Csk Regulates Blood Pressure by Controlling the Synthetic Pathways of Aldosterone

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Background: Blood pressure is regulated by a network of diverse physiological pathways. The C-terminal Src kinase (CSK) locus (15q24) is associated with blood pressure in various ethnic groups. It was recently reported that *Csk* insufficiency increases blood pressure through Src. The mechanisms of hypertension in *Csk*^{+/-} mice are examined further in this study.

Methods and Results: To identify a causal component responsible for hypertension in *Csk*^{+/-}, the heart rate was measured by electrocardiogram and plasma volume by Evans blue dilution. Plasma volume increased in *Csk*^{+/-} compared with wild-types, while the heart rate did not change. Plasma sodium and aldosterone levels rose consistently in *Csk*^{+/-} vs. wild-types, and spironolactone, a mineralocorticoid receptor antagonist, reduced blood pressure. The amounts of Sgk1 and Na⁺/K⁺-ATPase (NKA) increased in the kidney of *Csk*^{+/-} compared with wild-types. It was also found that Cyp11b2 (aldosterone synthase) was upregulated in the adrenal glands of *Csk*^{+/-}, and that Csk was enriched in the zona glomerulosa of adrenals, the major site of aldosterone production in the normal mouse.

Conclusions: The results of the present study identify a physiological pathway by which blood pressure is regulated, in which the insufficiency of *Csk* induces aldosterone production with zonal specificity in the adrenal glands, increasing sodium reabsorption and plasma volume and thus resulting in hypertension.

Key Words: Adrenal gland; Aldosterone; Blood pressure; C-terminal Src kinase (CSK); Plasma volume

Blood pressure is a complex trait that is regulated by a network of physiological pathways that involve the modulation of heart rate, vascular tone, and blood volume.¹ Short-term regulation of blood pressure is attained via the effects of sympathetic/parasympathetic activation on vascular tone and cardiac contractility through the sensing of baroreceptors in large arteries, whereas long-term regulation occurs through the effects of the renin-angiotensin-aldosterone system (RAAS) on the kidney in controlling blood volume.² Despite their classical definition, short-term regulatory mechanisms are not easily dissected from long-term mechanisms, because the autonomic nervous system and RAAS permeate common target tissues and often relay signals through shared signaling pathways.

Genetic research, including genome-wide association studies (GWASs), has identified approximately 116 single-nucleotide polymorphisms (SNPs) and rare mutations that contribute to the genetic architecture of blood pressure and hypertension.^{3–7} It is now evident that hypertension is a polygenic trait, wherein rare syndromes of hypertension

represent extreme cases, because most hypertensive cases are not explained by a mutation in a single gene.³ Further, the finding of genes that cause monogenic syndromes highlights the significant functions of the kidney and adrenal gland in regulating blood pressure.^{2,3} Specifically, 10% of all monogenic forms of hypertension are estimated to have primary aldosteronism, suggesting that the homeostatic maintenance of aldosterone levels and the effect of aldosterone on kidney function are pivotal in keeping blood pressure within the normal range.³

Despite the increase in the discoveries of SNPs that are associated with blood pressure, few cases have revealed an association between a genetic variation and the causal pathway that leads to hypertension. The most notable studies have reported a variant (rs13333226) in the promoter region of the uromodulin gene (*UMOD*),⁸ a SNP in the promoter (rs3918226) near the endothelial nitric oxide synthase (*eNOS*) gene,^{9,10} a SNP (rs5068) in the 3' untranslated region of *NPPA*, the gene that encodes for atrial natriuretic peptide (ANP),^{11,12} and a SNP (rs17249754)

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near the plasma membrane calcium-transporting ATPase 1 gene (*ATP2B1*).^{13,14}

The 15q24 locus is one of many that have been identified by blood pressure GWASs in Asians and Europeans,^{11,15,16} and has been confirmed by GWASs in Japanese, Koreans, and Europeans.¹⁷ We recently reported that this locus harbors C-terminal Src kinase (*CSK*), a causative gene that is associated with changes in blood pressure and regulates blood pressure through Src.¹⁸

Csk represses the activity of Src family tyrosine kinases (Src, Fyn, and Lyn) by phosphorylating their negative regulatory tyrosine in vitro,^{19,20} and functions as their negative regulator in vivo.^{21,22} Mice embryos that are null for *Csk* die at embryonic day 9–10 and exhibit several developmental defects, including abnormal vascularization.^{21,23} Csk is also associated with blood pressure, as supported by a study of spontaneously hypertensive rats (SHRs) in which it was reported that low activation of Csk by angiotensin II (Ang II) upregulates Src compared with normotensive Wistar-Kyoto rats (WKY), accelerating Ang II-induced growth in the vascular smooth muscle cells (VSMCs) of SHRs.²⁴ A genetic analysis of crosses between *Csk* mutant mice and *Src* or *Fyn* mutant mice demonstrated that *Src*, but not *Fyn*, is in part epistatic to *Csk*, wherein some of the defects in *Csk*-null embryos were rescued in *Src/Csk* double-knockout embryos.²⁵

In contrast to the few studies on Csk, Src has been examined extensively with regard to blood pressure regulation, particularly in the RAAS in various tissues. As discussed, the activity of Src is important in Ang II-induced vasoconstriction of VSMCs. Ang II stimulates Src through angiotensin II type 1 receptor (AT1R) in rat VSMCs,²⁶ and Ang II-induced intracellular Ca^{2+} mobilization and vascular contraction are abolished in *Src*^{-/-} VSMCs and PP2-treated cells, respectively.²⁷ A study in hypertensive rats that were induced by the 2-kidney 1-clip (2K1C) mechanism showed that Src activation was greater in the hypothalamus of 2K1C rats than in sham-operated rats, and that inhibition of Src negated the Ang II-induced increase in sympathetic nerve activity and blood pressure of 2K1C, implicating Src in autonomic nerve regulation of blood pressure.²⁸

Src activation is also required for Ang II-mediated functions in the kidney. In the renal proximal tubules, Src mediates Ang II-induced fluid reabsorption through the apical Na^+/H^+ exchanger (NHE) and the basolateral $Na^+-HCO_3^-$ cotransporter (NBC), and *Csk* overexpression inhibits the activation of both NHE and NBC.^{29,30} Notably, Src modulates Ang II-induced aldosterone production in the adrenal gland. Inhibition of Src by PP2 increases *CYP17A1* mRNA levels, slowing the synthesis of aldosterone in Ang II-stimulated H295R adrenal cells.³¹ Further, the binding of Ang II to AT1R enhances the activity of SRC and protein kinase C (PKC), which upregulates *CYP11B2*.^{32,33}

Although many studies in animals, cells, and human populations have implicated SRC and CSK in blood pressure regulation, the mechanisms through which this occurs has not been determined. We have reported that knockdown and haploinsufficiency of *Csk* increase blood pressure through Src. Using the *Csk*^{+/-} mouse, we have established a regulatory pathway in which *Csk* insufficiency induces the synthesis of aldosterone through the upregulation of *Cyp11b2*, resulting in high blood pressure.

Figure 1. Increased plasma volume and sodium reabsorption result in hypertension dependent on Src in *Csk*^{+/-} mice. **(A)** Inhibition of Src by PP2 (50 μg/kg body weight) normalizes high blood pressure in *Csk*^{+/-} mice (n=5–19). The left panel (a line graph) shows blood pressure as the average value at each minute from 40 to 80 min after anesthesia, and the right panel (a bar graph) is the mean blood pressure in each group for 40 min. **(B)** Plasma volume increased significantly in *Csk*^{+/-}, and PP2 treatment lowered plasma volume in *Csk*^{+/-} but not in wild-types (no treatment, n=20–21; PP2, n=13). **(C)** The level of plasma sodium was significantly increased in *Csk*^{+/-} and normalized to wild-type levels on PP2 treatment (no treatment, n=20–21; PP2, n=10–13). **(D)** The level of plasma potassium did not differ between *Csk*^{+/-} and wild-type and was significantly reduced by PP2 in *Csk*^{+/-} (no treatment, n=20–21; PP2, n=10–12). The results are reported as mean ± SEM. *P<0.05, **P<0.01, and ***P<0.005 vs. respective controls.

Methods

Animal Research and Ethics Statement

All mice were housed and handled in a pathogen-free facility at the College of Pharmacy, Kyung Hee University, as per the Guide for the Care and Use of Laboratory Animals, fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice were maintained on a 12-h light/dark cycle at constant temperature with free access to food (LabDiet 5L79, St. Louis, MO, USA) and water. Every effort was made to minimize the number of animals that was sacrificed and their suffering. Animals were anesthetized by intraperitoneal (i.p.) injection of tribromoethanol (Avertin, 18 mL of working solution per kg body weight), the working solution of which was diluted to 25 μg/mL in 0.9% NaCl from the stock solution (1 g/mL 2,2,2-tribromoethanol dissolved in tertiary amyl alcohol), and euthanized by removal of the heart. The experiments were approved by the local committee for the Care and Use of Laboratory Animals, Kyung Hee University (license number: KHUASP(SE)-16-035).

Table. ECG Parameters of *Csk*^{+/-} and Wild-Types

ECG trait	<i>Csk</i> ^{+/+} (n=10)	<i>Csk</i> ^{+/-} (n=10)	P-value (Mann-Whitney U-test)
Heart rate (beats/min)	405.8±6.97	392.8±7.00	0.315
PR interval (ms)	58.0±1.35	58.3±1.23	0.661
QRS duration (ms)	12.5±0.37	12.6±0.34	0.796
QTc interval (ms)	70.3±5.44	64.5±1.43	0.971

Results are expressed as mean±SEM. ECG, electrocardiogram.

Mouse Genotyping

Csk knockout heterozygous mice, B6.129S-*Csk*^{tm1Sor}/J, were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Heterozygous and wild-type homozygous pairs were mated, and the progeny was genotyped by polymerase chain reaction (PCR) with the primer set using the wild type (5'-CGCAGTCTACGAGGTGATGA-3'), the mutant (5'-CCTTCTATCGCCTTCTTGACG-3'), and the common reverse primer (5'-GGGCTCAGTTCAA GTTCAGG-3').

Statistical Analysis

The statistical analysis was performed using SPSS (IBM SPSS Statistics 22.0). The data were analyzed with the genotype and experimental conditions blinded. Two-group comparisons were performed by using the Mann-Whitney U-test, because the U-test is generally considered to be more powerful than the t-test.³⁴ The parenthetical values in the legend indicate the number of mice that was used for each group. The results are reported as mean±SEM. Differences with *P*<0.05 were considered statistically significant. **P*<0.05, ***P*<0.01, and ****P*<0.005 vs. respective controls.

Further details regarding the methods used, including blood pressure measurement, reagents, electrocardiogram, plasma volume determination, plasma and urine analyses, quantitative real-time PCR, western blotting, immunohistochemistry and immunofluorescence, are described in the **Supplementary File 1**.

Results

Increased Plasma Volume and Sodium Reabsorption Cause Hypertension Through Src in *Csk*^{+/-} Mice

Csk modulates blood pressure through Src activation.¹⁸ Here, we confirmed that the administration of PP2 (50 µg/kg body weight), a Src inhibitor, significantly reduced blood pressure only in *Csk*^{+/-} mice, without a significant effect in wild-type mice (*Csk*^{+/+}) (**Figure 1A**).

Major components that regulate blood pressure involve cardiac output and blood volume.^{2,3,35} Cardiac output is affected by heart rate and stroke volume.^{3,36} To determine the contribution of cardiac output, we measured the heart rate by electrocardiogram (ECG) in *Csk*^{+/-} and wild-types. There was no significant change in ECG parameters, including heart rate, PR interval, QRS duration, and QTc interval (**Table**).

Then, we measured plasma volume using Evans blue dye in both groups.³⁷ Plasma volume rose significantly in *Csk*^{+/-} compared with wild-types (**Figure 1B**). Treatment with PP2 negated this increase in *Csk*^{+/-} but did not significantly alter levels in wild-type animals, which is consistent with its effect on blood pressure (**Figure 1B**). These results suggest

that elevations in plasma volume underlie hypertension in *Csk*^{+/-} and are attributable to *Csk*/Src signaling.

To determine whether the increase in plasma volume was caused by ion reabsorption, we measured the amounts of plasma Na⁺ (sodium) and K⁺ (potassium) using ion-selective electrodes (ISEs). Plasma Na⁺ concentrations rose significantly in *Csk*^{+/-} compared with wild-type mice and declined on treatment with PP2 (**Figure 1C**). Plasma K⁺ concentrations did not differ between *Csk*^{+/-} and wild-type mice and were decreased significantly with PP2 in *Csk*^{+/-} (**Figure 1D**). The consistent changes in plasma volume and Na⁺ levels suggest that the alteration in plasma volume is attributable to increased Na⁺ reabsorption due to *Csk* insufficiency.

Increased Plasma Aldosterone Causes Hypertension in *Csk*^{+/-}

Because plasma volume and Na⁺ levels are regulated directly by aldosterone, we measured the aldosterone content in mouse plasma and urine using an enzyme immunoassay (EIA) kit. Plasma aldosterone levels were significantly higher in *Csk*^{+/-} vs. wild-types (**Figure 2A**), whereas urine aldosterone levels did not differ between the groups (**Figure 2B**), indicating that aldosterone is a significant factor of hypertension in *Csk*^{+/-} mice. After PP2 treatment, plasma aldosterone levels failed to decrease in either group (**Figure 2A**), but urine aldosterone fell significantly by 45% in *Csk*^{+/-} (**Figure 2B**).

To examine the function of aldosterone in regulating blood pressure in *Csk*^{+/-}, we measured the effects of spironolactone, a mineralocorticoid receptor (MR) antagonist, via i.p. injection. Spironolactone decreased blood pressure similarly in *Csk*^{+/-} and wild-types (**Figure 2C**), suggesting MR-dependent hypertension.

To determine whether the increase in aldosterone influenced the expression of ion reabsorption-related molecules in the kidney, we performed western blot to estimate the levels of serum- and glucocorticoid-regulated kinase 1 (Sgk1) and the kidney channels: ROMK, a renal outer medullary potassium channel; ENaCa/β, an epithelial sodium channel; and NKAα, the Na⁺/K⁺-ATPase α subunit. ROMK co-localizes with Src in the kidney, and the stimulation of Src directly and indirectly induces endocytosis of ROMK, leading to the conservation of potassium.³⁸ ENaCa/β and NKAα are the 2 main effectors of sodium reabsorption in the cortical collecting duct of the kidney, and their activities and levels change in response to aldosterone.^{39,40} Sgk1 upregulates rapidly due to aldosterone in cortical collecting duct cells, increasing the number of active ENaCa/βs.⁴¹

In our results, the level of ROMK decreased significantly in the kidney of *Csk*^{+/-}, which is consistent with several reports that have suggested a direct function of Src in

Figure 2. Increased aldosterone leads to hypertension in *Csk*^{+/-}. **(A)** Plasma aldosterone was significantly higher in *Csk*^{+/-} than in wild-types and increased further by PP2 treatment in both groups (no treatment, n=40; PP2, n=19–21). **(B)** Urine aldosterone was unchanged between *Csk*^{+/-} and wild-types but decreased significantly in *Csk*^{+/-} on PP2 treatment (no treatment, n=22–23; PP2, n=20–27). **(C)** Spironolactone (Spiro), an MR inhibitor, was injected i.p. into mice (50mg/kg) 2h before measurement. Inhibition of MR lowered blood pressure in *Csk*^{+/-} and wild-types (no treatment, n=12–18; spironolactone, n=7–11). **(D)** Representative western blots of the kidney cortex proteins are shown in the left panel, and the graphs of the relative band density of each protein are presented in the right panels, as normalized to actin. The ROMK channel was significantly decreased in *Csk*^{+/-}, and PP2 treatment normalized its level (n=14–18). The Sgk1 and NKAα channel proteins increased significantly in *Csk*^{+/-} and were partially normalized by PP2 (Sgk1, n=16–18; NKAα, n=13–21). ENaCa and β channels were unchanged between groups (ENaCa, n=12–16; ENaCβ, n=4–10). The results are reported as mean±SEM. *P<0.05, **P<0.01, and ***P<0.005 vs. respective controls.

degrading ROMK in the kidney (Figures 2D,S1).³⁸ The amounts of Sgk1 and NKAα rose significantly in *Csk*^{+/-}, whereas the total amounts of ENaCa/β were unchanged (Figure 2D). PP2 partially normalized the increases in Sgk1 and NKAα (Figure 2D). These results suggest that aldosterone facilitates transcellular movement of sodium by upregulating Sgk1 and NKAα in *Csk*^{+/-}.

Altered Expression of Steroidogenic Enzymes Increases Aldosterone Production in the Adrenal Gland of *Csk*^{+/-}

To examine whether a deficiency in *Csk* affects the synthetic pathway of aldosterone, we measured the mRNA levels of 5 cytochrome P450 family enzymes that participate in steroidogenic pathways in the adrenal gland of mice: *StAR*, a steroidogenic acute regulatory protein; *Cyp11a1*, a cholesterol side-chain cleavage enzyme; *Hsd3b1*, 3β-hydroxysteroid dehydrogenase type 1 (the murine ortholog of human *HSD3B2*); *Cyp11b1*, 11β-hydroxylase; and *Cyp11b2*, aldosterone synthase (Figure 3C).

The mRNA levels of *StAR*, which transports cholesterol into the mitochondria, did not differ between *Csk*^{+/-} and wild-types but increased on PP2 treatment (Figure 3A). *Cyp11a1* declined significantly in *Csk*^{+/-} compared with wild-types and returned to wild-type levels after PP2 treatment (Figure 3A), implying that the overall pool of

precursors that enters the steroidogenic pathways is constrained in *Csk*^{+/-}. The effects of PP2 on *StAR* and *Cyp11a1* expression are consistent with a previous report in which the inhibition of Src increased mRNA levels of *StAR* and *CYP11A1*.⁴²

Hsd3b1 was unchanged in *Csk*^{+/-} and after PP2 treatment (Figure 3A). The mRNA level of *Cyp11b1*, which mediates the last step in corticosterone synthesis, increased in *Csk*^{+/-} compared with wild-types, but was unaffected by PP2 (Figure 3A). *Cyp11b2*, the last enzyme in the synthesis of aldosterone, rose significantly in *Csk*^{+/-} vs. wild-types and returned to basal levels with PP2 (Figure 3A).

To measure the changes in the aldosterone synthase levels in the adrenal gland, a western blot was performed using an antibody against Cyp11b2. Active Src increased significantly in *Csk*^{+/-}, whereas inactive Src declined (Figure 3B). The activation of Src was mitigated to wild-type levels by PP2 but did not normalize completely (Figure 3B). *Csk* remained significantly low in *Csk*^{+/-}, regardless of PP2 treatment (Figure 3B). Consistent with their mRNA levels, Cyp11b2 increased significantly in *Csk*^{+/-}, and normalized to the wild-type level with PP2 treatment (Figure 3B).

Figure 3. Altered expression of steroidogenic enzymes induces aldosterone production in the adrenal glands of *Csk*^{+/-}. (A) Total RNA from adrenal glands was analyzed by qRT-PCR. Data are presented as the average value of the relative mRNA levels of each group, normalized to *Gapdh* mRNA (n=12–27). (A) *StAR* mRNA did not differ between *Csk*^{+/-} and wild-types and increased on PP2 treatment. *Cyp11a1* transcripts fell in *Csk*^{+/-} and were normalized by PP2. *Hsd3b1* mRNA was unchanged between groups. *Cyp11b1* mRNA rose in *Csk*^{+/-} but was unchanged by PP2. The level of *Cyp11b2* mRNA increased in *Csk*^{+/-} and returned to wild-type levels by PP2. (B) Representative western blots of adrenal gland proteins and the graphs of the relative band density of each protein are presented in the left and right panel, respectively. Phosphorylated proteins are normalized to total proteins, and total proteins to actin. Phosphorylated Src^{Y416} (active) increased significantly in *Csk*^{+/-} (n=4–6), whereas phosphorylated Src^{Y527} (inactive) decreased (n=8–10). Activation of Src was partially normalized by PP2. Csk remained low in *Csk*^{+/-}, regardless of PP2 treatment (n=8). The amount of Cyp11b2 increased significantly in *Csk*^{+/-} (n=4), and reverted to wild-type levels by PP2 (n=4). The results are reported as mean ± SEM. *P<0.05, **P<0.01, and ***P<0.005 vs. respective controls. (C) Diagram of steroidogenic enzymes involved in aldosterone production of mice. Cholesterol is transferred into the mitochondria by StAR, which is a rate-limiting step in the production of steroid hormones, and is cleaved by Cyp11a1, resulting in pregnenolone. Consequently, pregnenolone is converted to progesterone by Hsd3b1. Cyp11b1 is the corticosterone synthesis enzyme in the zona fasciculata/zona reticularis (ZF/ZR), and Cyp11b2 is the aldosterone synthesis enzyme, expressed primarily in the zona glomerulosa (ZG).

Csk Abounds in the Zona Glomerulosa of the Mouse Adrenal Gland

To study the expression of Csk in the adrenal gland, we performed an immunohistochemical analysis using an antibody against Csk. The mammalian adrenal gland cortex comprises 3 concentric steroidogenic zones in which the 3 principal types of steroid hormones are produced: mineralocorticoids, such as aldosterone, in the zona glomerulosa (ZG, the outermost zone of the adrenal); glucocorticoids, such as cortisol and corticosterone, in the zona fasciculata (ZF, the middle zone between the glomerulosa and reticularis); and androgens in the zona reticularis (ZR, the innermost zone).^{43,44}

Csk was expressed in the mouse adrenal gland at higher levels in the ZG than in the ZF and ZR, as shown by densitometry of the positive-stained areas (Figure 4A). The zone-specific pattern of Csk expression was then confirmed by immunofluorescence. Csk was clearly visible as red fluorescence, which was strong in the ZG and moderate in the ZR, but absent from the ZF (Figure 4C). In contrast, Src

was ubiquitously expressed throughout all zones (Figure 4B).

These results suggest that a deficiency in *Csk* influences the synthetic pathway of aldosterone by altering Cyp11b2 expression with zonal specificity, as illustrated in Figure 4D.

Discussion

Our data have identified Csk as a key modulator of blood pressure, demonstrating that a deficiency in *Csk* increases plasma volume and sodium reabsorption through greater synthesis of aldosterone in the adrenal gland, ultimately resulting in high blood pressure.

Our 2 principal findings are as follows. Csk regulates blood volume, which is among the major factors that affect blood pressure, such as cardiac output (heart rate × stroke volume), and peripheral vessel resistance. A contribution of heart rate to blood pressure has been ruled out, because the electrocardiogram of *Csk*^{+/-} showed no change in heart rate. The intrinsic resistance of blood vessels was not assessed in our study. The function of Csk in the vasculature

has been presumed to contribute to the generation of hypertension, based on reports in *Csk*-null embryos and VSMCs of SHRs.^{23,24,45} Thus, to further examine the function of *Csk* in the vasculature, it might be more appropriate to study the in vitro vascular reactivity of resistant arteries by using a VSMC-specific *Csk* knockout mouse. In addition to the vasculature, the intrinsic function of *Csk* in other tissues, such as the kidney and brain, must be examined in a mouse with tissue-specific deficiency of *Csk* to rule out the effects of variations in aldosterone.

Our second major finding is the regulatory function of *Csk* in aldosterone formation in the adrenal gland. Our results demonstrate that the synthetic pathway of aldosterone is enhanced in the adrenal gland of *Csk*^{+/-}, as depicted in **Figure 4D**. Plasma aldosterone rises through greater *Cyp11b2* expression in the adrenal gland and exerts its effects on *Sgk1* and the *NKAα* channel in the kidney, increasing sodium reabsorption.

In the kidney, the amount of ROMK channels decreased, but the effect of this reduction on hypertension remained unknown, because plasma potassium levels were unchanged in the kidney of *Csk*^{+/-}. The increase in *Sgk1* suggests that higher aldosterone levels act on kidney channels through the induction of *Sgk1* in *Csk*^{+/-}. Of the 2 main effectors of sodium reabsorption, *NKAs* increase in activity and number in response to aldosterone, whereas *ENaCs* undergo early activation but experience a slow rise in number.^{39,40} In our results, the total amount of *ENaCa/β* did not increase in *Csk*^{+/-}, implying that aldosterone-induced *Sgk1* affects only the activity of *ENaCs*, not their expression level.⁴¹ The upregulation of *NKAα* suggests that transcellular movement of sodium is facilitated by aldosterone-induced *NKAs* in *Csk*^{+/-}.⁴⁰ Treatment with PP2 partially reversed the increase in *Sgk1* and *NKAα* to wild-type levels, likely due to a delayed effect throughout the synthesis of steroidogenic enzymes and the degradation of accumulated proteins.

The regulation of *CYP11B2* by SRC has been supported by several reports. SRC induces members of the nuclear receptor superfamily/nerve growth factor-induced (NGFI-B) family of transcription factors, which are required for the generation of *CYP11B2* transcripts.^{33,46} Recent studies suggest that SRC-induced protein kinase D (PKD/PKCμ) mediates aldosterone production and upregulates *CYP11B2* in the ZG. Constitutively active PKD constructs upregulate *CYP11B2* in human adrenocortical cells, and treatment with PP2 and Src-I, selective Src family kinase inhibitors, represses the activation of PKD, consequently impeding Ang II-induced aldosterone production in primary bovine adrenal glomerulosa cells.^{47,48} Once activated, PKD phosphorylates activating transcription factor (ATF)/cAMP response element binding protein (CREB) members, which are also important for *CYP11B2* transcription.⁴⁹ Notably, we found that *Csk* was enriched in the ZG, whereas *Src* is expressed throughout the adrenal cortex, implying that the regulation of aldosterone production by *Csk* may converge in the ZG. As with steroidogenic enzymes, the adrenal gland exhibits a zone-specific expression pattern of *AT1R* in the ZG,⁵⁰ indicating that the negative regulation of *Cyp11b2* by *Csk* is a safeguard that maintains the normal range of aldosterone production in Ang II-controlled situations.

Our study demonstrates a novel pathway of blood pressure regulation whereby *Csk* influences aldosterone production, therefore modulating sodium reabsorption

Figure 4. *Csk* is expressed in the zona glomerulosa (ZG) of the adrenal gland. The adrenal gland of a wild-type mouse was analyzed for *Csk* and *Src* by immunohistochemistry (**A** and **B**) and immunofluorescence (**C**). The square inset in the upper panel (scale bar, 100 μm) is enlarged in the lower panel (scale bar, 25 μm for **A** and **B**; 10 μm for **C**). (**A**) The expression of *Csk* is more concentrated in the ZG than in the zona fasciculata/zona reticularis (ZF/ZR) in *Csk*-stained adrenal sections and was significantly high in the ZG, based on measurements of the staining density per unit area (n=8). In the lower panel, the black line denotes the border between the capsule and ZG; the red line, the border between ZG and ZF; and the blue line, the border between ZR and medulla. The results are reported as mean ± SEM. *P<0.05 vs. ZG. (**B**) The expression of *Src* was distributed throughout the 3 zones. (**C**) The expression pattern of *Csk* is clearly seen in the ZG and ZR but not ZF. (**D**) The regulatory function of *Csk* in the adrenal gland ZG is depicted. In normal situations, such as in the wild-type mouse, *Csk* represses *Src* activity and therefore maintains the synthetic level of aldosterone at homeostasis by not inducing *Cyp11b2*. However, in such a situation as in *Csk*^{+/-}, the low level of *Csk* increases *Src* activity and induces the synthesis of aldosterone by upregulating *Cyp11b2*.

and plasma volume. Thus, our findings increase our understanding of the mechanisms of aldosterone production in the adrenal gland and aldosterone-inducible hypertension.

Study Limitations

We have used a mouse model with global *Csk* insufficiency, rather than a mouse with an adrenal gland-specific *Csk* deficiency. As *Csk* and *Src* were not only expressed in various tissues including vasculature, kidney, and brain, but their regional functions were also known to contribute to the generation of hypertension; our study should not be overestimated beyond a role of *Csk* in the adrenal gland. Future research is warranted for the important roles of *Csk* in diverse tissues.

Another weakness in our study was that plasma aldo-

sterone levels rose in *Csk*^{+/-} than wild-types, but further increased by PP2 treatment (Figure 2A). We assumed that it might be somewhat explained by the lowered urinary excretion of aldosterone after PP2 treatment (Figure 2B), although the change in urinary excretion was unexplainable in this study. It might be helpful to distinguish the major metabolites (dihydroaldosterone and tetrahydroaldosterone) of aldosterone in mice to understand the effect of PP2 treatment, which was not possible by using an EIA kit.

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Supplementary Files

Supplementary File 1

Supplementary Methods

Figure S1. Src activation is dependent on Csk and inhibited by PP2 treatment in the kidney cortex.

Table S1. Primers for quantitative real-time polymerase chain reaction

Table S2. Lists of antibodies used for WB, IHC, and IF

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