Csk Regulates Blood Pressure by Controlling the Synthetic Pathways of Aldosterone

Sung-Moon Kim; Ji-One Kang, PhD; Ji Eun Lim, PhD; Sue-Yun Hwang, PhD; Bermseok Oh, PhD

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. 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. 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. 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. Further, the finding of genes that cause monogenic syndromes highlights the significant functions of the kidney and adrenal gland in regulating blood pressure. 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.

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), a SNP in the promoter (rs3918226) near the endothelial nitric oxide synthase (eNOS) gene, a SNP (rs5068) in the 3’ untranslated region of NPPA, the gene that encodes for atrial natriuretic peptide (ANP), and a SNP (rs17249754)
near the plasma membrane calcium-transporting ATPase 1 gene (ATP2B1)\textsuperscript{13,14}

The 15q24 locus is one of many that have been identified by blood pressure GWASs in Asians and Europeans\textsuperscript{11,15,16} and has been confirmed by GWASs in Japanese, Koreans, and Europeans.\textsuperscript{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.\textsuperscript{18}

Csk represses the activity of src family tyrosine kinases (Src, Fyn, and Lyn) by phosphorylating their negative regulatory tyrosine in vitro\textsuperscript{19,20} and functions as their negative regulator in vivo\textsuperscript{21,22}. Mice embryos that are null for Csk die at embryonic day 9–10 and exhibit several developmental defects, including abnormal vascularization.\textsuperscript{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 it was reported that low activation of Csk by angiotensin II (Ang II) upregulates Src compared with normotensive II (Ang II) upregulates Src compared with normotensive SHR.\textsuperscript{24}

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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 vascular smooth muscle cells (VSMCs) of Wistar-Kyoto rats (WKY), accelerating Ang II-induced growth in the vascular smooth muscle cells (VSMCs) of SHR.\textsuperscript{25} 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.\textsuperscript{26}

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\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) and the basolateral Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{−} cotransporter (NBC), and Csk overexpression inhibits the activation of both NHE and NBC.\textsuperscript{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.\textsuperscript{31} Further, the binding of Ang II to AT1R enhances the activity of SRC and protein kinase C (PKC), which upregulates CYP11B2.\textsuperscript{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\textsuperscript{+/-} 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.

**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 25μg/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).
**Mouse Genotyping**

Csk knockout heterozygous mice, B6.129S-Csk<sup>tm1Sor</sup>/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. 44 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, electrophrocardiogram, 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 Aldosterone Causes Hypertension in Csk<sup>−/−</sup> 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<sup>−/−</sup> mice, without a significant effect in wild-type mice (Csk+/-) (Figure 1A).

Major components that regulate blood pressure involve cardiac output and blood volume. 23,35 Cardiac output is affected by heart rate and stroke volume. 36 To determine the contribution of cardiac output, we measured the heart rate by electrocardiogram (ECG) in Csk<sup>−/−</sup> 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<sup>−/−</sup> compared with wild-types (Figure 1B). Treatment with PP2 negated this increase in Csk<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>+</sup> (sodium) and K<sup>+</sup> (potassium) using ion-selective electrodes (ISEs). Plasma Na<sup>+</sup> concentrations rose significantly in Csk<sup>−/−</sup> compared with wild-type mice and declined on treatment with PP2 (Figure 1C). Plasma K<sup>+</sup> concentrations did not differ between Csk<sup>−/−</sup> and wild-type mice and were decreased significantly with PP2 in Csk<sup>−/−</sup> (Figure 1D). The consistent changes in plasma volume and Na<sup>+</sup> levels suggest that the alteration in plasma volume is attributable to increased Na<sup>+</sup> reabsorption due to Csk insufficiency.

### Table. ECG Parameters of Csk<sup>−/−</sup> and Wild-Types

| ECG trait                  | Csk<sup>−/−</sup> (n=10) | Csk<sup>+</sup>/− (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.
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decorating ROMK in the kidney (Figures 2D,S1). The amounts of Sgk1 and NKAα rose significantly in Csk+/−, whereas the total amounts of ENaCα/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.

**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).
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...
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In our results, the total amount of ENaC \( \alpha \) Csk \( ^{+/−} \), implying that aldosterone-induced Sgk1 induced NKAs in cellular movement of sodium is facilitated by 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 NKAa 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.

In our results, the total amount of ENaC/\( \beta \) did not increase in Csk \( ^{+/−} \), implying that aldosterone-induced Sgk1 affects only the activity of ENaCs, not their expression level. The upregulation of NKAs suggests that transcellular movement of sodium is facilitated by aldosterone-induced NKAs in Csk \( ^{+/−} \). Treatment with PP2 partially reversed the increase in Sgk1 and NKAs 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. Recent studies suggest that SRC-induced protein kinase D (PKD/PKC\( \mu \) 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. Once activated, PKD phosphorylates activating transcription factor (ATF)/cAMP response element binding protein (CREB) members, which are also important for CYP11B2 transcription. 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, 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 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 the adrenal gland and aldosterone-inducible hypertension. 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

Please find supplementary file(s): [http://dx.doi.org/10.1253/circj.CJ-17-0080](http://dx.doi.org/10.1253/circj.CJ-17-0080)