Aldosterone Is Essential for Angiotensin II-Induced Upregulation of Pendrin

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ABSTRACT

The renin-angiotensin-aldosterone system has an important role in the control of fluid homeostasis and BP during volume depletion. Dietary salt restriction elevates circulating angiotensin II (AngII) and aldosterone levels, increasing levels of the Cl⁻/HCO₃⁻ exchanger pendrin in β-intercalated cells and the Na⁺-Cl⁻ cotransporter (NCC) in distal convoluted tubules. However, the independent roles of AngII and aldosterone in regulating these levels remain unclear. In C57BL/6J mice receiving a low-salt diet or AngII infusion, we evaluated the membrane protein abundance of pendrin and NCC; assessed the phosphorylation of the mineralocorticoid receptor, which selectively inhibits aldosterone binding in intercalated cells; and measured BP by radiotelemetry in pendrin-knockout and wild-type mice. A low-salt diet or AngII infusion upregulated NCC and pendrin levels, decreased the phosphorylation of mineralocorticoid receptor in β-intercalated cells, and increased plasma aldosterone levels. Notably, a low-salt diet did not alter BP in wild-type mice, but significantly decreased BP in pendrin-knockout mice. To dissect the roles of AngII and aldosterone, we performed adrenalectomies in mice to remove aldosterone from the circulation. In adrenalectomized mice, AngII infusion again upregulated NCC expression, but did not affect pendrin expression despite the decreased phosphorylation of mineralocorticoid receptor. By contrast, AngII and aldosterone coadministration markedly elevated pendrin levels in adrenalectomized mice. Our results indicate that aldosterone is necessary for AngII-induced pendrin upregulation, and suggest that pendrin contributes to the maintenance of normal BP in cooperation with NCC during activation of the renin-angiotensin-aldosterone system by dietary salt restriction.

Received March 5, 2017. Accepted July 18, 2017. Published online ahead of print. Publication date available at www.jasn.org.

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Pendrin, encoded by SLC26A4, is a Cl⁻/HCO₃⁻ exchanger expressed specifically in β-intercalated cells and regulates acid-base balance by excreting HCO₃⁻ in the urine. In addition to its role in HCO₃⁻ excretion,
several studies have demonstrated that pendrin promotes reabsorption of Cl\(^-\),\(^1\) and increases Na\(^+\) reabsorption in cooperation with the Na\(^+\)-dependent Cl\(^-\)/HCO\(_3\)\(^-\) exchanger.\(^2\)\(^-\)\(^1\)\(^2\)\(^-\)\(^1\)\(^9\)\(^-\)\(^1\)\(^2\)\(^-\)\(^1\)\(^9\)\(^-\)\(^1\)\(^0\) Recently, we identified a phosphorylation site in the ligand-binding domain of the mineralocorticoid receptor (MR) at S843 (pMR-S843), which prevents ligand binding and MR signaling in intercalated cells.\(^9\) In addition to the ligand–receptor relationship in MR activation, there are several factors influencing pendrin expression; plasma AngII and aldosterone levels and potassium concentration ([K\(^+\)]), all of which exert effects upon the other. Indeed, AngII upregulates pendrin expression,\(^2\)\(^,\)\(^8\)\(^,\)\(^9\)\(^,\)\(^1\)\(^9\)\(^-\)\(^1\)\(^2\)\(^-\)\(^1\)\(^9\)\(^-\)\(^1\)\(^0\) which is associated with dephosphorylation of pMR-S843; however, the independent roles of AngII and aldosterone in regulating pMR-S843 levels and pendrin expression remain unclear.

With respect to the physiologic role of pendrin in BP regulation, pendrin overexpression in intercalated cells results in salt-sensitive hypertension,\(^1\)\(^3\) whereas acute deletion of pendrin lowers BP.\(^1\)\(^4\) Moreover, there has been growing interest in pendrin and NCC interactions associated with BP maintenance. However, the precise mechanisms associated with the way in which pendrin contributes to BP maintenance in cooperation with NCC remain obscure. Recent studies indicate that single deletion of pendrin or NCC does not result in a salt-losing phenotype\(^1\)\(^0\)\(^,\)\(^1\)\(^5\)\(^,\)\(^1\)\(^6\); however, double-knockout mice demonstrate severe salt loss and hypotension.\(^1\)\(^0\) Pendrin expression is elevated in the kidneys of NCC-knockout mice,\(^6\)\(^,\)\(^1\)\(^0\) raising the possibility that pendrin or NCC compensates for the loss of the other through their increased expression and activity associated with regulating NaCl reabsorption.

Given the previous finding indicating upregulation of pendrin expression during dietary salt restriction and aldosterone infusion,\(^1\)\(^7\)\(^,\)\(^1\)\(^8\) in this study using pendrin-knockout (PDS\(^{−/−}\)) mice, we evaluate the role of pendrin in the regulation of BP in cooperation with NCC during RAAS activation induced by changes in salt intake from a high-salt (HS) diet to a low-salt (LS) diet. In addition, we investigate the independent roles of AngII and aldosterone on MR phosphorylation and pendrin expression in vivo compared with NCC expression. This comparison resulted in detailed insight into pendrin regulation, given that the role of the RAAS in NCC regulation has been more extensively studied.\(^3\)\(^,\)\(^4\)\(^,\)\(^1\)\(^9\)\(^,\)\(^2\)\(^0\)

**RESULTS**

**Activation of RAAS by NaCl Restriction Increases Pendrin and Decreases pMR-S843 Levels**

During volume depletion, the activated RAAS contributes to maintaining fluid volume through NaCl reabsorption. To modulate intravascular volume by dietary salt intake, mice were fed either an HS (8% NaCl) or LS (0.03% NaCl) diet. As expected, NaCl restriction by an LS diet increased plasma renin activity (30.8±3.2 ng/ml per hour versus 18.8±1.8 ng/ml per hour; P<0.004) (Figure 1A) and plasma aldosterone concentrations (1513±190 pg/ml versus 152±48 pg/ml; P<0.001) (Figure 1B), suggesting activation of the endogenous RAAS. Plasma [K\(^+\)] was not affected by these diets (3.96±0.15 mmol/L versus 4.02±0.05 mmol/L; P=0.66) (Figure 1C). Additionally, an LS diet increased membrane-protein abundance of pendrin by 1.5-fold (P=0.004), NCC by 2.1-fold (P=0.003), and phosphorylation of NCC at threonine-53 (pNCC-T53) by 3.1-fold (P<0.01) (Figure 1, D–G). Furthermore, pMR-S843 levels, but not total MR levels (Figure 1, H and I) in whole kidney lysates were reduced by 61% (P=0.001), despite no changes in plasma [K\(^+\)].

**Dietary Salt Restriction Decreases BP in PDS\(^{−/−}\) Mice**

Activation of the RAAS by dietary salt restriction increased pendrin as well as NCC during volume depletion.\(^1\)\(^5\)\(^,\)\(^1\)\(^6\) To evaluate the physiologic roles of pendrin in maintaining BP during volume depletion, we investigated the effect of dietary salt restriction on BP using PDS\(^{−/−}\) and wild-type (WT) mice. Averaged mean arterial pressures (MAPs) at baseline for an HS diet were similar in both groups (PDS\(^{−/−}\): 124.1±0.8 mm Hg versus WT: 124.3±0.8 mm Hg) (Figure 1, J and K). WT mice showed no significant difference between HS and LS diets (Figure 1, J and K), but PDS\(^{−/−}\) mice exhibited significantly lower MAPs on an LS diet compared with those observed on an HS diet (Figure 1, J and K). These differences between HS and LS diets were significantly greater in PDS\(^{−/−}\) mice relative to those observed in WT mice (PDS\(^{−/−}\): −11.0±0.7 mm Hg versus WT: −1.6±0.6 mm Hg; P<0.001) (Figure 1L), indicating that deletion of the pendrin gene resulted in NaCl loss and resultant BP reduction. On the basis of the previous findings of NCC upregulation in PDS\(^{−/−}\) mice with an LS diet,\(^1\)\(^8\) our results suggest that activation of pendrin contributed to maintaining normal BP in cooperation with NCC during volume depletion with an LS diet, in contrast to the small role of pendrin in the regulation of BP during volume repletion with an HS diet.

**AngII Infusion Increases Pendrin and Decreases pMR-S843 Levels**

Next, we examined whether exogenous AngII also increased pendrin and decreased pMR-S843 levels. AngII infusion increased plasma aldosterone concentrations (257±60 pg/ml versus 65±19 pg/ml; P=0.01) (Figure 2A), whereas plasma...
was not affected by AngII infusion at day 7 (4.01±0.10 mmol/L versus 3.89±0.04 mmol/L; P=0.31) (Figure 2B). Of note, AngII infusion increased membrane-protein abundance of pendrin by 1.4-fold (P=0.001), NCC by 1.8-fold (P=0.01), and pNCC-T53 by 3.0-fold (P=0.01) (Figure 2, C–F), and reduced pMR-S843 levels by 25% (P=0.01) (Figure 2, G and H). These results suggest that both endogenously and exogenously activated RAAS increased membrane-protein abundance of pendrin, NCC, and pNCC-T53 without altering plasma [K⁺], which was associated with MR dephosphorylation.

To investigate whether AngII-induced pendrin upregulation is mediated by MR activation associated with MR dephosphorylation, we administered the MR antagonist (MRA) spironolactone to AngII-treated mice. Spironolactone administration significantly decreased membrane-protein abundance of pendrin by 35% (P<0.001)
AngII Increases NCC, but Not Pendrin Expression in Adrenalectomized Mice Despite MR Dephosphorylation

To dissect the roles of AngII and aldosterone, we performed adrenalectomies to remove aldosterone from the circulation of blood in mice, resulting in complete abolishment of aldosterone in plasma (Figure 3A). In adrenalectomized mice, plasma [K⁺] was unaffected by AngII infusion (4.59 ± 0.15 mmol/L versus 4.64 ± 0.19 mmol/L; P = 0.83) (Figure 3B). AngII infusion upregulated membrane-protein abundance of NCC by 1.9-fold (P = 0.04) and pNCC-T53 by 2.8-fold (P = 0.03), but not that of pendrin (a 1.1-fold change; P = 0.25) in adrenalectomized mice (Figure 3, C–F), although pMR-S843 levels were again reduced in the absence of aldosterone (a 30% reduction; P = 0.04) (Figure 3, G and H). These results strongly supported our hypothesis that aldosterone was necessary for AngII-mediated pendrin expression, despite aldosterone-independent MR dephosphorylation induced by AngII (Figure 2, C and D).

AngII and Aldosterone Coadministration in Adrenalectomized Mice Increases Pendrin Expression along with MR Dephosphorylation

To confirm that aldosterone is essential for AngII-induced pendrin upregulation, we supplemented adrenalectomized mice with either vehicle (ADx-Veh) or AngII with aldosterone (ADx-AngII-Aldo). Aldosterone levels during exogenous supplementation were similar to those in mice on an LS diet (Figures 1B and 4A). Additionally, plasma [K⁺] was significantly lowered (2.64 ± 0.88 mmol/L versus 4.25 ± 0.13 mmol/L; P < 0.001) (Figure 4B). Of note, AngII and aldosterone coadministration to adrenalectomized mice markedly upregulated levels of pendrin by 1.9-fold (P < 0.001), NCC by 5.0-fold (P < 0.001), and pNCC-T53 by 10.1-fold (P < 0.001) (Figure 4, I–L) and (H) quantification of pMR-S843 levels in the whole-kidney lysates of Sham and AngII-infused mice. (I) Representative Western blots and quantifications of (J) pendrin, (K) NCC, and (L) pNCC-T53 in the membrane fraction of kidneys from mice treated with AngII infusion and AngII infusion with spironolactone (n = 6 per group). Equal loading was confirmed by parallel Coomassie-stained gels (see Supplemental Figure 3). Statistical comparisons were performed using an unpaired t test. Data represent the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. PAC, plasma aldosterone concentration.

Figure 2. AngII infusion increases pendrin and decreases pMR-S843 levels. (A) PAC and (B) plasma [K⁺] in C57BL/6J mice undergoing either a sham operation (Sham; n = 8) or AngII infusion (n = 9). (C) Representative Western blots and quantifications of (D) pendrin, (E) NCC, and (F) pNCC-T53 in the membrane fraction of kidneys from Sham and AngII-infused mice (n = 6 per group). Equal loading was confirmed by parallel Coomassie-stained gels (see Supplemental Figure 3). (G) Representative Western blots and (H) quantification of pMR-S843 levels in the whole-kidney lysates of Sham and AngII-infused mice. (I) Representative Western blots and quantifications of (J) pendrin, (K) NCC, and (L) pNCC-T53 in the membrane fraction of kidneys from mice treated with AngII infusion and AngII infusion with spironolactone (n = 6 per group). Equal loading was confirmed by parallel Coomassie-stained gels (see Supplemental Figure 3). Statistical comparisons were performed using an unpaired t test. Data represent the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. PAC, plasma aldosterone concentration.
4, C–F), along with a 53% reduction in pMR-S843 levels (P=0.001) (Figure 4, G and H). To further evaluate whether AngII and aldosterone act synergistically to stimulate pendrin or NCC, we tried to compare membrane-protein abundance of pendrin and NCC in the kidney of aldosterone-treated adenalec-tomized mice with or without AngII infusion; expressions of both pendrin and NCC were significantly higher in aldosterone-treated adenalec-tomized mice with AngII infusion (Figure 5, C–F), associated with comparable plasma [K+] (Figure 5B). Aldosterone levels during exogenous supplementation were almost two times higher than those in mice on an HS diet (Figures 5A and 2A). When high-dose and low-dose aldosterone were administered to AngII-treated adenalec-tomized mice, aldosterone-induced increases in pendrin expression occurred in a dose-dependent fashion (Figure 6, C–F), in accompaniment with increased plasma aldosterone and decreased plasma [K+] (Figure 6, A and B), suggesting that there was a synergy between AngII and aldosterone to promote expressions of pendrin as well as NCC.

AngII and Aldosterone Coadministration in Adrenalectomized Mice Increases Immunostaining of Pendrin in β-Intercalated Cells

We then performed immunofluorescence analysis by costaining mouse kidneys with pendrin or pMR-S843 and aquaporin 2 (AQP2), a marker of principal cells in the connecting tubule and collecting duct. Immunofluorescence results showed increased pendrin labeling in β-intercalated cells from ADx-AngII-Aldo mice compared with that observed from ADx-Veh mice (Figure 7A). Importantly, in contrast to the diffuse cytoplasmic and weak apical-membrane pendrin-labeling signals observed in β-intercalated cells from ADx-Veh mice, intense pendrin labeling was observed in the apical regions of β-intercalated cells from ADx-AngII-Aldo mice (Figure 7A). These results suggested that AngII and aldosterone coadministration promoted membrane-trafficking processes and increased membrane-protein abundance of pendrin. No immunostains of pMR-S843 in principal cells stained with AQP2 confirmed that pMR-S843 existed exclusively in intercalated cells,9 and that pMR-S843 labeling was markedly reduced in ADx-AngII-Aldo mice (Figure 7B), consistent with the results of Western blots. Along with pendrin staining, pNCC-T53 labeling was significantly increased in ADx-AngII-Aldo mice (Figure 7C). These data indicated that AngII was capable of upregulating NCC independent of aldosterone, whereas aldosterone was essential for AngII-induced pendrin upregulation.

Given that the well known genomic action of aldosterone is mediated by nuclear translocation of the aldosterone-MR complex in principal cells, we have evaluated whether cytoplasmic MR in β-intercalated cells is also translocated into the nucleus when AngII and aldosterone are coadministered to adenalectomized mice. We found the nuclear translocation of MR in β-intercalated cells (Figure 8) as well as in principal cells (Supplemental Figure 1) with AngII and aldosterone coadministration, whereas it was not affected by AngII alone.

Figure 3. AngII increases NCC, but not pendrin levels in adre-nalec-tomized mice. (A) PAC and (B) plasma [K+] in adrenalecto-mized C57BL/6J mice treated with either vehicle (ADx-Veh; n=5) or AngII infusion (ADx-AngII; n=7). (C) Representative Western blots and quantifications of (D) pendrin, (E) NCC, and (F) pNCC-T53 in the membrane fraction of kidneys in ADx-Veh (n=5) or ADx-AngII (n=7) mice. Equal loading was confirmed by parallel Coomassie-stained gels (see Supplemental Figure 3). (G) Representative Western blots and (H) quantification of pMR-S843 levels in whole-kidney lysates from ADx-Veh (n=5) or ADx-AngII (n=7) mice. Statistical comparisons were performed using an unpaired t test. Data represent the mean±SEM. *P<0.05. PAC, plasma aldosterone concentration; U.D., undetectable.
DISCUSSION

In this study, we demonstrated that pendrin expression in β-intercalated cells was regulated by the combination of AngII and aldosterone. In DCT cells, AngII increased NCC expression independent of aldosterone; however, in β-intercalated cells, AngII alone was insufficient to increase pendrin levels, although it promoted MR dephosphorylation. In adrenalectomized mice, AngII alone did not increase pendrin levels despite dephosphorylation of pMR-S843, until the addition of aldosterone. These results clearly show that aldosterone is essential to increase pendrin expression. As a consequence, during volume depletion, pendrin contributed to maintaining normal BP in cooperation with NCC through NaCl reabsorption (Figure 9).

Mice with genetic deletion of pendrin and humans carrying inactivating mutations in the pendrin gene do not demonstrate salt wasting under basal conditions.10,15,21 Similarly, NCC-knockout mice also do not demonstrate salt wasting under basal conditions;10,16 however, NCC-knockout mice,16 as well as PDS−/− mice,15 demonstrate salt wasting and resultant volume depletion during NaCl restriction. Furthermore, a recently identified pendrin inhibitor has no effect on the basal condition when administered alone, although this compound increases the natriuretic effects of furosemide.22 These findings indicate that pendrin and NCC are predominantly active during volume depletion associated with RAAS activation.

Volume depletion increases AngII levels, which stimulate the adrenal gland to produce aldosterone in vivo.23 Under these conditions, kidneys maximize NaCl reabsorption in response to AngII and aldosterone. Recently, in DCT cells, the independent roles of AngII and aldosterone on NCC expression and activity were reported; in contrast to the direct effect of AngII on NCC,4,19 the effect of aldosterone is indirect. Aldosterone-induced activation of NCC is mediated by hypokalemia, on the basis of the inverse correlation between plasma [K+] and p-NCC expression.20 In both mice receiving an LS diet and AngII infusion, with no changes in plasma [K+], pendrin as well as NCC were upregulated (Figures 1 and 2). In adrenalectomized mice, however, AngII-induced upregulation of pendrin was not observed (Figure 3), despite the definite increase of NCC expression, suggesting that aldosterone was indispensable for AngII-induced upregulation of pendrin expression, in contrast to aldosterone-independent activation of NCC induced by AngII. Thus, on an LS diet, increased AngII decreases phosphorylation of pMR-S843, and in turn, increased the ability of MR binding to aldosterone, resulted in the potentiation of pendrin expression. Moreover, our results suggest that AngII and aldosterone act synergistically to stimulate pendrin or NCC (Figures 5 and 6). However, further studies are needed to clarify how MR phosphorylation is involved in aldosterone-induced upregulation of pendrin.

To evaluate the physiologic role of pendrin in BP regulation during volume depletion with an LS diet, we evaluated BP by radiotelemetry. Despite no difference in MAPs between PDS−/− mice with genetic deletion of pendrin and humans carrying inactivating mutations in the pendrin gene do not demonstrate salt wasting under basal conditions.10,15,21 Similarly, NCC-knockout mice also do not demonstrate salt wasting under basal conditions;10,16 however, NCC-knockout mice,16 as well as PDS−/− mice,15 demonstrate salt wasting and resultant volume depletion during NaCl restriction. Furthermore, a recently identified pendrin inhibitor has no effect on the basal condition when administered alone, although this compound increases the natriuretic effects of furosemide.22 These findings indicate that pendrin and NCC are predominantly active during volume depletion associated with RAAS activation.

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and WT mice on an HS diet, an LS diet significantly decreased MAPs in PDS$^{2/2}$ mice but not in WT mice (Figure 1), as firstly shown by Wall and colleagues.\textsuperscript{17,18} Notably, under basal conditions BP is similar or slightly reduced in PDS$^{2/2}$ relative to WT mice,\textsuperscript{17,18} which is consistent with the clinical study showing that basal levels of BP in people with pendrin syndrome are lower than in controls.\textsuperscript{24} Thus, NaCl intake must be high to eliminate the fall in BP by pendrin gene ablation observed in our study, and this depressor effect of pendrin gene ablation is greater when dietary NaCl is restricted. Taken together, activation of RAAS by volume depletion with an LS diet increased pendrin expression, and in turn, activated pendrin contributed to maintaining normal BP in cooperation with NCC through NaCl reabsorption.

Plasma $[K^+]$, with hypokalemia allowing aldosterone-induced pendrin expression associated with MR dephosphorylation.\textsuperscript{26} In our study, neither an LS diet (Figure 1) nor AngII infusion (Figures 2 and 3) altered plasma $[K^+]$, as previously reported.\textsuperscript{3,27} Thus, dephosphorylation of pMR-S843 induced by an LS diet and AngII infusion might be attributable solely to elevated AngII. As a result, activated MR binding to aldosterone, its agonist, resulted in increased pendrin expression. Moreover, this finding of nuclear MR translocation by AngII and aldosterone coadministration in $\beta$-intercalated cells as well as in principal cells leads to the assumption that upregulation of pendrin expression might be attributable to MR-dependent transcription of pendrin gene, although the precise mechanism of nuclear MR translocation in $\beta$-intercalated cells is still unclear.
Figure 7. AngII and aldosterone coadministration in adrenalectomized mice increases and decreases immunostaining of pendrin and pMR, respectively, in β-intercalated cells and increases immunostaining of pNCC in DCT cells. (A) Immunofluorescence analyses for detection of pendrin (green), AQP2 (red), and a merged image in kidneys from adrenalectomized C57BL/6J mice treated with either vehicle (ADx-Veh) or AngII and aldosterone (1.0 μg/d) coadministration (ADx-AngII-Aldo). (B) Immunofluorescence analyses for detection of pMR-S843 (green), AQP2 (red), and a merged image in kidneys from ADx-Veh or ADx-AngII-Aldo mice. (C) Immunofluorescence analyses for detection of pNCC-T53 (green), nuclei (DAPI; blue), and a merged image in kidneys from ADx-Veh or ADx-AngII-Aldo mice. Scale bars represent 50 μm. DAPI, 4′,6-diamidino-2-phenylindole.
Both AngII and aldosterone enhance NCC expression and activity.\(^3,4,28\) Recently, Terker\(^{20,29}\) et al. reported that hypokalemia increased NCC independent of aldosterone-MR signaling. In our study, endogenously elevated AngII and aldosterone (by LS diet) and exogenously elevated AngII and aldosterone (by AngII infusion) increased NCC levels and NCC phosphorylation, despite unchanged plasma [K\(^+\)]. Additionally, AngII-induced upregulation of NCC and NCC phosphorylation was not reversed by blocking aldosterone-MR signaling after spironolactone treatment. Furthermore, AngII in the absence of aldosterone increased NCC and NCC phosphorylation. These results suggest that elevated AngII by an LS diet increases NCC levels and NCC phosphorylation in an aldosterone- and potassium-independent manner.

In both LS diet-fed mice and AngII-administered mice, we observed increases in both pendrin and NCC expression accompanied by no changes in plasma [K\(^+\)]; however, adrenalectomized mice coadministered AngII and aldosterone exhibited phenotypes associated with hypokalemia. According to hypokalemia-induced NCC expressions, both hypokalemia and AngII contributed to marked increases in NCC (5.0-fold) and p-NCC (10.1-fold) levels in adrenalectomized mice coadministered AngII and aldosterone (Figure 4). Given levels of pMR-S843 and pendrin expressions affected by plasma [K\(^+\)],\(^{26}\) the greater decrease in pMR-S843 levels in the adrenalectomized mice coadministered AngII and aldosterone as compared with those administered AngII alone (53% [Figure 4] versus 30% [Figure 3]) might be a consequence of hypokalemia, whereas we observed no difference in pMR-S843 levels between mice administered the same dosages of AngII with and without adrenalectomy (30% [Figure 3] versus 25% [Figure 2]). Moreover, despite similar plasma aldosterone levels, we observed a larger increase in pendrin expression in the adrenalectomized mice coadministered AngII and aldosterone compared with that observed in mice fed an LS diet (1.9 fold [Figure 4] versus 1.5 fold [Figure 1]) as a result of hypokalemia (plasma [K\(^+\)]; 2.64 mmol/L versus 3.96 mmol/L). These observations indicate that several factors influence pendrin; plasma AngII, aldosterone, and [K\(^+\)], all of which exert effects on the other. Further studies are needed to clarify how these factors are involved in the abnormal functions of pendrin and NCC related to disturbances in fluid homeostasis and hypertension.

As a clinical implication, pseudohypoaldosteronism type II model mice showed volume-dependent hypertension associated with NCC activation, but no changes in pendrin expression despite dephosphorylation of pMR-S843\(^9\) because of intrarenal AngII signal activation but decreased plasma aldosterone levels. According to the dissociation of pendrin expression from AngII signaling by suppression of plasma aldosterone during volume expansion, our results involving adrenalectomized mice clearly revealed that aldosterone was essential for AngII-induced upregulation of pendrin expression. Furthermore, our findings associated with PDS\(^{-/-}\) mice provide evidence supporting the critical role played by pendrin in maintaining normal BP during volume depletion, but not during volume expansion. Taken together, these findings offer respective therapeutic targets of NCC and pendrin for treating volume-dependent hypertension with intrarenal activation of AngII signaling, and disturbances in fluid balance with increased activity of circulating RAAS.

**CONCISE METHODS**

**Animals and Experimental Design**

Animal care and treatment complied with the standards described in the Guidelines for the Care and Use of Laboratory Animals of the University of Tokyo (Tokyo, Japan). All studies were approved by the
In protocol 7, aldosterone-treated (0.1 µg/d) adrenalectomized C57BL/6J mice received either vehicle (ADx-Aldo; n=5) or AngII infusion (ADx-Aldo-AngII; n=5) for 7 days.

In protocol 8, AngII-treated adrenalectomized C57BL/6J mice received either vehicle (ADx-AngII; n=5), low-dose (0.3 µg/d) aldosterone (ADx-AngII-Aldo-L; n=4) or high-dose (1.0 µg/d) aldosterone (ADx-AngII-Aldo-H; n=5).

**Adrenalectomy**

In protocols 5–8, bilateral adrenalectomy was performed through a dorsal incision under isoflurane anesthesia as previously described. Mice were fed an HS diet until the end of the studies to compensate for natriuresis after adrenalectomy. After a 7-day recovery from surgery, all animals were implanted with osmotic minipumps (ALZET) to administer either vehicle, AngII, aldosterone, or AngII and aldosterone coadministration. All minipumps also contained dexamethasone (12 µg/kg per day) for glucocorticoid replacement. The doses of aldosterone and dexamethasone were chosen to provide near-physiologic replacement, on the basis of published studies.31,32

**BP Measurements in Conscious Mice**

Radiotelemetry devices were inserted into PDS−/− and WT mice by arterial catheterization of the left carotid artery under isoflurane anesthesia, with the telemetry body positioned in a subcutaneous pocket on the right flank. Throughout the measurements, arterial BP in a conscious mouse was directly monitored by radiotelemetry using a PA-C10 transmitter, RPC-1 receiver, APR-1 ambient-pressure monitor, and a Data-Quest-ART-Silver 4.2 acquisition system (Data Sciences International, New Brighton, MN). Continuous measurement of MAPs in 10-second intervals was recorded every 15 minutes, and hourly MAPs were calculated by averaging four sequential MAP records. Baseline measurements were recorded for three consecutive days (days 0–3) in mice fed an HS diet. At day 7, post-treatment measurements were recorded for the final three consecutive days of the LS diet (days 7–10).

**Blood Collection and Electrolyte Measurements**

At the end of the experiments, we extracted blood from the inferior vena cava under anesthesia. Blood was immediately transferred into heparinized tubes, and 80 µl was loaded into an EC8+ cartridge for electrolyte measurement using an i-STAT®-1 analyzer (Abbott, Abbott Park, IL). The remaining blood volume was centrifuged at 5000 rpm for 15 minutes at 4°C, and plasma was removed and stored at −20°C. Plasma aldosterone concentrations and plasma renin activities were measured by radioimmunoassay (SRL, Tokyo, Japan).

In protocol 1, C57BL/6J mice were fed either an HS (8% NaCl) diet (n=9) or an LS (0.03% NaCl) diet (n=8) for 13 days.

In protocol 2, BP was measured by radiotelemetry in PDS−/− mice (n=5) and WT mice (n=5). Mice were fed an HS diet for 10 days (days 0–7). At day 3, mice began eating an LS diet, which continued for 7 days.

In protocol 3, C57BL/6J mice received either a sham operation (n=8) or AngII infusion (n=9) for 7 days.

In protocol 4, C57BL/6J mice received either AngII infusion (n=7) or AngII infusion with the MRA spironolactone (0.2 g/kg chow; n=6) for 7 days.

In protocol 5, adrenalectomized C57BL/6J mice received either vehicle (ADx-Veh; n=5) or AngII infusion (ADx-AngII; n=7) for 7 days.

In protocol 6, adrenalectomized C57BL/6J mice received either vehicle (ADx-Veh; n=6) or AngII and aldosterone (1.0 µg/d) coadministration (ADx-AngII-Aldo; n=8) for 7 days.

**Animal Diets**

In protocols 1 and 2, mice were fed either an HS or LS diet, prepared by modifying the AIN-76A semipurified diet (Oriental Yeast, Tokyo, Japan). In protocols 3–8, mice were fed an HS diet (Oriental Yeast).

**AngII Infusion**

In protocols 3–8, osmotic minipumps (ALZET model 2002; DURECT Corporation, Cupertino, CA) were implanted subcutaneously to infuse AngII at a dose of 400 µg/kg per day.4

**Institutional Animal Care and Use Committee of the University of Tokyo (RAC 150402). Male C57BL/6J mice were obtained from Tokyo Laboratory Animals Science (Tokyo, Japan). PDS−/− mice with a 129/Sv background were developed by Everett and colleagues17,30 and were obtained from The Jackson Laboratory (Bar Harbor, ME). WT mice with a 129/Sv background were also obtained from The Jackson Laboratory. All mice had free access to drinking water and diet under temperature-controlled conditions and a 12-hour light/dark cycle. All mice used for experiments were from 8- to 14-weeks-old.

In protocol 1, C57BL/6J mice were fed either an HS (8% NaCl) diet (n=9) or an LS (0.03% NaCl) diet (n=8) for 13 days.

In protocol 2, BP was measured by radiotelemetry in PDS−/− mice (n=5) and WT mice (n=5). Mice were fed an HS diet for 10 days (days 0–7). At day 3, mice began eating an LS diet, which continued for 7 days.

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In protocol 6, adrenalectomized C57BL/6J mice received either vehicle (ADx-Veh; n=6) or AngII and aldosterone (1.0 µg/d) coadministration (ADx-AngII-Aldo; n=8) for 7 days.
Western Blot Analysis
Kidneys were removed, snap frozen in liquid nitrogen, and stored at -80°C until homogenization. Total membrane fraction was isolated using a Minute plasma membrane-protein isolation kit (Invent Biotechnologies, Eden Prairie, MN) according to the manufacturer’s instructions. Enrichment of membrane proteins, but not cytoplasmic proteins, was validated in the laboratory (see Supplemental Figure 2).

Briefly, kidney tissues were lysed in buffer A containing protease inhibitors and phosphatase inhibitors (Roche Diagnostics, Basel, Switzerland) and placed in a filter cartridge. After centrifugation at 14,000 rpm for 30 seconds, pellets were resuspended and centrifuged at 3000 rpm for 1 minute. The supernatant was collected and centrifuged again at 14,000 rpm for 30 minutes, and the resulting pellet was resuspended in buffer containing 40 mM Tris (pH 7.9), 260 mM sucrose, and 1% Triton X-100 and used as the total membrane-protein sample. Total cell lysate was isolated with extraction buffer containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1% IGEPAL CA630, 1 mM EDTA, and protease inhibitors (Complete; Roche Diagnostics) and phosphatase inhibitors (PhosSTOP; Roche Diagnostics) and centrifuged at 14,000 rpm for 30 minutes to obtain the cellular proteins in the supernatant. Protein concentrations were determined using a BCA protein assay (Pierce, Rockford, IL) in duplicate.

Western blotting was performed as previously described, with some modifications. Briefly, equal amounts of protein were mixed with 2X Laemmlı sample buffer, boiled for 10 minutes (or incubated for 30 minutes at room temperature for membrane proteins), separated on polyacrylamide gels, and transferred to polyvinylidene fluoride membranes. The membranes were blocked with polyvinylidene fluoride–blocking reagent (Toyobo, Osaka, Japan) for 30 minutes at room temperature and incubated with primary and peroxidase-conjugated secondary antibodies, followed by imaging using enhanced chemiluminescence reagents (GE Healthcare, Waukesha, WI). Gluteraldehyde 3-phosphate dehydrogenase (GAPDH; for total cell lysates) and Coomassie brilliant blue (Bio-Rad, Hercules, CA) staining were used as the total membrane-protein sample. Western blotting and 1:100 for immunofluorescence staining; a gift from Peter Aronson, Yale University, New Haven, CT), NCC (1:3000 for Western blotting; Millipore, Bedford, MA), pNCC-T53 (1:500 for Western blotting and 1:500 for immunofluorescence staining; a gift from Celso Gomez-Sanchez, University of Mississippi Medical Center, Jackson, MS), AQP2 (1:200 for immunofluorescence staining; Santa Cruz Biotechnology, Dallas, TX), and GAPDH (1:5000 for Western blotting; Abcam, Cambridge, UK). The antibody for pMR-S843 (1:1000 for Western blotting and 1:100 for immunofluorescence staining) was created and characterized as described previously.

Statistical Analyses
Data are presented as the mean±SEM. Statistical comparisons were performed using the unpaired t test or ANOVA as indicated in the figure legends. P<0.05 was considered statistically significant.

ACKNOWLEDGMENTS
The authors thank Peter Aronson for the pendrin antibody, Johannes Loffing for the phosphorylated NCC antibody, and Celso Gomez-Sanchez for the MR antibody.

This work was supported by JSPS KAKENHI (grants 15H05788 and 17K16074), the AMED-CREST from Japan Agency for Medical Research and development, AMED, and the Charitable Trust Araki Medical and Biochemical Research Memorial Fund.

DISCLOSURES
None.

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This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2017030243/-/DCSupplemental.