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The circadian clock protein Period 1 regulates expression of the renal epithelial sodium channel in mice

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The mineralocorticoid aldosterone is a major regulator of sodium transport in target epithelia and contributes to the control of blood pressure and cardiac function. It specifically functions to increase renal absorption of sodium from tubular fluid via regulation of the α subunit of the epithelial sodium channel (αENaC). We previously used microarray technology to identify the immediate transcriptional targets of aldosterone in a mouse inner medullary collecting duct cell line and found that the transcript induced to the greatest extent was the circadian clock gene Period 1. Here, we investigated the role of Period 1 in mediating the downstream effects of aldosterone in renal cells. Aldosterone treatment stimulated expression of Period 1 (Per1) mRNA in renal collecting duct cell lines and in the rodent kidney. RNA silencing of Period 1 dramatically decreased expression of mRNA encoding αENaC in the presence or absence of aldosterone. Furthermore, expression of αENaC-encoding mRNA was attenuated in the renal medulla of mice with disruption of the Per1 gene, and these mice exhibited increased urinary sodium excretion. Renal αENaC-encoding mRNA was expressed in an apparent circadian pattern, and this pattern was dramatically altered in mice lacking functional Period genes. These results suggest a role for Period 1 in the regulation of the renal epithelial sodium channel and more broadly implicate the circadian clock in control of sodium balance.

Introduction
Sodium (Na) is the principal extracellular cation and plays a vital role in maintaining extracellular fluid volume and therefore blood pressure. The mineralocorticoid hormone aldosterone acts directly on the distal nephron and collecting duct to increase Na reabsorption, which in turn increases blood volume, vascular reactivity, and blood pressure (1, 2). Indeed, most known forms of monogenic hypertension are the result of a defect in the downstream signaling targets of aldosterone (3). In target epithelia, aldosterone increases the absorption of Na from the tubular fluid into the extracellular space by increasing the number and activity of epithelial sodium channels (ENaCs) at the apical membrane and the Na+/K+-ATPase at the basolateral membrane. Transepithelial Na and H2O reabsorption increases blood volume and vascular reactivity and consequently systemic blood pressure. Whereas these late effects of aldosterone action have been well characterized, the mediators of this action have not.

We have shown that SV40-transformed mouse inner medullary collecting duct (mIMCD-3) cells, derived from the terminal third of the inner medullary collecting duct (IMCD), represent a model for aldosterone action (4). These cells express 11β-hydroxy-steroid dehydrogenase type 2, which confers mineralocorticoid specificity; αENaC, the regulated and rate-limiting subunit of renal ENaC function (5, 6); and the mineralocorticoid receptor (MR). These transcripts are markers of aldosterone-responsive cells. In order to identify the acute transcriptional effects of aldosterone, we previously used a microarray analysis to evaluate changes in gene expression in mIMCD-3 cells after an acute exposure to aldosterone (4). The most highly induced transcript in the study was Period 1 (Per1), previously described as period homolog. The Per1 gene encodes a member of the basic helix-loop-helix–Period-Arnt-SIM (bHLH-PAS) domain-containing family of transcription factors that recognize E-box motifs in the promoters of target genes. Per1 is an important component of the circadian clock; its discovery and characterization provided essential insight into the mechanism behind regulation of circadian patterns (7). Many physiological processes are governed by or exhibit a circadian pattern, including the sleep-wake cycle, heartbeat, body temperature, hormone secretion, renal blood flow, and renal electrolyte excretion (8, 9). The circadian clock pacemaker is the suprachiasmatic nucleus (SCN), located in the ventral hypothalamus. The SCN processes information from “peripheral clocks,” including the liver and kidney. Positive and negative regulatory feedback loops make up the central circadian clock. In the positive loop, 2 bHLH transcription factors, Clock and Bmal1, heterodimerize to drive transcription of Per1, Per2, and cryptochrome (Cry). In the negative loop, Cry/Per oligomers shuttle between the cytoplasm and nucleus to inhibit Clock/Bmal1, thereby decreasing their own transcription. Transcript and protein levels of these circadian transcription factors rise and fall over the course of an approximately 24-hour period.
RNA interference was used to evaluate the effect of Per1 knockdown and blood pressure are not understood. mechanisms for the circadian fluctuation in electrolyte excretion were first reported more than 100 years ago, by Zadek in 1881 and Vogel in 1854, respectively (reviewed in ref. 10). A diurnal rhythm of urinary sodium, potassium, and chloride excretion has been established for many decades (11–13). In normal individuals, electrolyte excretion and urine production are greater during the day than at night. Alteration of this circadian pattern is often associated with pathophysiological conditions, including hypertension and cardiovascular disease (14, 15). Whereas these clinical observations are well established, the underlying molecular mechanisms for the circadian fluctuation in electrolyte excretion and blood pressure are not understood.

Here, we report upregulation of Per1 by aldosterone and further confirm the upregulation of Per1 in the kidney in vivo. To characterize the aldosterone-mediated regulation of Per1, we cloned and characterized the Per1 promoter and used RNA interference to analyze the role of the MR and the glucocorticoid receptor (GR) in the regulation of Per1 by aldosterone. In turn, RNA interference was used to evaluate the effect of Per1 knockdown on aldosterone action. Per1 knockdown prevented the induction of αENaC by aldosterone. Interestingly, knockdown of Per1 in the absence of aldosterone also led to a decrease in αENaC levels, which suggests that Per1 contributes to the basal regulation of this subunit. Furthermore, αENaC was downregulated in the inner and outer medulla in vivo in Per1-deficient compared with wild-type mice. Finally, we show for the first time to our knowledge that αENaC mRNA is expressed in a circadian pattern similar to Per1 and that this expression profile is altered in mice lacking functional Period genes. These data clearly demonstrate the importance of the circadian rhythm gene Per1 in the regulation of αENaC expression. Such findings provide the first evidence of a direct molecular link between the circadian clock and a mediator of Na balance.

**Results**

*Per1 is induced by aldosterone in vivo.* In order to evaluate the in vivo regulation of Per1, we injected rats with vehicle (ethanol) or aldosterone. Animals were euthanized, and the inner medulla was dissected at intervals up to 6 hours following the injection of hormone or vehicle. Aldosterone-induced changes in Per1 mRNA levels were determined relative to vehicle-treated control animals. Per1 expression increased more than 3-fold in animals exposed to aldosterone for 6 hours (Figure 1). This result suggests that Per1 is subject to aldosterone regulation in vivo.

*Circadian rhythm genes are upregulated by aldosterone.* Aldosterone induced 2 circadian rhythm genes in our earlier microarray studies: period homolog 1 (Per1) and period homolog 2 (Per2) (4). Per1 was upregulated to a greater extent than any other gene in the microarray study. To extend the study of the regulation of Per1 by aldosterone and to consider the effect of aldosterone on Per2, we treated mIMCD-3 cells with vehicle (ethanol) or 1 μM aldosterone for 1 or 24 hours (Figure 2A). As a control for the efficacy of aldosterone treatment, expression of serum- and glucocorticoid-regulated kinase 1 (Sgk1) was measured. Sgk1 is a well-characterized aldosterone target and regulator of ENaC (16). As expected, Per1 and Sgk1 mRNA levels increased more than 4-fold and 2-fold, respectively, after 1 hour of hormone treatment. These results were entirely consistent with our previous report (4). Interestingly, Per2 was not noticeably upregulated at 1 hour, even though the results of our microarray indicated a nearly 2-fold increase in Per2 at that time point. However, Per2 expression increased more than 2-fold after 24 hours of aldosterone treatment. Thus, we focused our attention on the more highly regulated transcript, Per1.
To address the issue of the concentration of aldosterone used in these experiments, we preformed a dose-response study in mIMCD-3 cells (data not shown). Per1 was significantly increased by aldosterone at 0.1 μM (2.8 ± 0.06-fold increase, n = 3, P < 0.001) and at 0.01 μM (1.24 ± 0.04-fold increase, n = 3, P < 0.001). These results indicate that the induction of Per1 by aldosterone occurs at sub-micromolar doses of the hormone.

The transcriptional upregulation of the Scnn1a gene (hereafter referred to as αENaC) by aldosterone is a well-documented effect (17, 18), but the effect of aldosterone on αENaC mRNA levels had not been studied in mIMCD-3 cells. αENaC mRNA levels were unchanged after 1 hour of aldosterone treatment (Figure 2A). This was expected, because the known transcriptional effects of aldosterone on αENaC occur only after several hours of hormone exposure (17). However, after 24 hours of aldosterone treatment in mIMCD-3 cells, αENaC expression increased by nearly 15-fold. The results validated mIMCD-3 cells as an appropriate in vitro model for investigation of aldosterone action on αENaC gene expression. To confirm that aldosterone increased Per1 protein levels, we performed Western blot analysis on total cell lysates from mIMCD-3 cells treated with vehicle or aldosterone for 6 hours (Figure 2B). Per1 protein levels increased after aldosterone treatment.

**Aldosterone stimulates Per1 transcription.** Two approaches were taken to determine whether aldosterone action on Per1 was a direct result of transcriptional activation of the Per1 gene. A BAC clone containing part of mouse chromosome 11 was used as template for a PCR-based approach to clone the Per1 promoter (data not shown). A 2,002-bp fragment of the Per1 promoter, up to but not including the translation start site, was cloned in-frame with the luciferase cDNA to generate the plasmid Per1/luc. mIMCD-3 cells were transfected with pGL3 or Per1/luc for 24 hours and then treated with vehicle (ethanol) or 1 μM aldosterone for 6 hours. Aldosterone treatment had no effect on the negative control pGL3 but increased Per1/luc luciferase activity by only 20% (Figure 3A) (P < 0.05). Although this difference was statistically significant, the magnitude of increase was less than what was observed by using quantitative real-time RT-PCR (QPCR) to measure steady-state Per1 mRNA levels in the presence of aldosterone in mIMCD-3 cells. Compared with a pGL3 control vector containing the SV40 promoter, the level of luciferase activity of Per1/luc was 2- to 3-fold higher (data not shown). This high background may have obscured the effect of external stimulation on the Per1 luciferase construct. Therefore, we tested direct transcriptional activity from the endogenous Per1 gene.

Analysis of the short-lived heterogeneous nuclear RNA (hnRNA) is a direct measure of transcriptional activity (19). Primers were designed to amplify a region of the Scnn1a gene (hereafter referred to as αENaC) by aldosterone is a well-documented effect (17, 18), but the effect of aldosterone on αENaC mRNA levels had not been studied in mIMCD-3 cells. αENaC mRNA levels were unchanged after 1 hour of aldosterone treatment (Figure 2A). This was expected, because the known transcriptional effects of aldosterone on αENaC occur only after several hours of hormone exposure (17). However, after 24 hours of aldosterone treatment in mIMCD-3 cells, αENaC expression increased by nearly 15-fold. The results validated mIMCD-3 cells as an appropriate in vitro model for investigation of aldosterone action on αENaC gene expression. To confirm that aldosterone increased Per1 protein levels, we performed Western blot analysis on total cell lysates from mIMCD-3 cells treated with vehicle or aldosterone for 6 hours (Figure 2B). Per1 protein levels increased after aldosterone treatment.

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mIMCD-3 cells (Table 1). Transfection with MR10 siRNA yielded the best knockdown in MR mRNA levels, a nearly 90% reduction. Transfection with GR-10 siRNA yielded a comparable knockdown in GR mRNA levels.

MR10 or GR10 siRNA was transfected into mIMCD-3 cells 24 hours prior to 1-hour treatment with vehicle or aldosterone in order to determine the effect of receptor knockdown on Per1 expression (Figure 4). MR knockdown inhibited the response of Per1 to aldosterone by approximately 30%, while GR knockdown essentially prevented hormonal induction of Per1. These results are consistent with our previous report using spironolactone and mifepristone, pharmacological inhibitors of MR and GR, respectively (4). These data indicate that both MR and GR contribute to aldosterone action in mIMCD-3 cells.

Per1 knockdown prevents the aldosterone-mediated induction of αENaC expression. ENaC is a heteromeric channel, thought to consist of equal numbers of α, β, and γ subunits (21). In the kidney, αENaC is the aldosterone-responsive subunit and acts as the rate-limiting component for channel formation (5, 22). To test the hypothesis that Per1 mediates the downstream action of aldosterone, we determined the effect of Per1 knockdown on the aldosterone-mediated induction of αENaC (Figure 5). mIMCD-3 cells were transfected with a non-target siRNA or each of 4 independent Per1-specific siRNAs. Twenty-four hours later, cells were treated with vehicle or aldosterone for an additional 24 hours. Fold changes in mRNA levels were calculated relative to the non-target siRNA–transfected cells treated with vehicle. Importantly, Per1 was induced by aldosterone in the non-target siRNA–transfected cells, demonstrating that siRNA transfection did not negatively affect hormone responsiveness. However, the presence of Per1-specific siRNA effectively prevented the aldosterone-mediated induction of Per1 (Figure 5A). Transfection with each of the 4 independent Per1 siRNAs resulted in effective knockdown of Per1 expression. Indeed, expression levels of Per1 in Per1 siRNA–transfected, aldosterone-treated cells were not significantly different from those in the non-target siRNA–transfected, vehicle-treated control.

Next, the effect of Per1 knockdown on Sgk1 was evaluated (Figure 5B). As was observed with Per1, transfection with the non-target siRNA did not affect the induction of Sgk1 by aldosterone. Interestingly, the level of Sgk1 expression in aldosterone-treated

Figure 5
Per1 knockdown attenuates the response of αENaC to aldosterone. Mouse IMCD-3 cells were transfected with Per1 siRNA sequences 24 hours prior to vehicle or aldosterone treatment. QPCR was used to analyze changes in gene expression of Per1 (A), Sgk1 (B), and αENaC (C) after Per1 knockdown in the presence of aldosterone compared with vehicle-treated non-target siRNA–transfected cells. Fold change values were normalized against actin and are presented as mean ± SEM; n = 4 (n = 3 for Per1-5 and Per1-6). *P < 0.05 versus non-target siRNA vehicle control; NS, not significant versus non-target siRNA–transfected cells treated with vehicle; ‡not significant versus non-target siRNA aldosterone-treated sample.
mIMCD-3 cells was equivalent in non-target siRNA–transfected cells and Per1 siRNA–transfected cells, indicating that knockdown of Per1 had no effect on the aldosterone-mediated increase in Scnn1a mRNA expression. This result was consistent with each of the 4 independent Per1-specific siRNAs.

As was observed previously (see Figure 2A), the increase in αENaC mRNA levels in response to 24 hours of aldosterone treatment was nearly 15-fold (Figure 5C). Transfection with a non-targeting siRNA control did not interfere with the response of αENaC to aldosterone. Per1 knockdown resulted in a dramatic reduction in αENaC mRNA levels. Indeed, levels of αENaC in Per1 siRNA–transfected cells were not statistically different from those in the non-target siRNA–transfected, vehicle-treated control cells. Each of the Per1-specific siRNAs had an equivalent effect: knockdown of Per1 effectively prevented the induction of αENaC by aldosterone.

**Effect of Per1 on αENaC occurs at the level of transcription.** In order to determine the mechanism of the effect of Per1 knockdown on αENaC expression, we examined αENaC hnRNA levels (Figure 6A). Three independent sets of vehicle- or aldosterone-treated samples from mIMCD-3 cells transfected with a non-target siRNA or the most effective (see Figure 5A) Per-specific siRNA, Per1-8 siRNA, were used as template. Primers were designed to cross the boundary from mIMCD-3 cells transfected with a non-target siRNA or the Per1 expression vector. Data are presented as mean ± SEM; n = 4. ∗P < 0.05 versus αENaC/luc plus vector.

**Figure 6**

Effect of Per1 knockdown on αENaC is transcriptional. (A) Top panel: Primers were designed to amplify a 238-bp region of the Scnn1a (αENaC) gene between exon 8 and intron 8 in order to measure hnRNA as an indicator of transcriptional activity. Templates from 3 (nos. 1–3) independent sets of non-target siRNA– or Per1-8 siRNA–transfected mIMCD-3 cells treated with vehicle or aldosterone were used in PCR reactions; n = 3. V, non-target siRNA–transfected cells plus vehicle; A, non-target siRNA–transfected cells plus aldosterone; P, Per1-8 plus aldosterone. Bottom panel: An 874-bp GAPDH product was amplified as a PCR control. (B) αENaC promoter luciferase activity was measured as described in Figure 3. Mouse IMCD-3 cells were cotransfected with pGL3 or αENaC/luc and empty pCMVSPORT6 vector or Per1 expression vector. Data are presented as mean ± SEM; n = 4. ∗P < 0.05 versus αENaC/luc plus vector.
aldosterone treatment, while Per2 expression was upregulated only after 24 hours of aldosterone treatment (Supplemental Figure 1A). αENaC expression was upregulated more than 10-fold after 24 hours of hormone treatment. These results were consistent with what was observed in mIMCD-3 cells (see Figure 2A).

Since the effect of Per1 knockdown on the aldosterone response of αENaC mRNA in mIMCD-3 cells was so dramatic, a similar experiment was performed in mIMCD-K2 cells to test the effect in an independent cell line (Supplemental Figure 1B). As was observed in mIMCD-3 cells, transfection with any of 4 independent Per1-specific siRNAs prevented the induction of Per1 by aldosterone. Sgk1 mRNA levels were increased in Per1-knockdown cells. However, in contrast to what was observed in mIMCD-3 cells, the magnitude of Sgk1 induction in Per1-specific siRNA-transfected mIMCD-K2 cells was less than that in the aldosterone-treated non-target siRNA–transfected cells. The effect of Per1 knockdown on αENaC was equivalent in mIMCD-3 cells and mIMCD-K2 cells. The results thus suggest that Per1 knockdown inhibits the response of αENaC to aldosterone in mIMCD-K2 cells.

**Figure 7**

Effect of Per1 knockdown on αENaC occurs in the early phase of aldosterone action. Mouse IMCD-3 cells were transfected with a non-target siRNA or Per1-8 siRNA for 24 hours and then treated with vehicle or aldosterone for 2, 4, or 6 hours. QPCR was used to analyze changes in gene expression of Per1 (A), Sgk1 (B), and αENaC (C) after Per1 knockdown in the presence of aldosterone compared with control (vehicle treated, non-target siRNA–transfected cells; data not shown). Fold change values were normalized against actin relative to the non-target siRNA–transfected, vehicle-treated control. Data are presented as mean ± SEM; n = 3. *P < 0.05 versus control; †P < 0.05 versus non-target siRNA–transfected cells treated with aldosterone.

**Figure 8**

αENaC expression is inhibited by Per1 knockdown in the absence of aldosterone. Mouse IMCD-3 cells were transfected with a non-target siRNA or Per1-8 siRNA. Forty-eight hours later, total RNA was isolated and processed for QPCR. Fold changes in Sgk1 and αENaC were calculated relative to non-target siRNA–transfected cells with vehicle. In non-target siRNA–transfected cells treated with aldosterone, Per1 mRNA levels increased more than 3-fold at 2, 4, or 6 hours of hormone exposure (Figure 7A, solid line). The increase in Per1 by aldosterone was prevented in cells transfected with the Per1-8 siRNA (Figure 7A, dashed line). Sgk1 mRNA

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expression increased at all time points of aldosterone treatment (Figure 7B). The increase in Sgk1 by aldosterone was not affected by Per1 knockdown. Indeed, Sgk1 levels were slightly higher in aldosterone-treated cells transfected with Per1-8 siRNA. Surprisingly, αENaC mRNA levels were significantly increased following 2 hours of aldosterone treatment. The aldosterone-induced increase in αENaC mRNA levels was observed after 4 or 6 hours of hormone treatment (Figure 7C). Interestingly, the early increase in αENaC mRNA levels was prevented after siRNA-mediated knockdown of Per1 (Figure 7C). These data indicate that the effect of Per1 knockdown on the regulation of αENaC by aldosterone occurs in the early phase of aldosterone action.

αENaC expression is attenuated in the inner medulla of Per1-deficient mice. Total RNA was isolated from the inner medullas of wild-type (129/Sv) or Per1-deficient mice. QPCR was used to analyze changes in gene expression of Sgk1 (gray bars) and αENaC (black bars) in Per1-deficient compared with wild-type control animals. Fold change values were normalized against actin relative to wild-type control mice. Data are presented as mean ± SEM; n = 3. *P < 0.05 versus wild-type.

Figure 9
αENaC expression is attenuated in the inner medulla of Per1-deficient mice. Total RNA was isolated from the inner medullas of wild-type (129/Sv) or Per1-deficient mice. QPCR was used to analyze changes in gene expression of Sgk1 (gray bars) and αENaC (black bars) in Per1-deficient compared with wild-type control animals. Fold change values were normalized against actin relative to wild-type control mice. Data are presented as mean ± SEM; n = 3. *P < 0.05 versus wild-type.

αENaC expression is inhibited by Per1 knockdown in the absence of aldosterone. The results of the time course experiment indicated that the effect of Per1 knockdown on αENaC occurred within the first 2 hours after aldosterone treatment. This result led to the hypothesis that Per1 contributes to the basal regulation of αENaC expression. To test this, we analyzed changes in αENaC expression after Per1 knockdown in the absence of aldosterone stimulation (Figure 8). mIMCD-3 cells were transfected with a non-target siRNA or Per1-8 siRNA. Per1 mRNA levels were significantly decreased in these cells (83% ± 3.5%). Fold change values were calculated relative to the non-target siRNA-transfected control. Sgk1 levels were unaffected by Per1 knockdown. However, αENaC expression was decreased more than 2-fold after Per1 knockdown in the absence of aldosterone. These data suggest a role for Per1 in the basal regulation of αENaC transcription.

Figure 10
Per1 mediates regulation of αENaC expression in the outer medulla. (A) The induction of αENaC by aldosterone is attenuated in OMCD1 cells after Per1 knockdown. OMCD1 cells were grown, siRNA-transfected, and treated with vehicle or aldosterone as described for mIMCD-3 cells. *P < 0.05 versus non-target siRNA-transfected cells treated with vehicle; **P < 0.05 versus non-target siRNA-transfected cells treated with aldosterone; n = 4. (B) In the absence of aldosterone, Per1 knockdown results in a 2-fold decrease in αENaC mRNA levels in OMCD1 cells. *P < 0.05 versus non-target siRNA–infected cells; n = 6. (C) Per1-deficient mice exhibit reduced αENaC expression in the outer medulla. *P < 0.05 versus wild-type; n = 3. All data are presented as mean ± SEM.
Per1 knockdown resulted in a statistically significant decrease in αENaC mRNA levels (Figure 10B). To evaluate the role of Per1 in the regulation of αENaC in the outer medulla in vivo, we analyzed αENaC mRNA levels in the outer medulla of wild-type and Per1-deficient mice. Figure 10C shows that αENaC mRNA levels were reduced more than 2-fold in mice lacking Per1 compared with wild-type mice. These data are consistent with the in vitro and in vivo results from the inner medulla. Thus, there appears to be a role for Per1 in the in vivo regulation of αENaC expression in the inner and outer medulla.

Per1 mediates regulation of αENaC expression in mpkCCD, cells. To evaluate whether the Per1-mediated regulation of αENaC expression extended to the cortex, we tested the effect of Per1 knockdown in an in vitro model of the cortical collecting duct (CCD). Murine mpkCCD, cells (30) were grown and transfected with siRNA and treated with vehicle or aldosterone as described for mIMCD-3 cells. Consistent with the work of Bens et al. (30), the mRNA expression of αENaC was increased by aldosterone in these cells (Figure 11A). As we observed in mIMCD-3 and OMCD, cells, Per1 knockdown inhibited the induction of αENaC by aldosterone. Next, the effect of Per1 knockdown on αENaC expression was tested in the absence of hormone treatment. Again, consistent with our results in mIMCD-3 and OMCD, cells, knockdown of Per1 led to a nearly 3-fold decrease in αENaC mRNA levels (Figure 11B).

Mice lacking functional Per1 excrete more urinary Na than wild-type mice. To evaluate renal Na handling in Per1-deficient mice, we performed a metabolic cage study. Twenty-four-hour urine collections were made from Per1-deficient mice or wild-type control mice (129/Sv) maintained on normal laboratory chow. Body weight was not significantly different between wild-type (34.6 ± 1.6 g) and Per1-deficient (32.8 ± 1.9 g) mice (P = 0.5). Food and water intake tended to be higher in Per1-deficient mice (5.7 ± 1.1 g; 6.5 ± 1.4 ml) compared with wild-type mice (4.3 ± 0.6 g; 4.8 ± 0.5 ml), but this trend did not reach significance (P = 0.32 for food intake; P = 0.25 for water intake). Urinary Na and chloride (Cl) excretion were evaluated (Figure 12, A and B), and total urinary volume was measured (Figure 12C). Cl excretion tended to be higher in Per1-deficient mice compared with control animals, but this difference was not significant. Importantly, statistically significant increases in urinary Na excretion and urine output were observed in mice lacking Per1 compared with wild-type mice. This observation is consistent with our in vivo data demonstrating decreased renal αENaC expression in Per1-deficient mice.

mRNA expression profile of αENaC is altered in Period-deficient mice. Perhaps the best test of the hypothesis that αENaC is regulated by the circadian clock in vivo is to evaluate the 24-hour expression profile of αENaC in wild-type compared with Period-deficient mice. To our knowledge, the 24-hour expression profile of αENaC mRNA has never been measured in the kidney. Kidneys from wild-type mice or mice lacking functional Per1, Per2, and Per3 (triple knocked out, or TKO) were collected every 4 hours over a 24-hour period. The TKO mice have been described (31) and provide the best tool for evaluating the role of this family of circadian clock proteins in the regulation of αENaC. We show that αENaC mRNA expression fluctuates with an apparent circadian pattern that is similar to that of Per1 in the inner medulla (Figure 13A), the outer medulla (Figure 13B), and the cortex (Figure 13C) of wild-type mice. At some time points, αENaC expression was higher in the TKO compared with wild-type animals (ΔCt is an inverse measure of relative expression). Because this experiment does not distinguish among the effects of Per1, Per2, and Per3, the implications of this outcome are unclear. Interestingly, the peak of αENaC mRNA expression was shifted by 12 hours in each segment of the kidney in TKO mice compared with wild type. In the inner medulla, wild-type αENaC expression peaked at circadian time 10 (CT10), while TKO αENaC peaked at CT22. In the outer medulla, αENaC expression peaked at CT18 in wild-type mice compared with CT6 in the TKO mice. αENaC mRNA levels peaked at CT2 in the cortex of TKO mice versus CT14 in the wild-type mice.

To evaluate differences in the 24-hour expression profile of renal αENaC in mice lacking functional Period genes compared with wild-type mice, we generated scatter plots using the mean ΔCt values at each time point for αENaC expression in each renal segment for wild-type and TKO mice. Waveform functions were then fitted to the data (Supplemental Figure 2). The results for the inner medulla and outer medulla show that loss of the Period genes resulted in an inversion of the expression profile for αENaC. The expression profile for αENaC in the wild-type cortex was very similar to that in the inner and outer medulla. In the TKO cortex,
Together, these data demonstrate that the 24-hour renal Na transport. Per1 knockdown prevented the response of representing the IMCD, OMCD, and CCD. In addition, molecular link between the circadian clock and a mediator of evidence supporting the hypothesis that the circadian clock regulates. Most strikingly, we show that Per1-deficient mice exhibited higher urinary Na excretion. However, the pattern appeared to be reversed in the time period tested. Together, these data demonstrate that the 24-hour αENaC expression profile is dramatically altered in the inner medulla, the outer medulla and the cortex of mice lacking the 3 Period genes compared with wild-type mice. This result provides compelling evidence supporting the hypothesis that the circadian clock regulates in vivo αENaC mRNA expression.

Discussion
Here we show for the first time to our knowledge that αENaC mRNA expression is regulated by the aldosterone-responsive circadian clock gene Per1 in the kidney. These data provide a direct molecular link between the circadian clock and a mediator of renal Na transport. Per1 knockdown prevented the response of αENaC to aldosterone in several renal collecting duct cell lines representing the IMCD, OMCD, and CCD. In addition, αENaC levels were decreased after Per1 knockdown in the absence of hormonal stimulation in these cell lines. Furthermore, αENaC mRNA levels were reduced in the inner medulla and outer medulla in vivo in Per1-deficient mice. Consistent with this observation, the Per1-deficient mice exhibited higher urinary Na excretion. Most strikingly, we show that αENaC mRNA appears to be expressed in a pattern very similar to that of the circadian rhythm gene Per1 in wild-type mice and that this expression profile is altered in mice lacking functional Period genes. This novel observation could have major implications for renal Na handling, and possibly blood pressure control.

Our report of the upregulation of Per1 by aldosterone is consistent with in vitro work concerning regulation of Per1 by the renin angiotensin aldosterone system (RAAS). Microarray analysis in a human adrenocortical cell line after angiotensin II treatment demonstrated that Per1 was upregulated 3-fold compared with control (24). Per1 and Per2 were induced by aldosterone in cardiomyoblasts, an effect that was blocked by spironolactone (32). Another connection between hormone signaling and Per1 was demonstrated by Balsalobre et al. They found that glucocorticoids reset circadian timing in peripheral tissues, including the kidney, by inducing the expression of Per1 (33). Of particular note, Sgk1 mRNA levels were unaffected by Per1 knockdown in mIMCD-3 cells or in the inner medulla of Per1-deficient mice and were only slightly decreased following Per1 knockdown in mIMCD-K2 cells. Sgk1 is considered a canonical regulator of ENaC, yet evidence suggests that Sgk1 is not solely responsible for mediating aldosterone action on ENaC. For example, Sgk1-knockout mice have a much milder Na wasting phenotype than MR-knockout mice (34–37). Furthermore, ENaC function is fully upregulated by chronic aldosterone exposure in Sgk1-knockout mice (37), suggesting that other mediators of aldosterone action can stimulate ENaC. Together with our demonstration of the role of Per1 in regulating αENaC expression, these in vivo studies suggest that Per1 action is not likely to be downstream of Sgk1. Since Sgk1 mRNA levels are not affected by Per1 knockdown, it seems unlikely that Sgk1 is directly downstream of Per1 action. It remains to be seen whether Per1 action is truly independent of Sgk1. Given the results in the mIMCD-K2 cells, which are derived from a more proximal region than mIMCD-3 cells, it is possible that the sequence of regulatory events could differ depending on the specific region of the nephron.

Perhaps the most intriguing implication of these findings concerns the known role of ENaC in the maintenance of blood pressure. Underscoring the importance of ENaC in blood pressure regulation is the fact that gain-of-function mutations in ENaC subunits cause the hypertensive disorder Liddle syndrome. Conversely, loss-of-function mutations in ENaC subunits result in the hypotensive disorder pseudohypoaldosteronism type 1a. Aldosterone is known to be secreted with a diurnal pattern, as it increases during sleep (38). The connection among the circadian clock, aldosterone signaling, and αENaC expression is especially interesting given the well-characterized diurnal pattern of blood pressure. Blood pressure increases in the early morning, and this is followed by a plateau during active daytime and then a “dip” in values during sleep (39). Patients who do not exhibit this dipping pattern are designated “non-dippers.” There is extensive evidence sup-

Figure 12
Mice lacking Per1 excrete more sodium compared with wild-type mice. Per1 mutant mice (n = 3) or wild-type mice (n = 4) were housed in metabolic cages and maintained on a normal laboratory chow diet with free access to water for 48 hours. Food and water intake and body weight were monitored. Two 24-hour urine collections were made. Urine was analyzed for Na (U\textsubscript{Na}\textsubscript{V}; A), Cl (U\textsubscript{Cl}\textsubscript{V}, B), and total volume (C). #P < 0.05 versus wild-type. Data are presented as mean ± SEM.
The mechanism of how the circadian clock affects physiological processes in the kidney and Na homeostasis is an active area of investigation. Gene profiling experiments in tissues such as liver and heart have shown that 6%–8% of expressed genes are subject to circadian control (reviewed in ref. 9). Presumably, this involves regulation of targets by circadian clock proteins or their direct effectors. The importance of the circadian clock in regulation of physiological processes such as renal and cardiac function is clear. However, the underlying molecular mechanisms are largely unknown. It was recently demonstrated that hamsters with a disrupted circadian rhythm display severe renal and cardiac disease (48). These hamsters have a mutation in the gene for casein kinase–1ε, a kinase that directly phosphorylates Per1, leading to Per1 degradation (8).

In conclusion, we have shown for the first time to our knowledge that Per1 is regulated by aldosterone and in turn mediates aldosterone action on the expression of αENaC mRNA. Importantly, the effect of Per1 knockdown on αENaC occurred even in the absence of aldosterone stimulation and was observed in Per1-deficient mice as well. These data suggest a new pathway for regulation of αENaC by the circadian clock. This observation could lead to the first characterization of a molecular regulator of circadian blood pressure patterns.

Methods

Animals. All animal experiments were approved by the University of Florida Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (290–320 g; Harlan) were given an i.p. injection (1 ml/kg) of aldosterone (1 mg/kg) or vehicle (20 μl ethanol/kg in saline). After 1, 2, or 6 hours, rats were anesthetized with isoflurane, and the aorta was cannulated with a 19G winged infusion set. After blood collection into an EDTA-coated syringe, the kidneys were immediately flushed by in vivo aortic perfusion of PBS (pH 7.4) with the vena cava vented. The right kidney was removed and the inner medulla dissected before being flash frozen in liquid nitrogen.

Kidneys from Per1-deficient mice, TKO mice, and wild-type controls were collected at the University of Massachusetts Medical School. These animals have been described previously (28, 31).

Metabolic study. Metabolic cage studies were performed at the University of Massachusetts Medical School with approval from and in accordance with the local IACUC. Per1-deficient mice and 129/Sv mice were acclimated to metabolic cages (Lab Products) for 3 days before two 24-hour urine samples were collected. Animals had free access to standard laboratory chow and water. Body weight and food and water intake were measured. Urinary Na and Cl content were measured using a Nova 16 Electrolyte/Chemistry Analyzer (Nova Biomedical).

Cell culture. mIMCD-3 cells were purchased from ATCC. Mouse IMCD-K2 cells were a gift from Bruce Stanton (Dartmouth Medical School, Hanover, New Hampshire, USA) (27). OMCD cells were a gift from Thomas DuBose (Wake Forest University, Winston-Salem, North Carolina, USA) (29). Alain Vandewalle (INSERM, Paris, France) provided the mpkCCD 141cells (30).

Cells were maintained in DMEM-F12 plus 10% FBS and 50 μg/ml gentamicin. For aldosterone treatments, 600,000 cells were plated in each well of a 24-well Corning Costar Transwell dish. Twenty-four hours after cells reached 100% confluency, the medium was changed to phenol red–free DMEM-F12 (Invitrogen) plus 10% charcoal/dextran-treated FBS to deprive the cells of steroid hormones. Another 24 hours later, cells were treated with vehicle (ethanol) or 1 μM aldosterone for varying time intervals. Final ethanol concentration in both vehicle and aldosterone-treated cells was 0.1%.

RNA isolation and qPCR. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA samples (10 μg) were reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega). Reaction conditions were 42°C, 50 minutes; then 93°C, 5 minutes; and 70°C, 5 minutes. qPCR was performed in a 96-well optical plate with Fast SYBR green reagent (Applied Biosystems) and a Bio-Rad iCycler. A reference cycle was performed, followed by 40 amplification cycles. Data are presented as mean ± SEM; n = 3 for each data point. *P < 0.05, wild-type αENaC expression versus TKO.
Table 2
Taqman assays used for quantitative real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reporter sequence</th>
<th>Assay ID&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per1</td>
<td>CCTCAGCCCTGTCGGCACACTAGG</td>
<td>Mm00501813_m1</td>
<td>106</td>
</tr>
<tr>
<td>Per2</td>
<td>CACCTCTAATCTGCAAGGCCTCTC</td>
<td>Mm00478113_m1</td>
<td>73</td>
</tr>
<tr>
<td>Actin</td>
<td>TACTGAGCTGGTTTTACACCTTT</td>
<td>Mm00607939_s1</td>
<td>69</td>
</tr>
<tr>
<td>Ssn1a (eNAC)</td>
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<td>Mm00803386_m1</td>
<td>68</td>
</tr>
<tr>
<td>Nr3c1 (GR)</td>
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<td>Mm00433832_m1</td>
<td>92</td>
</tr>
<tr>
<td>Nr3c2 (MR)</td>
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<td>Mm01241597_m1</td>
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<tr>
<td>Sgk1</td>
<td>CAAAACGCTGAAGTTTGGAAGTCTT</td>
<td>Mm00441380_m1</td>
<td>68</td>
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<sup>a</sup>Purchased from Applied Biosystems.

The authors acknowledge Robert Dallmann for collection of kidneys from Per-deficient mice; Bruce Stanton for the mIMCD-K2 research article

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Statistics. One-way ANOVA was performed with the Bonferroni test for error protection, with the exception of the time course data in Figure 13, for which a 2-way ANOVA with the Tukey test was used. Data are presented as mean ± SEM or SD as designated. P values less than 0.05 were considered significant.

research article
cells; Thomas DuBoise for the OMCD; cells; Alain Vandewalle for the mpkCCD14 cells; Damian Romero for the Per1 expression construct; and Christie Thomas for the εENaC promoter luciferase construct. This work was supported by grants from the NIDDK (R01 DK049750 to C.S. Wingo and T32 DK-07518 and AHA 0825467E to M.L. Gumz) and by the Department of Veterans Affairs (C.S. Wingo and M.L. Gumz).


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