; 359:158–165. [PubMed: 18614783]
19. Lynch AI, Claas SA, Arnett DK. A review of the role of atrial natriuretic peptide gene polymorphisms in hypertension and its sequelae. Curr Hypertens Rep. 2009; 11:35–42. [PubMed: 19146799]
20. Yan W, Sheng N, Seto M, Morser J, Wu Q. Corin, a mosaic transmembrane serine protease encoded by a novel cDNA from human heart. J Biol Chem. 1999; 274:14926–14935. [PubMed: 10329693]
21. Jiang J, et al. Ectodomain shedding and autocleavage of the cardiac membrane protease corin. J Biol Chem. 2011; 286:10066–10072. [PubMed: 21288900]
22. Rockwell NC, Krysan DJ, Komiyama T, Fuller RS. Precursor processing by kex2/furin proteases. Chem Rev. 2002; 102:4525–4548. [PubMed: 12475200]
23. Beaubien G, et al. The distinct gene expression of the pro-hormone convertases in the rat heart suggests potential substrates. Cell Tissue Res. 1995; 279:539–549. [PubMed: 7736551]
24. Wang W, et al. Impaired sodium excretion and salt-sensitive hypertension in corin-deficient mice. Kidney Int. 2012; 82:26–33. [PubMed: 22418978]
25. Constam DB, Robertson EJ. SPC4/PACE4 regulates a TGFβ signaling network during axis formation. Genes Dev. 2000; 14:1146–1155. [PubMed: 10809672]
26. Wang W, et al. Salt-sensitive hypertension and cardiac hypertrophy in transgenic mice expressing a corin variant identified in blacks. Hypertension. 2012; 60:1352–1358. [PubMed: 22987923]
27. Dong N, et al. Corin mutations K317E and S472G from preeclamptic patients alter zymogen activation and cell surface targeting. J Biol Chem. 2014; 289:17909–17916. [PubMed: 24828501]
28. Taneda H, Andoh K, Nishioka J, Takeya H, Suzuki K. Blood coagulation factor Xa interacts with a linear sequence of the kringle 2 domain of prothrombin. J Biochem. 1994; 116:589–597. [PubMed: 7852276]

29. Guipponi M, et al. The transmembrane serine protease (TMPRSS3) mutated in deafness DFNB8/10 activates the epithelial sodium channel (ENaC) *in vitro*. Hum Mol Genet. 2002; 11:2829–2836. [PubMed: 12393794]
30. Chan JC, et al. Hypertension in mice lacking the proatrial natriuretic peptide convertase corin. Proc Natl Acad Sci USA. 2005; 102:785–790. [PubMed: 15637153]
31. Chen HH. Heart failure: a state of brain natriuretic peptide deficiency or resistance or both! J Am Coll Cardiol. 2007; 49:1089–1091. [PubMed: 17349889]
32. Chen S, et al. Protease corin expression and activity in failing hearts. Am J Physiol Heart Circ Physiol. 2010; 299:H1687–H1692. [PubMed: 20802129]
33. Dries DL. Process matters: Emerging concepts underlying impaired natriuretic peptide system function in heart failure. Circ Heart Fail. 2011; 4:107–110. [PubMed: 21406676]
34. Gladysheva IP, et al. Corin overexpression improves cardiac function, heart failure, and survival in mice with dilated cardiomyopathy. Hypertension. 2013; 61:327–332. [PubMed: 23232642]
35. Li JP, et al. The association between paired basic amino acid cleaving enzyme 4 gene haplotype and diastolic blood pressure. Chin Med J (Engl). 2004; 117:382–388. [PubMed: 15043778]
36. Cohen JC, Hobbs HH. Genetics. Simple genetics for a complex disease. Science. 2013; 340:689–690. [PubMed: 23661745]
37. Horton JD, Cohen JC, Hobbs HH. PCSK9: a convertase that coordinates LDL catabolism. J Lipid Res. 2009; 50(suppl):S172–S177. [PubMed: 19020338]
38. Seidah NG, Awan Z, Chretien M, Mbikay M. PCSK9: a key modulator of cardiovascular health. Circ Res. 2014; 114:1022–1036. [PubMed: 24625727]
39. Davidson MH. Dyslipidaemia: PCSK9 antibodies: a dividend of the genomics revolution. Nat Rev Cardiol. 2013; 10:618–619. [PubMed: 24019032]
40. Seidah NG, Prat A. The biology and therapeutic targeting of the proprotein convertases. Nat Rev Drug Discov. 2012; 11:367–383. [PubMed: 22679642]
41. Liao X, Wang W, Chen S, Wu Q. Role of glycosylation in corin zymogen activation. J Biol Chem. 2007; 282:27728–27735. [PubMed: 17660514]
42. Knappe S, Wu F, Masikat MR, Morser J, Wu Q. Functional analysis of the transmembrane domain and activation cleavage of human corin: design and characterization of a soluble corin. J Biol Chem. 2003; 278:52363–52370. [PubMed: 14559895]
43. Wu F, Yan W, Pan J, Morser J, Wu Q. Processing of pro-atrial natriuretic peptide by corin in cardiac myocytes. J Biol Chem. 2002; 277:16900–16905. [PubMed: 11884416]
44. Qi X, Jiang J, Zhu M, Wu Q. Human corin isoforms with different cytoplasmic tails that alter cell surface targeting. J Biol Chem. 2011; 286:20963–20969. [PubMed: 21518754]
45. Dinter A, Berger EG. Golgi-disturbing agents. Histochem Cell Biol. 1998; 109:571–590. [PubMed: 9681636]
46. Malfait AM, et al. A role for PACE4 in osteoarthritis pain: evidence from human genetic association and null mutant phenotype. Ann Rheum Dis. 2012; 71:1042–1048. [PubMed: 22440827]
47. Jiang J, Pristera N, Wang W, Zhang X, Wu Q. Effect of sialylated O-glycans in pro-brain natriuretic peptide stability. Clin Chem. 2010; 56:959–966. [PubMed: 20348402]
48. Vasudevan NT, et al. $G_{\beta\gamma}$-independent recruitment of G-protein coupled receptor kinase 2 drives tumor necrosis factor α-induced cardiac β-adrenergic receptor dysfunction. Circulation. 2013; 128:377–387. [PubMed: 23785004]
49. Dong N, et al. Plasma-soluble corin in patients with heart failure. Circ Heart Fail. 2010; 3:207–211. [PubMed: 20061521]

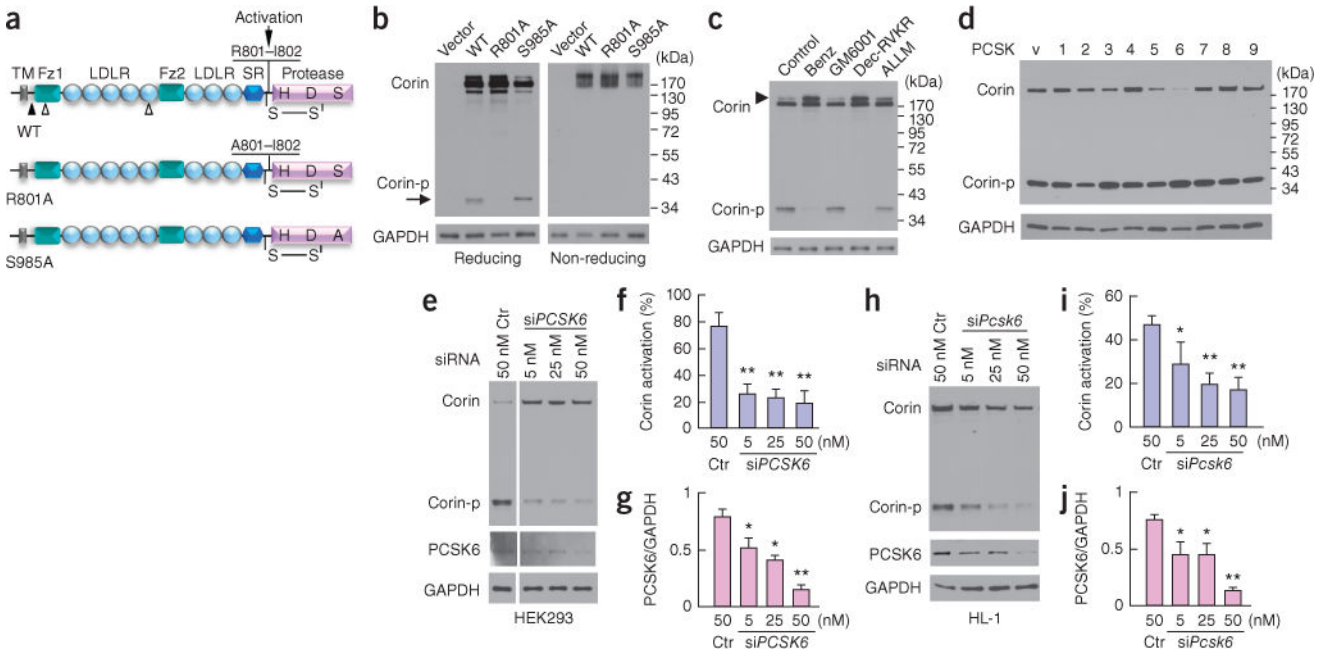

Figure 1.

Corin activation cleavage. (**a**) Corin domains and variants. WT corin and the R801A and S985A variants are diagrammed. An arrow indicates the corin activation site. Filled and open arrowheads indicate ADAM-mediated shedding and corin autocleavage sites, respectively. A disulfide bond (S-S) connects the propeptide and the protease domain. TM, transmembrane; Fz, frizzled; LDLR, LDL receptor; SR, scavenger receptor. His (H), Asp (D) and Ser (S) indicate catalytic residues. (**b**) Western blot analysis to assess corin activation in HEK293 cells transiently transfected with the indicated plasmids under reducing and non-reducing conditions. The zymogen form of corin (Corin) and the cleaved and activated form of corin (Corin-p, arrow) were detected using an antibody against a V5 tag in plasmid-encoded corin. (**c**) Western blot analysis of corin activation cleavage in HEK293 cells stably transfected with WT corin and cultured with the protease inhibitors benzamidine (Benz), GM6001, dec-RVKR-cmk (Dec-RVKR) or ALLM. The arrowhead indicates the corin zymogen band on the cell surface (see Supplementary Fig. 3). (**d**) Western blot analysis of corin activation in HEK293 cells transfected with plasmids encoding PCSK1–PCSK9 or an empty vector (v). (**e–j**) Western blot analysis of corin activation (Corin-p versus Corin) in HEK293 (**e–g**) and HL-1 (**h–j**) cells transfected with siRNAs targeting the human *PCSK6* (si*PCSK6*) and mouse *Pcsk6* (si*Pcsk6*) genes, respectively. Endogenous PCSK6 levels, normalized to GAPDH levels, were measured. **P* < 0.05; ***P* < 0.01 versus control siRNA (Ctr) by one-way ANOVA (ANOVA) and Tukey's *post hoc* test. Values are mean ± s.d. (*n* = 4 per group). In **e**, non-adjacent lanes from the same western blot were used, as indicated.

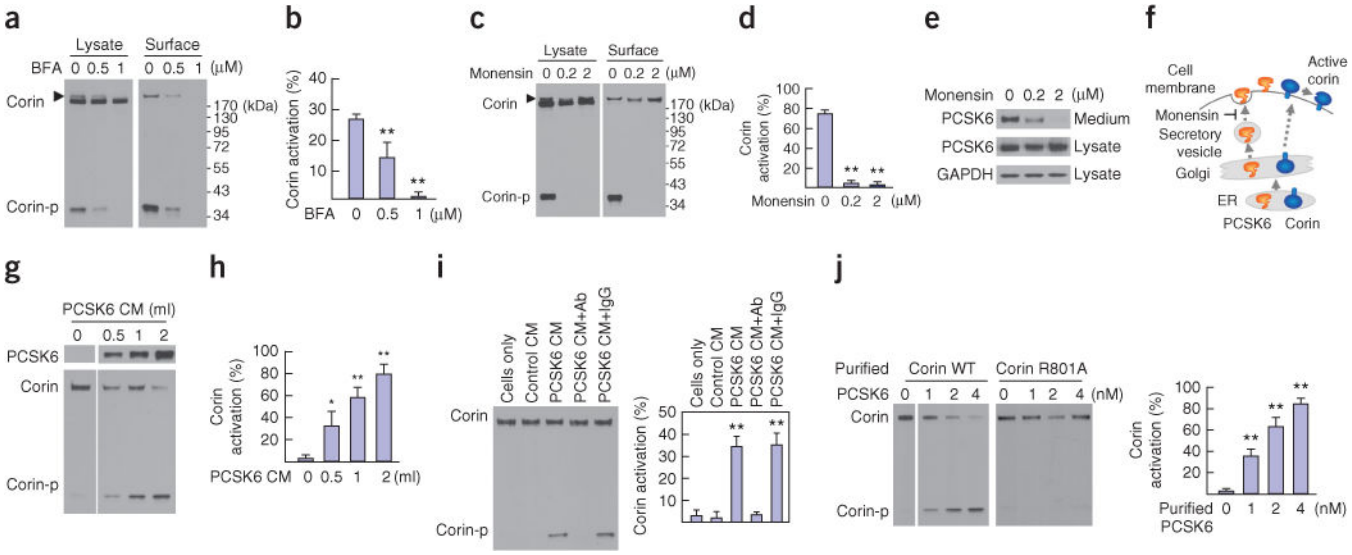

Figure 2.

Cellular mechanism of PCSK6-mediated corin activation. Western blot analysis of corin activation in cell lysates (left) and surface-labeled proteins (right) in HEK293 cells treated with BFA (**a,b**) or monensin (**c,d**). Arrowheads indicate corin zymogen bands on the cell surface. Corin-p indicates the cleaved protease domain fragment. (**b,d**) Percentages of corin activation were calculated on the basis of densitometry from western blots of cell lysates. Values are mean ± s.d. (n = 3 per group). **P < 0.01 versus control by one-way ANOVA and Tukey's *post hoc* test. (**e**) Western blot analysis of PCSK6 in the culture medium and lysate from monensin-treated HEK293 cells. (**f**) A proposed model, in which PCSK6 and corin traffic separately to the cell surface, where PCSK6 activates corin. Monensin blocks PCSK6 secretion via secretory vesicles. (**g,h**) Western blot analysis of corin activation (bottom) in transfected HEK293 cells treated with increasing amounts of PCSK6-containing conditioned medium (PCSK6 CM, top). *P < 0.05; **P < 0.01 versus control (n = 3 per group). (**i**) Western analysis of corin activation with PCSK6 CM with or without immunodepletion using a PCSK6-specific antibody (Ab) or control IgG. Corin-transfected HEK293 cells without PCSK6 CM treatment (cells only) or treated with CM from untransfected HEK293 cells (control CM) were used as controls. **P < 0.01 versus control CM (n = 3 per group). (**j**) Western blot analysis of corin activation by increasing amounts of purified PCSK6. Activation was tested for WT corin (left) and the cleavage site variant R801A (right). Percentages of corin activation were calculated on the basis of densitometry from western blots of WT corin. **P < 0.01 versus control in corin WT (left) (n = 3 per group). In **g** and **j** (left), non-adjacent lanes from the same western blot were used, as indicated. Values are mean ± s.d.

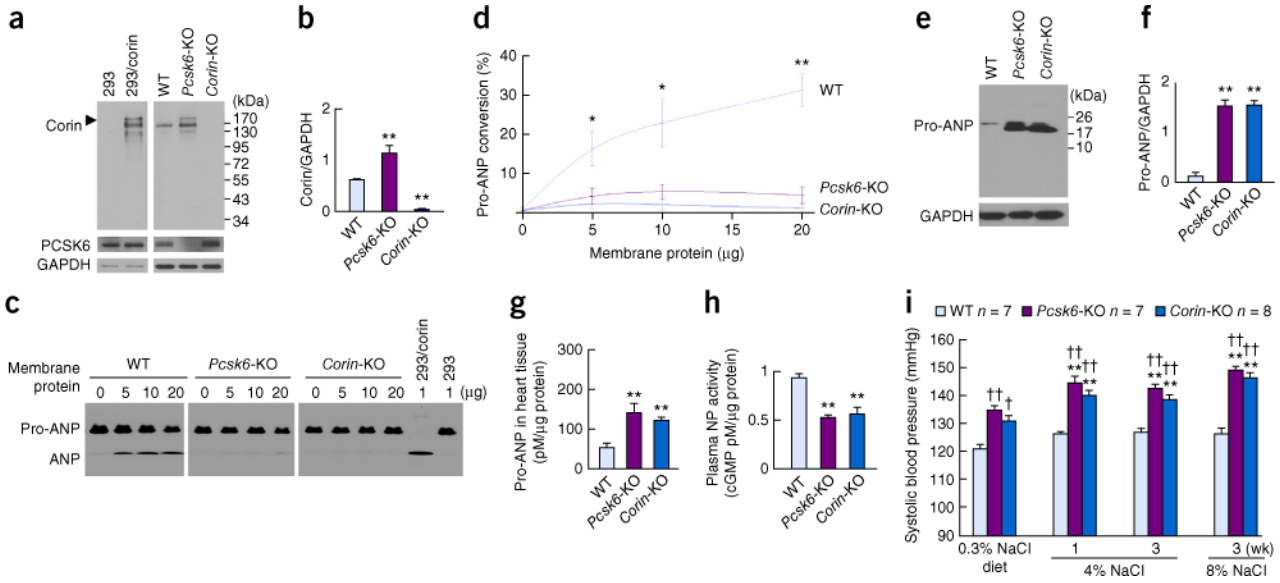

Figure 3.

Analyses in *Pcsk6*-KO mice. (**a**) Western blot analysis of corin and PCSK6 in WT, *Pcsk6*- and *Corin*-KO mouse hearts. Untransfected (293) and corin-transfected (293/corin) HEK293 cells were used as controls. An arrowhead indicates the corin zymogen band. The anti-corin antibody used in this experiment does not recognize the cleaved protease domain fragment. (**b**) Levels of corin protein in mouse hearts, normalized to GAPDH levels, were calculated. $^{**}P < 0.01$ versus WT ($n = 3$ per group). (**c,d**) Pro-ANP processing activity of cell membranes from mouse hearts, as indicated by pro-ANP conversion to ANP in western blots. HEK293 cell samples were used as controls. Percentages of pro-ANP conversion were calculated on the basis of densitometry from western blots. $^{*}P < 0.05$; $^{**}P < 0.01$ versus *Pcsk6*-KO and *Corin*-KO mice ($n = 3$ per group). (**e,f**) Western blot analysis of pro-ANP in mouse hearts. Levels of pro-ANP in mouse hearts, normalized to GAPDH levels, were calculated. $^{**}P < 0.01$ versus WT ($n = 3$ per group). (**g**) ELISA analysis of pro-ANP levels in mouse hearts. $^{**}P < 0.01$ versus WT ($n = 5$ per group). (**h**) Plasma natriuretic peptide (NP) activity. $^{**}P < 0.01$ versus WT ($n = 5$ per group). In **d,f–h**, the results for *Pcsk6*- and *Corin*-KO mice were not significantly different. (**i**) Systolic blood pressure in mice on normal-salt (0.3% NaCl) and high-salt (4% and 8% NaCl) diets at 1 and 3 weeks (wk). $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus WT for groups on the same diet; $^{**}P < 0.01$ versus mice of the same genotype on normal-salt diet by two-way ANOVA and Bonferroni *post hoc* test. The results for *Pcsk6*- and *Corin*-KO mice were not significantly different on either the normal-salt or high-salt diets. Values are mean ± s.d.

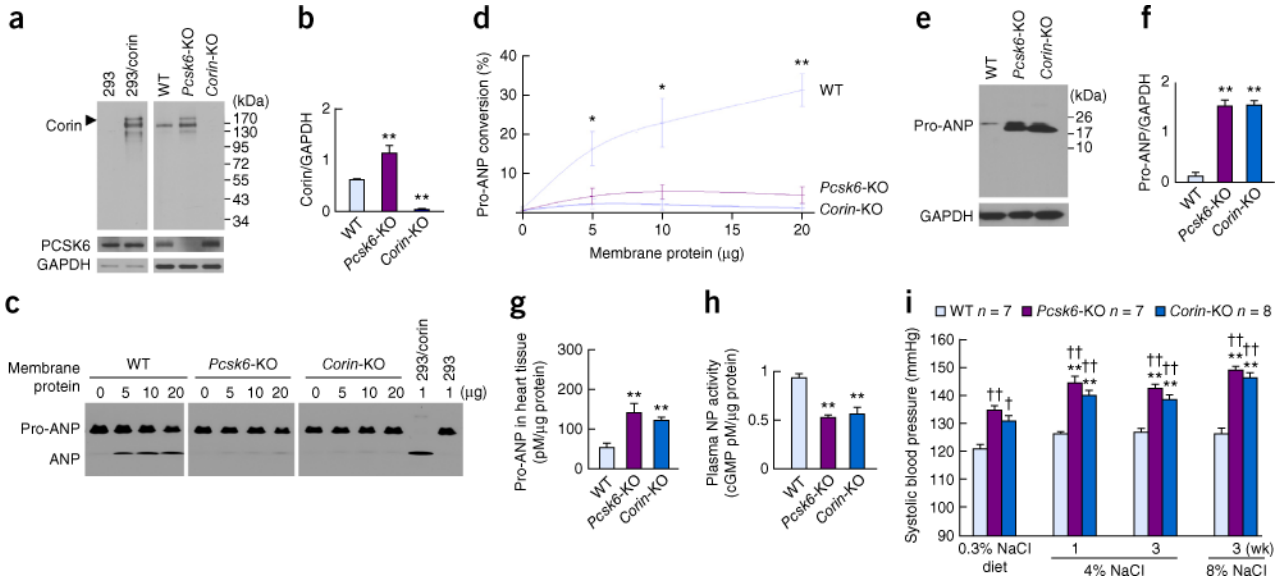

Figure 4.

PCSK6-mediated activation of human corin variants and analysis of the D282N PCSK6 variant. (**a**) Schematic indicating the location of the amino acid substitutions in the corin variants examined. (**b**) Western blot analysis of corin activation in HEK293 cells stably overexpressing PCSK6 (HEK293- PCSK6). WT corin and the activation cleavage site mutant R801A were used as positive and negative controls, respectively. (**c**) Western blot analysis of corin activation in HEK293-PCSK6 cells expressing the indicated corin variants. (**d**) DNA sequencing traces detecting a G/A mutation in a hypertensive individual. This mutation causes an Asp282Asn (D282N) substitution in the PCSK6 catalytic domain. (**e**) The PCSK6 signal peptide (Sp) and the pro, catalytic, P and cysteine (Cys)-rich domains are indicated. (**f**) Western blot analysis of corin activation in HEK293 cells transfected with plasmids expressing WT PCSK6 and the D282N variant or the vector (Vec). Cropped sections of a western blot from the same experiment were used. (**g**) Percentages of corin activation (Corin-p versus Corin) were calculated on the basis of densitometry of western blots in **f**. **P* < 0.05; ***P* < 0.01 versus vector (Vec) by one-way ANOVA and Tukey's *post hoc* test (*n* = 3 per group). (**h**) Western blot analysis of PCSK6 expression in the culture medium (CM) and lysate from HEK293 cells expressing WT PCSK6 or the D292N variant. (**i,j**) Western analysis of corin activation in HEK293 cells transfected with WT corin incubated with increasing amounts of conditioned medium (CM) containing either WT PCSK6 or the D282N variant. **P* < 0.05; ***P* < 0.01 versus vector (Vec) (*n* = 3 per group). Values are mean ± s.d.