Hypokalemia and Pendrin Induction by Aldosterone

Ning Xu, Daigoro Hirohama, Kenichi Ishizawa, Wen Xiu Chang, Tatsuo Shimosawa, Toshiro Fujita, Shunya Uchida, Shigeru Shibata

Abstract—Aldosterone plays an important role in regulating Na-Cl reabsorption and blood pressure. Epithelial Na+ channel, Na+-Cl− cotransporter, and Cl-/HCO3− exchanger pendrin are the major mediators of Na-Cl transport in the aldosterone-sensitive distal nephron. Existing evidence also suggests that plasma K+ concentration affects renal Na-Cl handling. In this study, we posited that hypokalemia modulates the effects of aldosterone on pendrin in hyperaldosteronism. Chronic aldosterone infusion in mice increased pendrin levels at the plasma membrane, and correcting hypokalemia in this model almost completely blocked pendrin upregulation. However, hypokalemia induced by a low-K+ diet resulted in pendrin downregulation along with reduced plasma aldosterone levels, indicating that both hypokalemia and aldosterone excess are necessary for pendrin induction. In contrast, decreased plasma K+ levels were sufficient to increase Na+-Cl− cotransporter levels. We found that phosphorylation of mineralocorticoid receptor that prevents aldosterone binding in intercalated cells was suppressed by hypokalemia, which resulted in enhanced pendrin response to aldosterone, explaining the coordinated action of aldosterone and hypokalemia in pendrin regulation. Finally, to address the physiological significance of our observations, we administered aldosterone to mice lacking pendrin. Notably, plasma K+ levels were significantly lower in pendrin knockout mice (2.7±0.1 mmol/L) than in wild-type mice (3.0±0.1 mmol/L) after aldosterone infusion, demonstrating that pendrin alleviates hypokalemia in a state of aldosterone excess. These data indicate that the decreased plasma K+ levels promote pendrin induction by aldosterone, which, in concert with Na+-Cl− cotransporter, counteracts the progression of hypokalemia but promotes hypertension in primary aldosterone excess. (Hypertension. 2017;69: 855-862. DOI: 10.1161/HYPERTENSIONAHA.116.08519.)

Key Words: aldosterone ■ blood pressure ■ hypertension ■ hypokalemia ■ potassium

Accumulating data suggest that aldosterone plays a major role in the development of hypertension. Clinical studies have demonstrated that primary aldosteronism (PA) affects 5% to 20% of individuals with hypertension.1,2 Moreover, increased aldosterone levels predispose normotensive individuals to hypertension.3 Aldosterone and its receptor, the mineralocorticoid receptor (MR), regulate extracellular fluid volume and plasma K+ levels by controlling ion transport processes in the distal nephron. Classical targets of aldosterone in this segment are the principal cells of the collecting duct, which express the amiloride-sensitive, epithelial Na+ channel (ENaC).4,5 Aldosterone regulates ENaC by increasing its apical membrane levels and channel activity. The latter process requires the proteolytic cleavage of the inhibitory domain in the extracellular loop by aldosterone-induced serine proteases.4,6,7 The reabsorption of Na+ through ENaC generates a lumen-negative transepithelial voltage, which provides the driving force for Cl− reabsorption and K+ secretion.5,8 In the collecting duct, transcellular Cl− transport occurs through pendrin in renal intercalated cells,9-11 another cell type that comprises the collecting duct.

Pendrin, encoded by SLC26A4, is a Cl-/HCO3− exchanger expressed specifically in β-intercalated cells. Pendrin has been shown to regulate the acid/base balance by excreting HCO3− into the urine.9 In addition to its role in alkali secretion, however, accumulating studies have demonstrated the key role of pendrin in maintaining extracellular fluid volume homeostasis by regulating Cl− reabsorption.12-13 Importantly, mice lacking the pendrin gene (Pds−/−) show volume contraction, low blood pressure, and increased Cl− excretion compared with wild-type mice when they are on a low Na-Cl diet.14,15 Cl− reabsorption in the cortical collecting duct disappears in Pds−/− mice, explaining the impaired ability to retain fluid.13 In addition, double knockout of pendrin and Na+-Cl− cotransporter (NCC) present in the distal convoluted tubules (DCT) leads to severe volume depletion and hypotension,10 demonstrating that NCC and pendrin play compensatory roles in Cl− and fluid volume conservation. Conversely, mice overexpressing pendrin show salt-sensitive hypertension and are very sensitive to Cl− intake.17

Mineralocorticoids can upregulate pendrin,12 and Pds−/− mice show blunted pressor responses to deoxycorticosterone12
and aldosterone, indicating that pendrin mediates fluid volume regulation by aldosterone. We previously showed that this process is modulated by MR phosphorylation at S843 in the ligand-binding domain (MR<sup>843-P</sup>), which prevents ligand binding and MR signaling in intercalated cells. The pathophysiological roles of MR<sup>843-P</sup> in hypertension have remained unknown.

The plasma K<sup>+</sup> concentration is increasingly recognized as an important regulator of renal ion transport mechanisms. In DCT cells, plasma K<sup>+</sup> levels alter NCC phosphorylation levels independently of aldosterone, which is implicated in the inverse relationship between dietary K<sup>+</sup> intake and blood pressure levels. Stimulation of NCC by hypokalemia also plays a pathogenic role in PA. In addition to NCC, previous findings have implicated K<sup>+</sup> intake in regulating pendrin expression, and we have shown that dietary K<sup>+</sup> loading decreases pendrin levels.

To date, the role of the plasma K<sup>+</sup> concentration in regulating pendrin remains unclear, especially in the context of hyperaldosteronism. In this study, we tested the hypothesis that low plasma K<sup>+</sup> levels potentiate the effect of aldosterone on pendrin expression, thereby counteracting the progression of hypokalemia in a state of aldosterone excess.

**Methods**

**Animals**

This study was approved by the institutional review board in the Teikyo University Review Board No. 14-018. Male C57BL/6 mice and Sprague–Dawley rats were obtained from Tokyo Laboratory Animals Science (Japan). Pdr<sup>−/−</sup> mice with 129/Sv background were developed by Everett et al. and were obtained from Jackson Laboratory (United States). Wild-type mice with 129/Sv background were also obtained from Jackson Laboratory. The details of the study protocol are described in Figure S1 in the online-only Data Supplement and are also available in the extended Materials and Methods in the online-only Data Supplement.

**Western Blot**

Western blotting was performed as described previously. Plasma membrane fraction was purified from total kidneys using plasma membrane isolation kit (Minute, Invent Biotechnologies, United States). Enrichment of plasma membrane proteins but not cytoplasmic proteins was validated in the laboratory (Figure S2). Tubulin (for total cell lysates) and Coomassie brilliant blue staining of the gel (for plasma membrane proteins; Figure S3) were used to monitor the identical loading of different samples. The rest of the Methods is available in the online-only Data Supplement.

**Statistical Analysis**

The data are summarized as means±SEM. Unpaired <i>t</i> test was used for comparisons between 2 groups. For multiple comparisons, statistical analysis was performed by ANOVA followed by Tukey post hoc tests. <i>P</i> values <0.05 was considered statistically significant.

**Results**

**Pendrin and NCC Induction by Aldosterone Is Ameliorated by K<sup>+</sup> Supplementation**

Excessive aldosterone production in PA is associated with decreased plasma K<sup>+</sup> levels. Interestingly, however, it is also known that only a subpopulation of PA patients actually develops hypokalemia. We investigated whether a plasma K<sup>+</sup> decrease mediates the effects of aldosterone in the distal nephron. Mice received a continuous infusion of aldosterone (60 ng/h) for 2 weeks; 1 group of mice received K<sup>+</sup> supplementation via drinking water to prevent a decrease in plasma K<sup>+</sup> levels (study 1). Aldosterone infusion for 2 weeks caused a significant decrease in plasma K<sup>+</sup> levels (3.1±0.1 mmol/L in aldosterone-infused mice versus 4.5±0.1 mmol/L in controls; <i>P</i>&lt;0.001), an effect that was blocked by K<sup>+</sup> supplementation (4.6±0.1 mmol/L; not significant versus control group; Figure 1A). Water intake and urinary volumes increased, whereas urinary pH decreased by K<sup>+</sup> supplementation in aldosterone-infused mice (Table S1).

ENaC activation by aldosterone involves proteolytic cleavage of the inhibitory domain, which reduces the molecular weight of ENaCγ from 85 kDa (uncleaved form) to 70 kDa (active, cleaved form). Western blot analysis of an isolated plasma membrane fraction revealed that the levels of pendrin, NCC, and the active, cleaved form of ENaCγ were elevated in aldosterone-infused mice (by 1.9-, 2.3-, and 2.2-fold for pendrin, NCC, and cleaved ENaCγ, respectively; <i>P</i>&lt;0.001 versus control; Figure 1B). Notably, the correction of hypokalemia by K<sup>+</sup> supplementation almost completely abolished the upregulation of pendrin (1.1-fold increase; <i>P</i>=0.471 versus control and <i>P</i>&lt;0.001 versus aldosterone infusion group; Figure 1B). In aldosterone-infused mice, K<sup>+</sup> intake also reduced plasma membrane NCC levels. In contrast, K<sup>+</sup> loading did not decrease but instead enhanced ENaCγ cleavage by 3.4-fold versus control; <i>P</i>&lt;0.001). Immunofluorescence analysis revealed that pendrin was strongly expressed at the apical membrane of intercalated cells in the collecting duct in aldosterone-infused mice, which was profoundly attenuated by K<sup>+</sup> supplementation (Figure 1C; Figure S4). These data indicate that high aldosterone is sufficient to activate ENaCγ, whereas the decrease in plasma K<sup>+</sup> is important for the induction of pendrin and NCC induction by aldosterone.

We also evaluated whether the well-described, blood pressure–lowering effects of oral K<sup>+</sup> intake were associated with the changes in pendrin and NCC, using a rat model of hypertension. Uninephrectomized rats that received aldosterone (0.75 μg/h) showed a progressive increase in blood pressure, which was ameliorated by preventing hypokalemia (170±2 mmHg in aldosterone-infused rats versus 155±1 mmHg in aldosterone-infused rats that received K<sup>+</sup> at 4 weeks; <i>P</i>&lt;0.01; Figure S5A), consistent with previous observations using similar models. In addition, urinary albumin excretion tended to be reduced by K<sup>+</sup> supplementation (4.5±1.6 versus 1.4±0.3 mg/d; <i>P</i>=0.079). In the plasma membrane fraction of the kidney, we found that K<sup>+</sup> supplementation abolished the increases in pendrin and NCC expression, but not in cleaved ENaCγ in this model (Figure S5B).

**Reversal of Hypokalemia by Amiloride Abrogates Pendrin Induction by Aldosterone**

The downregulation of pendrin by K<sup>+</sup> supplementation in the aldosterone infusion model suggests the involvement of plasma K<sup>+</sup> levels or oral K<sup>+</sup> intake. Previous studies have also suggested a role for Cl<sup>−</sup> in pendrin regulation. To clarify the mechanism underlying our observations and to test whether changes in the plasma K<sup>+</sup> concentration mediate the effects of
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Aldosterone on pendrin, aldosterone-infused mice were treated with amiloride, a K⁺-sparing diuretic (15 mg/L in drinking water) that inhibits ENaC but does not directly affect pendrin (study 2). As expected, amiloride prevented the decrease in the plasma K⁺ levels in aldosterone-infused mice (3.4±0.3 mmol/L in aldosterone-infused mice versus 4.7±0.1 mmol/L in aldosterone plus amiloride group; \( P < 0.001 \)). In the plasma membrane fraction, pendrin upregulation in aldosterone-infused mice was fully blocked by amiloride (Figure S6). These data demonstrate that hypokalemia mediates the induction of pendrin by aldosterone.

Hypokalemia Induced by K⁺ Restriction Increases NCC but Decreases Pendrin Expression

The results described above indicate that aldosterone and plasma K⁺ play a role in the regulation of pendrin. Given that hypokalemia increases NCC phosphorylation independently of aldosterone, we tested whether hypokalemia alone could directly induce pendrin expression in the absence of hyperaldosteronism by feeding mice a low-K⁺ diet (by 1.5-fold; \( P = 0.0121 \); Figure 2B and 2C). In contrast, a low-K⁺ diet resulted in the significant decrease in pendrin levels (\( P < 0.001 \)). Consistent with the changes in pendrin levels, urinary pH tended to be decreased by the low-K⁺ diet (7.32±0.24 in normal-K⁺ diet group versus 6.82±0.07 in low-K⁺ diet group; \( P = 0.083 \)). The levels of cleaved ENaCγ and the ratio of cleaved versus total ENaCγ were also decreased in the low-K⁺ group. Low-K⁺ diet resulted in the significant decrease in plasma aldosterone levels (Figure 2D), likely explaining the reduced levels of pendrin and cleaved ENaCγ. Accordingly, immunofluorescence microscopy revealed that the apical staining of NCC was intensified whereas that of pendrin was reduced in mice fed a low-K⁺ diet relative to controls (Figure 2E and 2F; Figure S4). These data indicate that the decrease in plasma K⁺ not only induces NCC phosphorylation, but also enhances plasma membrane NCC expression. In contrast, hypokalemia alone is not sufficient to induce pendrin expression, which also requires hyperaldosteronism.

Hypokalemia Decreases MR Phosphorylation at S843

Our results support a mechanism in which low plasma K⁺ levels potentiate the effect of aldosterone in intercalated cells, resulting in pendrin induction. We previously demonstrated that phosphorylation of MR at S843 (MRS843-P) prevents ligand binding selectively in intercalated cells, regulating MR signaling. Given that this phosphorylation is increased by hyperkalemia, we expected that K⁺ depletion would lead to

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**Figure 1.** Pendrin and Na⁺-Cl⁻ cotransporter (NCC) induction by aldosterone is mediated by hypokalemia. A, Plasma K⁺ concentrations in the indicated animals. B, Effects of aldosterone and K⁺ supplementation on plasma membrane levels of the indicated proteins in the kidney. Blots show biological replicates. Bar graphs show the results of quantification. The amount of protein loaded on the gel was monitored using Coomassie brilliant blue (CBB) staining. C, Kidney sections stained for α-pendrin (green) and DAPI (blue) in the indicated animals. Pendrin staining is increased in the apical membrane in aldosterone-infused mice, which is blocked by K⁺ supplementation (see also Figure S4, which shows low-power field images). Bar represents 50 μm. Data are expressed as means±SEM; **\( P < 0.01 \). Aldo indicates mice received aldosterone infusion (n=9); Aldo+K, mice received aldosterone and 1% KCl in the drinking water (n=10); Ctrl, control mice received sham operation (n=7); and ENaC, epithelial Na⁺ channel.
dephosphorylation of MR, producing receptor competence. To test this hypothesis, we evaluated MR S843-P levels in mice eating a low-K+ diet. Western blot analysis revealed that the low-K+ diet caused a significant decrease in MR S843-P (a 46% reduction; \( P = 0.004 \); Figure 3), with no change in total MR.

**Low Plasma K+ Levels Potentiate Pendrin Induction by Aldosterone**

Given that K+ depletion reduced the phosphorylation of MR, converting to the active, competent form, we expected that aldosterone-mediated pendrin induction would be increased under these conditions. However, the biological effect might not be apparent in mice that do not exhibit elevated aldosterone levels. We therefore evaluated the effects of exogenous aldosterone infusion on pendrin levels in mice eating a low-K+ diet (study 4). Because changes in plasma K+ levels during the experiment could alter MR S843-P levels and thereby confound the analysis, we performed experiments to determine the dose of aldosterone that did not alter the plasma K+ levels. As shown in Figure 4A, the plasma K+ levels were not affected 7 days after the low-dose aldosterone (12 ng/h) in mice eating a normal-K+ diet or on a low-K+ diet. However, the low-dose aldosterone infusion significantly increased plasma aldosterone levels both in normal-K+ and low-K+ groups (Figure 4B).

In the plasma membrane fraction of the kidney, the low-dose aldosterone produced a modest but significant increase in cleaved ENaCγ levels in mice fed normal-K+ and low-K+ diets (Figure 4C and 4D), confirming the effects of aldosterone in the distal nephron. The magnitude of this effect was comparable between the groups. In contrast, although low-dose aldosterone increased pendrin expression by as much as 2.8-fold in mice on a low-K+ diet (\( P = 0.008 \); Figure 4C and 4E), it did not alter pendrin levels in mice on a normal-K+ diet (\( P = 0.31 \)). Moreover, MR S843-P levels were lower in the low-K+ group of aldosterone-infused mice (Figure S7). These data demonstrate that pendrin induction by aldosterone is influenced by plasma K+ levels and are consistent with hypokalemia promoting the

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**Figure 2.** K+ restriction increases Na+-Cl− cotransporter (NCC), whereas it decreases pendrin. A, Plasma K+ concentrations in mice eating a normal-K+ or a low-K+ diet (n=9). B, Effect of low-K+ diet on plasma membrane expression of the indicated proteins in the kidney. Blots show biological replicates. The amount of protein loaded on the gel was monitored using Coomassie brilliant blue (CBB) staining. C, Quantification of the expression levels described in (B). D, Plasma aldosterone concentrations in mice eating a normal-K+ or a low-K+ diet. E and F, Kidney sections stained for α-NCC (E, red) or α-pendrin (F, green) and DAPI (blue) in the indicated animals. Apical staining of NCC is increased, whereas that of pendrin is decreased in mice eating a low-K+ diet (see also Figure S4). Bars represent 50 μm. Data are expressed as means±SEM; *\( P < 0.05 \); **\( P < 0.01 \). ENaC indicates epithelial Na+ channel.

**Figure 3.** K+ restriction suppresses mineralocorticoid receptor (MR) phosphorylation at S843. MR phosphorylated at S843 (MR S843-P) and total MR levels in the kidneys of wild-type mice fed a normal-K+ or low-K+ diet determined by Western blot in biological replicates. Bar graphs show the results of densitometric quantification (n=9). Data are expressed as means±SEM; **\( P < 0.01 \).
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Recent studies indicate that the stimulation of NCC by hypokalemia serves as a mechanism to conserve K⁺ by reducing distal Na⁺ reabsorption and K⁺ excretion. However, compensatory actions of pendrin and NCC in renal K⁺ handling have been implicated by findings in subjects with Pendred syndrome. This autosomal recessive disorder results from a loss-of-function mutation in SLC26A4 (encoding pendrin) and is characterized by sensorineural hearing loss and thyroid abnormality. Remarkably, treatment with thiazide induces severe hypokalemia in this disorder, indicating the role of pendrin (as well as NCC) in controlling plasma K⁺ levels. Given these data, we expected that pendrin induction in a state of aldosterone excess might act to prevent the progression of hypokalemia.

To test this possibility, wild-type and pendrin knockout (Pds⁻/⁻) mice (which show normokalemia at baseline) received continuous infusion of aldosterone (60 ng/h) for 2 weeks (study 5). Urinary pH was significantly lower in Pds⁻/⁻ mice receiving aldosterone than that in wild-type mice receiving aldosterone (Figure 5A), consistent with the role of pendrin in excreting HCO₃⁻. Of note, after 2 weeks of aldosterone infusion, Pds⁻/⁻ mice showed significantly lower levels of plasma K⁺ than did wild-type mice (2.7±0.1 versus 3.0±0.1 mmol/L; P=0.01; Figure 5B). These data are consistent with pendrin counteracting the progression of hypokalemia in hyperaldosteronism.

Discussion

We demonstrate that pendrin expression in intercalated cells is regulated by the interaction of aldosterone and plasma K⁺ concentrations. An increase in plasma aldosterone levels stimulates ENaC in principal cells. In DCT cells, hypokalemia directly increases NCC levels independently of aldosterone. However, unlike the regulation of ENaC or NCC, we found that neither aldosterone nor hypokalemia alone was sufficient to induce pendrin; hypokalemia allowed the pendrin induction
by aldosterone in intercalated cells (Figure 6), which was associated with MR\(^{5843-P}\) dephosphorylation. Our model indicates that variations in plasma K\(^+\) levels have a major effect on the response of pendrin to aldosterone and that pendrin induction prevents the progression of hypokalemia in hyperaldosteronism (as discussed below).

Recent studies have demonstrated that pendrin is a key component of the electroneutral Na-Cl reabsorption machinery in the collecting duct.\(^{16,19,37–39}\) In this segment, Cl\(^-\) transport through pendrin is coupled to Na\(^+\) reabsorption through ENaC (Figure S8).\(^{13,15,18}\) Moreover, evidence indicates that pendrin stimulates ENaC activity and abundance.\(^{13,15,18}\) In addition to this mechanism, pendrin can also work in tandem with Na\(^+\)-dependent, Cl\(^-/\)HCO\(_3^-\) exchanger in \(\beta\)-intercalated cells,\(^{37,39}\) partly mediating Na-Cl reabsorption in this segment. Electroneutral Na-Cl reabsorption in the collecting duct eliminates the lumen-negative potential that drives the K\(^+\) secretion through renal outer medullary K\(^+\) channel, thereby reducing renal K\(^+\) secretion.\(^{38,40}\)

These data also have important clinical implication for patients with PA. In this study, we showed that the coexistence of hyperaldosteronism and low plasma K\(^+\) levels stimulates pendrin, and that the genetic knockout of pendrin exacerbates hypokalemia in aldosterone infusion model. These data, together with the previous evidence that \(Pds^{-/-}\) mice show a blunted pressor response to aldosterone,\(^{18}\) indicate that the pendrin induction prevents the progression of plasma K\(^+\) decrease, but instead promotes hypertension in aldosterone excess. In subjects with PA, it has been demonstrated that only a subgroup of cases show hypokalemia.\(^2\) Recent studies have suggested a role of NCC in this process, which decreases distal Na\(^+\) reabsorption and K\(^+\) excretion.\(^{25,35}\) In addition to NCC, however, our data highlight the key role of pendrin in counteracting hypokalemia. This study suggests that the decrease in plasma K\(^+\) levels potentiates pendrin induction in hyperaldosteronism, which, in concert with NCC, alleviates the progression of hypokalemia. In addition to the effects of pendrin and NCC in counteracting hypokalemia, decreased plasma K\(^+\) levels may blunt aldosterone secretion in PA, mitigating ENaC activation and renal K\(^+\) secretion.

A potential limitation of this study is that we did not demonstrate the mechanisms by which plasma K\(^+\) levels modulate MR\(^{5843-P}\) levels in intercalated cells. In DCT cells, hypokalemia alters the membrane potential by influencing K\(^+\) exit likely through the inwardly rectifying K\(^+\) channel Kir4.1 (encoded by \(KCNJ10\)).\(^{23,41}\) Given that there is scant evidence for K\(^+\) conductive pathways at the basolateral membrane in \(\beta\)-intercalated cells,\(^{42}\) distinct mechanisms are likely to be involved. Another potential limitation is that it is unclear from the study what levels of plasma aldosterone induce pendrin expression in hypokalemia, although the data demonstrate that both aldosterone and hypokalemia are necessary for pendrin upregulation.

**Perspectives**

Our study demonstrates that pendrin expression in intercalated cells is regulated by the interaction of aldosterone and plasma K\(^+\). This mode of regulation is distinct from those of NCC in DCT cells or ENaC in principal cells, illustrating the diverse mechanisms underlying renal Na-Cl transport. Reduced plasma K\(^+\) levels potentiate pendrin induction by aldosterone, which, along with NCC induction by low plasma K\(^+\), counteracts the progression of hypokalemia. Given the role of pendrin
and NCC in fluid volume regulation, these mechanisms prevent plasma K+ decrease but promote hypertension. These data are also relevant to the well-described, however, poorly characterized antihypertensive effects of high K+ intake. Hyperaldosteronism is a complex disorder associated with hypokalemia and metabolic alkalosis. The pathogenic role of acid/base disturbance in a state of aldosterone excess may require further evaluation. The identity of the kinase regulating MR\textsuperscript{S451P} is the area of future research.

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S. Shibata designed research; N. Xu, D. Hirokama, K. Ishizawa, T. Shimosawa, and S. Shibata conducted research; W.X. Chang helped in acquisition of data; N. Xu, T. Fujita, S. Uchida, and S. Shibata analyzed and interpreted the data; S. Shibata wrote the article; T. Fujita and S. Uchida helped in critical revision of the article. We thank Hiromi Yamaguchi, Emiko Kuribayashi-Okuma, Shoko Baba, and Ayumi Koyanagi for their excellent technical supports.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- Cl-/HCO3- exchanger pendrin is regulated by the interaction of aldosterone and plasma K+ levels.
- Hypokalemia potentiates pendrin induction by aldosterone likely through dephosphorylating mineralocorticoid receptor.
- Deficiency of pendrin aggravates hypokalemia in hyperaldosteronism.

**What Is Relevant?**

- In a state of primary aldosterone excess, the decrease in plasma K+ levels promotes hypertension by increasing the levels of pendrin and Na-Cl cotransporter. These mechanisms represent physiological adaptation to prevent the progression of hypokalemia.

**Summary**

Aldosterone directly stimulates epithelial Na+ channel in principal cells, whereas hypokalemia induces Na-Cl cotransporter NCC independently of aldosterone in distal convoluted tubule cells of the kidney. However, pendrin in renal intercalated cells is coordinately regulated by both aldosterone and plasma K+ levels. Hypokalemia enhances the pendrin induction by aldosterone, which, along with NCC induction by low plasma K+, counteracts the progression of hypokalemia but promotes hypertension.
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Supplementary Information

Hypokalemia and pendrin induction by aldosterone

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Expanded Materials and Methods

Animal Study Protocol

The present study was approved by the institutional review board (IRB) in the Teikyo University Review Board #14-018. Male C57BL/6 mice at 6 weeks of age were obtained from Tokyo Laboratory Animals Science (Japan). Pendrin knockout (Pds<sup>-/-</sup>) mice with 129/Sv background were developed by Everett et al.,<sup>1,2</sup> and were obtained from Jackson Laboratory. Wild-type mice with 129/Sv background were also obtained from Jackson Laboratory. They were fed ad libitum and housed under a 12-h light cycle.

Main experiments consisted of 5 study protocols (Figure S1). In study 1, mice received subcutaneous infusion of aldosterone at 60 ng/hr for 14 days (n = 9). In one group, K<sup>+</sup> was supplemented via drinking water (1% KCl; n = 10).<sup>3</sup> Wild-type mice received sham operation served as a control group (n = 7). All the groups received normal salt diet.

In study 2, mice received subcutaneous infusion of aldosterone at 60 ng/hr for 14 days (n = 4). In one group, amiloride was given orally (15 mg/l in drinking water) (n = 6). We chose the dose because the mice were not tolerable to a higher dose (40 mg/l) of amiloride (three out of six mice died within 10 days likely because of hyperkalemia). Wild-type mice received sham operation served as a control group (n = 4). All the groups received normal salt diet.

In study 3, mice received either a low (0.005%; n = 9) or a normal (0.9%; n = 9) K<sup>+</sup> diet for seven days. Na-Cl content (0.3%) in the low-K<sup>+</sup> diet is identical to that in normal-K<sup>+</sup> diet.

In study 4, mice were kept on a normal (0.9%) or low (0.005%) K<sup>+</sup> diet. At day 5, six out of 12 mice on a normal K<sup>+</sup> diet and six out of 12 mice on a low-K<sup>+</sup> diet received subcutaneous infusion of aldosterone at 12 ng/hr (a low dose, which did not significantly alter plasma K<sup>+</sup> levels) for 7 days. All the mice were euthanized at day 12 (n = 6 each group).

In study 5, Pds<sup>-/-</sup> (n = 7) and wild-type mice (n = 12) (both with 129/Sv background) received subcutaneous infusion of aldosterone at 60 ng/hr for 14 days. Two out of seven Pds<sup>-/-</sup> mice died during the course of the experiment (presumably due to hypokalemia), and the remaining five mice were included in the analysis.

In additional experiments using rat model of hypertension, male Sprague-Dawley rats (weighing 250 g) received uninephrectomy under isoflurane anesthesia, and aldosterone (0.75 µg/hr)<sup>4</sup> was subcutaneously infused via an osmotic minipump for 4 weeks (n = 10). One group received K<sup>+</sup> supplementation (1% KCl) via drinking water (n = 9). Control rats received uninephrectomy without aldosterone infusion (n = 11). All the groups were kept on a standard, normal salt diet. Systolic blood pressure was measured using volumetric pressure recording (CODA non-invasive Blood Pressure System; Kent Scientific, USA), as described previously.<sup>5</sup> This method has been validated to provide accurate blood pressure measurement and highly correlates with telemetry method.<sup>6</sup>
Urine was collected using individual metabolic cages. Urinary pH was measured using glass electrode (Horiba, Japan). Animals were euthanized under anesthesia of inhaled isoflurane. Plasma K+ was obtained using iSTAT analyzer (Abbott Point of Care, Inc., USA) immediately after blood collection. Plasma aldosterone was measured by radioimmunoassay (SRL, Japan). Kidneys were removed, snap frozen, and stored at -80°C until use.

Western Blot

Western blotting was performed as described previously. Plasma membrane fraction was purified from total kidneys using plasma membrane isolation kit (Minute, Invent biotechnologies, USA). Enrichment of plasma membrane proteins but not cytoplasmic proteins was validated in the lab (Figure S2). For total cell lysates, kidneys were homogenized with TNE buffer. After the protein quantification, equal amounts of protein were mixed with Laemmli sample buffer. Tubulin (for total cell lysates) and Coomassie brilliant blue (CBB) staining of the gel (due to the lack of appropriate endogenous control for plasma membrane proteins; Figure S3) were used to monitor the identical loading of different samples. Primary antibodies included anti-MR^{S843-P} (1:3,000, created and characterized as described in Ref 10), anti-tubulin (1:4,000, Sigma, USA), anti-Na+/K+-ATPase (1:10,000, TEMECULA, USA), anti-pendrin (1:10,000; a gift from P. Aronson, Yale University), anti-NCC (1:1,000, created and characterized as described in Ref 9), and anti-ENaC_γ (1:1,000, StressMarq, Canada).

Immunostaining

Kidney tissues were fixed in 4% paraformaldehyde in PBS at 4°C. Tissues were incubated in 30% sucrose in PBS overnight at 4°C and mounted in OTC (Tissue-Tek) for sectioning. Tissue sections were blocked with 2% BSA, stained with the indicated primary antibodies and affinity-purified secondary antibodies conjugated to Alexa 488 fluorophore (Invitrogen). Nucleus was stained with DAPI.
Supplemental References


Supplemental Table

Table S1. Water intake, urinary volume and urinary pH from *study 1*.

<table>
<thead>
<tr>
<th>Biological Parameters</th>
<th>Control</th>
<th>Aldo</th>
<th>Aldo + K</th>
<th>ANOVA P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water intake, ml</td>
<td>2.2 ± 0.3</td>
<td>3.7 ± 0.8</td>
<td>10.0 ± 1.5*†</td>
<td>*P = 0.0003</td>
</tr>
<tr>
<td>Urinary volume, ml</td>
<td>0.53 ± 0.08</td>
<td>1.13 ± 0.27</td>
<td>5.74 ± 1.04*†</td>
<td>*P = 0.0001</td>
</tr>
<tr>
<td>Urinary pH</td>
<td>7.16 ± 0.12</td>
<td>7.64 ± 0.36</td>
<td>6.64 ± 0.16‡</td>
<td>*P = 0.0359</td>
</tr>
</tbody>
</table>

Values are means ± SEM, *P < 0.01 vs. control. †P < 0.01, ‡P < 0.05 vs. Aldo.
Supplemental Figures

A

Control

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Aldo

Aldosterone (80 ng/hr)

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Aldo+K

Aldosterone (80 ng/hr) + 1% KCl

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

B

Control

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Aldo

Aldosterone (80 ng/hr)

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Aldo+Ami

Aldosterone (80 ng/hr) + Amiloride (15 mg/l)

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

C

Normal K

0.9% K diet

Euthanize

0 1 2 3 4 5 6 7

Low K

0.005% K diet

Euthanize

0 1 2 3 4 5 6 7

D

Normal K

0.9% K diet

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12

Normal K + low aldol

Aldosterone (12 ng/hr)

0.9% K diet

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12

Low K

0.005% K diet

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12

E

Wild-type

Aldosterone (80 ng/hr)

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Pds"d

Aldosterone (80 ng/hr)

Euthanize

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

F

Uninephrectomy

Control

Euthanize

Day 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28

Uninephrectomy

Aldo

Aldosterone (0.75 µg/hr)

Euthanize

Day 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28

Uninephrectomy

Aldo+K

Aldosterone (0.75 µg/hr) + 1% KCl

Euthanize

Day 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Figure S1

Study protocol.

(A) In study 1, mice received subcutaneous infusion of aldosterone at 60 ng/hr for 14 days (Aldo; n = 9). One group received K+ supplementation *via* drinking water (1% KCl; Aldo+K group; n = 10). Wild-type mice that received sham operation served as a control (n = 7). All the groups received normal salt diet. (B) In study 2, mice received subcutaneous infusion of aldosterone at 60 ng/hr for 14 days (Aldo; n = 4). In one group, amiloride was given orally (15 mg/l in drinking water) (Aldo+Ami; n = 6). Wild-type mice that received sham operation served as a control group (n = 4). All the groups received normal salt diet. (C) In study 3, mice received either a low (0.005%; n = 9) or a normal (0.9%; n = 9) K+ diet for seven days. (D) In study 4, mice were kept on a normal (0.9%) or low (0.005%) K+ diet for 5 days. At day 5, a subgroup of mice on a normal K+ diet and those on a low-K+ diet received subcutaneous infusion of aldosterone (12 ng/hr; low aldo). All the mice (n = 6 for each group) were euthanized at day 12. (E) In study 5, pendrin knockout (Pds−/−) mice (n = 7) and wild-type mice (n = 12) with 129/Sv background received subcutaneous infusion of aldosterone at 60 ng/hr for 14 days. Two Pds−/− mice died during the experiment, and the remaining five mice were included in the analysis. (F) In an additional study, the effects of K+ supplementation were evaluated in a rat model of hypertension. Sprague-Dawley rats received uninephrectomy and subcutaneous infusion of aldosterone (0.75 μg/hr) for 4 weeks (Aldo group; n = 10). In Aldo+K group, uninephrectomized, aldosterone-infused rats received K+ supplementation (1% KCl) *via* drinking water (n = 9). Control rats received uninephrectomy without aldosterone infusion (n = 11). All the groups were maintained on a standard, normal salt diet.
Figure S2

**Purification of plasma membrane proteins.**

Plasma membrane fraction and cytoplasmic fraction were purified from wild-type mouse kidney using plasma membrane isolation kit (Minute, Invent biotechnologies). Each fractions were analyzed by Western blotting using the indicated antibodies. Total kidney lysates were extracted using lysis buffer containing 1% NP-40. The results show that plasma membrane proteins but not cytoplasmic proteins were highly enriched in the plasma membrane fraction.
Figure S3
Representative images for the Coomassie brilliant blue (CBB)-stained polyacrylamide gels.
(A-E) The amount of the plasma protein loaded on the gel was monitored by CBB staining in samples obtained from study 1 (A), study 2 (B), study 3 (C), study 4 (D), and an additional study using rat model of hypertension (E). A part of the image also appeared in the main figures.
Figure S4

Low-power images of the kidney stained for pendrin and NCC.

(A) Kidney sections stained for pendrin (green) in aldosterone-infused mice (left) and aldosterone-infused mice received K⁺ supplement (right). (B and C) Kidney sections stained for NCC (red; B) and pendrin (green; C) in mice on a normal-K⁺ diet (left) and a low-K⁺ diet (right). Bars represent 50 μm.
**Figure S5**

K⁺ supplementation prevents the upregulation of NCC and pendrin in aldosterone-infused rats, in association with blood pressure reduction.

(A) Systolic blood pressure was measured at 0, 2, and 4 weeks in the control rats (Ctrl), rats received aldosterone (Aldo), and rats received aldosterone and 1% KCl via drinking water (Aldo+K), using CODA Blood Pressure System (see Expanded Materials and Methods for detail). (B) Effects of K⁺ supplementation on plasma membrane levels of the indicated proteins in the kidney. Blots show biological replicates. Bar graphs show the results of quantitation. The amount of protein loaded on the gel was monitored using CBB staining. Data are expressed as means ± SEM; **P < 0.01.
Figure S6
Pendrin and NCC induction by aldosterone is blocked by amiloride.

(A) Plasma K⁺ concentrations in the indicated animals. Ami, amiloride. (B) Effects of amiloride on plasma membrane levels of the indicated proteins in the kidney of aldosterone-infused mice. Blots show biological replicates. The amount of protein loaded on the gel was monitored using CBB staining. Bar graphs show the results of densitometric analysis. Data are expressed as means ± SEM; **P < 0.01.
Figure S7
Low plasma K⁺ levels reduce MR\textsuperscript{S843-P} in aldosterone infusion model.
MR phosphorylated at S843 (MR\textsuperscript{S843-P}) and total MR levels in the kidneys of wild-type mice on a normal-K⁺ diet with low-dose aldosterone (12 ng/hr; low aldo), and a low-K⁺ diet with low-dose aldosterone were determined by Western blot in biological replicates. Bar graphs show the results of densitometric quantitation (n = 6 each group). Data are expressed as means ± SEM; **\( P < 0.01 \).
Figure S8

Proposed model for Na-Cl transport involving pendrin in the collecting duct.

In the collecting duct, Cl\(^-\) transport through pendrin is coupled to Na\(^+\) reabsorption through ENaC (left). HCO\(_3^\)\(^-\) excretion in exchange for Cl\(^-\) reabsorption results in the increased urinary pH. Apical H\(^+\)-ATPase present in the collecting duct limits the rise in HCO\(_3^\)\(^-\) concentration in the tubular lumen, indirectly promoting Na-Cl transport. Besides this mechanism, pendrin can work in tandem with Na\(^+\)-driven Cl\(^-\)/HCO\(_3^\)\(^-\) exchanger (NDCBE), partly contributing to electroneutral Na-Cl reabsorption in this segment (right). These electroneutral Na-Cl reabsorption mechanisms eliminate the lumen-negative voltage that drives K\(^+\) transport through renal outer medullary K\(^+\) channel (ROMK), decreasing urinary K\(^+\) excretion.