Low-Salt Diet and Circadian Dysfunction Synergize to Induce Angiotensin II–Dependent Hypertension in Mice


See Editorial Commentary, pp 493–495

Abstract — Blood pressure exhibits a robust circadian rhythm in health. In hypertension, sleep apnea, and even shift work, this balanced rhythm is perturbed via elevations in night-time blood pressure, inflicting silent damage to the vasculature and body organs. Herein, we examined the influence of circadian dysfunction during experimental hypertension in mice. Using radiotelemetry to measure ambulatory blood pressure and activity, the effects of angiotensin II administration were studied in wild-type (WT) and period isoform knockout (KO) mice (Per2-KO, Per2, 3-KO, and Per1, 2, 3-KO/Per triple KO [TKO] mice). On a normal diet, administration of angiotensin II caused nondipping blood pressure and exacerbated vascular hypertrophy in the Period isoform KO mice relative to WT mice. To study the endogenous effects of angiotensin II stimulation, we then administered a low-salt diet to the mice, which does stimulate endogenous angiotensin II in addition to lowering blood pressure. A low-salt diet decreased blood pressure in wild-type mice. In contrast, Period isoform KO mice lost their circadian rhythm in blood pressure on a low-salt diet, because of an increase in resting blood pressure, which was restorable to rhythmicity by the angiotensin receptor blocker losartan. Chronic administration of low salt caused vascular hypertrophy in Period isoform KO mice, which also exhibited increased renin levels and altered angiotensin 1 receptor expression. These data suggest that circadian clock genes may act to inhibit or control renin/angiotensin signaling. Moreover, circadian disorders such as sleep apnea and shift work may alter the homeostatic responses to sodium restriction to potentially influence nocturnal hypertension. (Hypertension. 2016;67:661-668. DOI: 10.1161/HYPERTENSIONAHA.115.06194.) • Online Data Supplement

Key Words: aging ■ angiotensin ■ circadian ■ hypertension ■ hypertrophy ■ sodium ■ renin

Hypertension remains a major risk factor for cardiovascular disease and death. Reducing sodium intake in the diet is important in blood pressure management. However, recent observations have emerged that challenge the absolute benefits of salt control in cardiovascular health. To date, the mechanisms and physiological interactions that underlie the potentially complex effects of sodium restriction and cardiovascular disease are unclear.

A significant characteristic of blood pressure is its circadian rhythm. In humans and animals, blood pressure rises and falls over 24 hours. The molecular mechanism responsible for circadian rhythms, the circadian clock, is important in the regulation of basal blood pressure. The circadian clock is driven by a heterodimeric interaction between the bHLH transcription factors Bmal1 and Clock or Npas2/MOP4. Bmal1/Clock or Bmal1/Npas2 heterodimers bind promoter elements of genes within the circadian clock loop (to control circadian rhythm) and outside of the clock loop to control function and physiology (output genes). The Period isoform genes (Per1, Per2, and Per3) and Cryptochrome genes (Cry1 and Cry2) comprise the negative feedback limb of the circadian clock loop. Single, double, and triple gene–targeted mutations of the Per isoforms, revealed that while Per1 and Per2 are critical for central locomotor behavioral rhythms, the Per isoform knockout (KO) mice (as well as the Clock mutant mice) exhibit a relatively normal locomotor rhythm in normal light cycle conditions, but lose locomotor rhythm during free running conditions in which 24 hours of constant dark/DD conditions are maintained, analogous to the Clock mutant mouse.

Recent evidence has demonstrated the importance of the circadian clock in key organs that are also involved in blood pressure regulation, including blood vessels, heart, and kidneys. Moreover, the significance of blood pressure rhythms in human disease is highlighted in human conditions where rhythms are absent, as in nondipper hypertensive patients that exhibit a raised incidence of cardiovascular morbidity. Moreover, the prevalence of nondipping blood pressure is not trivial, but may be as high as 45% in humans and may even further impact individuals with HIV, whereas targeting nondipping blood pressure with night-time...
administration of antihypertensives may even be beneficial in diabetes mellitus.\textsuperscript{35}

Herein, we undertook studies to examine the influence of circadian dysfunction during angiotensin II (Ang II), induced hypertension leading us to unexpected findings with regard to salt restriction.

**Material and Methods**

**Animals**

Care of the mice was according to institutional guidelines. Mice were kept on 12-hour light–12-hour dark cycles before and during portions of the time subjected to experimentation. Studies were performed on 4– to 6-month-old male littermate control (wild-type [WT]), Per2-KO, and Per1, 2, 3/triple KO (Per-TKO) mice as indicated. Period isof orm triple KO mice (Per-TKO) were raised to a colony (provided to us by Dr Weaver) in our laboratory. Studies were validated in the 3 different Per isof orm KO models of the mice. In these studies, we demonstrated that deletion of Per2-KO, Per2, 3-KO, and Per-TKO mice exhibited comparable phenotypes with regard to blood pressure and activity rhythms. These mice were originally generated by gene targeting in 129sv embryonic stem cells followed by chimeric males bred to isogenic 129sv females. Thus, the genetic background of the Per-TKO mice is 129S1/SvImJ. Detailed methods are available in the online-only Data Supplement.

**Statistical Analysis**

Comparisons were made with 2-way ANOVA and 1-way ANOVA with a Bonferroni post-test or with unpaired Student t tests. For studies, n=4–7 as indicated. SEM was used for error bars. Differences were considered statistically significant when P<0.05. Graphpad Prism (GraphPad Software Inc, La Jolla, CA) and cosinor analysis were used for analysis of data.

**Results**

**Blood Pressure Rhythm Is Sustained in Per2-KO Mice Despite Loss of Locomotor Rhythm in Free Running Conditions**

The circadian clock plays an important role in control of baseline blood pressure rhythm in standard light cycle conditions/ LD.\textsuperscript{10,26,36–38} Given the significant impact of free running conditions to worsen locomotor rhythm in circadian clock mutant mice, we sought to determine blood pressure rhythms in Per2-KO mice in conditions of constant darkness/DD. In LD, (12 hours light and 12 hours dark), blood pressure did not differ between WT and Per2-KO mice (Figure 1A). Similarly, locomotor activity in LD also exhibited a circadian rhythm in both WT and the Per2-KO mice (Figure 1B). DD conditions, however, effectively impaired the circadian rhythm in activity in the Per2-KO mice but not in the WT mice (Figure 1B). Despite the loss of activity rhythm in DD in Per2-KO mice, blood pressure remained intact in both WT and Per2-KO mice (Figure 1C). In 6 days of DD, resting (daytime) blood pressure of Per2-KO mouse did modestly increase averaging 119±2.34 mm Hg versus 114±2.99 mm Hg in WT mice (Figure 1C, inset). Also, although WT mice seemed to have a phase delay in blood pressure rhythm in response to DD, Per2-KO mice were absent in this response.

**Ang II Infusion Causes Nondipping Hypertension in Per2-KO Mice**

We next sought to determine if the response to experimentally induced hypertension was conditioned by circadian clock gene dysfunction. Thus, WT and Per2-KO mice were infused with Ang II. As observed in Figure 1A, baseline blood pressure and activity rhythms were not different between WT and Per2-KO mice (Figure 2A; Figure S1A). Exogenous administration of Ang II in LD caused a dramatic increase in blood pressure in the mice, but the elevation and rhythm of blood pressure was similar in WT and Per2-KO mice (Figure 2B) although activity was decreased in Per2-KO mice (Figure S1B). In contrast, in DD, Ang II caused a robust impairment in the blood pressure rhythm of Per2-KO mice (Figure 2C; WT=85.1% and Per2-KO=61.6%, robustness analyzed by Cosinor). The mean blood pressure over 24 hours was significantly higher in Ang II–infused Per2-KO mice (WT=140.8±3.88 mm Hg and Per2-KO=150.4±1.46 mm Hg; Figure 2D). The overall elevation in blood pressure in Per2-KO mice was because of nondipping blood pressure during the rest (day) period (subjective day mean arterial pressure; WT, 134.3±2.58 mm Hg; Per2-KO, 150.3±1.70 mm; Figure 2E). In DD, Locomotor activity was decreased, and arrhythmic as expected, in Per2-KO mice (Figure S1C). To determine if the day-time elevation in blood pressure caused an increase in blood vessel medial thickness, an index of vascular disease, aortas were harvested from vehicle-treated and Ang II–treated mice. WT and Per2-KO mice exhibited no differences in medial thickness under baseline conditions (Figure 2F). However, after the Ang II treatment, medial thickening was observed in WT mice but thickening was worsened in Per2-KO mice (Figure 2G). We expanded our studies to include more comprehensive Per isoform disrupted mice using both Per2, 3-KO mice (Figure S2) and Per1, 2, 3-KO/Per-TKO mice (Figure S3). Importantly, we were able to demonstrate a congruence of medial thickening phenotype among the isof orm KO of Per (Per2-KO, Per2,3-KO, and Per-TKO).

**Low-Salt Diet Causes Nondipping Hypertension During Circadian Dysfunction**

A low-salt diet is a potent stimulus to stimulate the endogenous renin–angiotensin–aldosterone system in humans\textsuperscript{39} and animals.\textsuperscript{40} To determine the impact of the endogenous Ang II pathway in the context of circadian dysfunction, we next undertook studies to stimulate the endogenous renin–angiotensin system by administration of a low-salt diet. In these studies, we implemented studies in Per-TKO mice, as these mice should be absent of any potential compensatory isoform function, although we did find congruence of phenotype among the single, double, and triple mutants. On a normal salt diet in LD, Per-TKO mice had similar blood pressure to WT mice (Figure 3A), and Per2-KO mice (Figures 1A and 2A). After mice were placed on a low-salt diet for 3 days, the WT mice exhibited a reduction in blood pressure, falling from ~130 mm Hg peak blood pressure (Figure 3A) to ~122 mm Hg (Figure 3B), with a comparable drop in blood pressure troughs (105–98 mm Hg), thus exhibiting the expected blood pressure drop in response to low-salt conditions. However, in Per-TKO mice the response to low-salt diet was completely different. The rhythm in blood pressure was abolished by the low-salt diet, resulting in nondipping blood pressure (Figure 3B) in Per-TKO, analogous to the effects of Ang II administration in Per2-KO mice (Figure
Regulation of the Renin–Angiotensin Pathway

Low-Salt Diet and Circadian Dysfunction Impair Restoration of Blood Pressure Rhythm

To determine if a low-salt diet caused misregulation of the renin–angiotensin pathway during circadian dysfunction, we measured renin levels in cardiovascular tissues in WT and Per-TKO mice on a normal salt diet displayed a similar pattern of renin mRNA expression in kidney (Figure S5B). On a low-salt diet, renin mRNA expression in kidney was increased 2-fold in Per-TKO compared with WT (Figure 4A). Plasma renin concentration was significantly elevated as well as measured by 2 distinct assays (Figure 4B; Figure S5A). Within the vasculature, renin protein expression in aorta also showed a significant increase in Per-TKO mice (Figure 4C). Thus, circadian clock dysfunction increased renin, which is a key enzyme that ultimately leads to the pro-proliferative and hypertensive peptide Ang II. Also, Per-TKO mice showed an 12-hour phase advance in expression of atrial natriuretic peptide in heart, a regulator of renin, compared with WT mice (Figure S9). We also sought to determine whether Period disruption affects AT1 receptors. We found that the temporal pattern of basal AT1 expression differs in Per-TKO mice compared with WT mice. Both WT and Per-TKO mice showed circadian oscillation, yet AT1 expression in Per-TKO mice displayed a phase delay in aorta (Figure 4D and kidney (Figure 4E) with higher day-time expression. To assess the direct effect of Per2 on AT1, we used an siRNA strategy to knockdown Per2 in human aortic smooth muscle cells (Figure 4F). Per2 knockdown in human aortic smooth muscle cells decreased AT1 expression, which was further exacerbated with Ang II treatment (Figure 4G). We also examined the effect of Ang II on Per2 using isolated peritoneal macrophages from circadian clock reporter mice (Per2-luciferase). These studies revealed Per2 promoter activity was decreased in Ang II–treated cells compared with control cells (Figure S10), suggesting there is a reciprocal relationship between angiotensin signaling and the circadian clock.

Discussion

Despite the numerous and effective therapies available, fewer than half of hypertensives under treatment have their blood pressure controlled to target levels. Among the confounding issues could be the prevalence of masked, resistant, and nondipping hypertension. The prevalence of nondipping hypertension (nondipping blood pressure in the Per-TKO mice caused by low salt was Ang II dependent, we administered the mice the angiotensin 1 (AT1) receptor antagonist losartan. Although losartan decreased blood pressure in both Per-TKO and WT mice as expected, losartan also restored the blood pressure rhythm in Per-TKO mice (Figure 3C). Blood pressure rhythm in Per-TKO mice was abolished with removal of losartan from the drinking water after 4 days (Figure S6A). In contrast to losartan, administration of the aldosterone antagonist spironolactone lowered blood pressure but was ineffective in restoring blood pressure rhythm (Figure S6B).

Although circadian clock mutant mice are crucial in elucidating our understanding of the role of the circadian clock in the cardiovascular system, circadian dysfunction induced in WT mice by light-cycle derangement may also mimic perturbed states, such as shift work and sleep disruption. Thus, we subjected WT mice that were administered a low-salt diet to a dramatically shortened light–dark cycle (4:4 hours LD conditions). After 18 days of the shortened light-cycle, rhythmic blood pressure was intact (Figure S7A), but as time progressed to 31 days (Figure S7B) and even longer to 43 days, rhythmic blood pressure was abolished in WT mice (Figure 3D), whereas the arrhythmic blood pressure also persisted in the long term in Per-TKO with the chronic low salt (Figure 3E). The effect of low salt in the long term was detrimental to the vasculature because aortas from Per-TKO mice (Figure 3F) showed significantly increased medial thickening. Per-TKO mice on a chronic low-salt diet also exhibited cardiac hypertrophy (Figure S8A) and decreased body weight (Figure S8B).

Low-Salt Diet and Circadian Dysfunction Impair Regulation of the Renin–Angiotensin Pathway

To determine if a low-salt diet caused misregulation of the renin–angiotensin pathway during circadian dysfunction, we measured renin levels in cardiovascular tissues in WT and Per-TKO. WT and Per-TKO mice on a normal salt diet exhibited a circadian rhythm in Per-TKO mice that was indistinguishable from WT mice in LD conditions, shown as blood pressure trace (left) and day/night quantification (inset). B, Locomotor activity was derived from radiotelemetry and quantified by, averaging values during peaks (night) and troughs (day) of activity traces in conditions of LD and DD; (n=6, *P<0.05, 1-way ANOVA). C, Placement of mice in DD conditions caused a phase change in blood pressure rhythm (peaks and troughs were temporally shifted), however, rhythm persisted (n=6, *P<0.05). One-way ANOVA was used to compare differences in blood pressure values. MAP indicates mean arterial pressure.

Figure 1. Basal blood pressure rhythm is maintained in Per2-knockout (KO) mice in standard light cycle conditions/LD and constant dark/DD conditions. Radioelectrometric transmitters were placed in wild-type (WT) and Per2-KO mice. A, In LD conditions, blood pressure exhibited a circadian rhythm in Per2-KO mice that was indistinguishable from WT mice in LD conditions, shown as blood pressure trace (left) and day/night quantification (inset). B, Locomotor activity was derived from radiotelemetry and quantified by, averaging values during peaks (night) and troughs (day) of activity traces in conditions of LD and DD; (n=6, *P<0.05, 1-way ANOVA). C, Placement of mice in DD conditions caused a phase change in blood pressure rhythm (peaks and troughs were temporally shifted), however, rhythm persisted (n=6, *P<0.05). One-way ANOVA was used to compare differences in blood pressure values. MAP indicates mean arterial pressure.
hypertension has recently been reported to be as high as 45% in individuals, and even higher in patients being treated with antihypertensives (53%). Indeed, it is established that non-dipping or nocturnal hypertension has a substantially negative influence on patient morbidity.

Studies in mice with genetically mutated circadian clocks have revealed the importance of the Bmal1 clock component in the control of basal blood pressure rhythm and the Cryptochrome clock component in high salt–sensitive hypertension. In addition, other studies showed that the circadian period of mean arterial pressure, heart rate, and locomotor activity is shortened in Per2 mutant mice in constant darkness under basal conditions. More recently, Bmal1 in vascular smooth muscle has been shown to play a key role in circadian blood pressure regulation. These studies have established that the circadian clock genes are fundamentally important in blood pressure regulation. In this study, with regard to basal blood pressure regulation, we find that DD may phase advance blood pressure in WT mice, comparable with the phase advance that occurs in WT mice in locomotor activity in DD, an effect absent in the Per2-KO mice. Moreover, we find that experimental hypertension induced by Ang II infusion caused nondipping hypertension in Period KO mice that worsened Ang II–induced pathological vascular remodeling in the aorta. Surprisingly, a low-salt diet, which is a known stimulus of the endogenous renin–angiotensin system, caused nondipping blood pressure and medial thickening in circadian clock KO mice but also in WT mice that were induced to circadian derangement by a shortened light cycle. In mice with intact circadian rhythm and function, low salt did as expected,
reduce blood pressure. Although the absolute levels of sodium reduction induced experimentally in these mouse studies may not be achievable in humans, it maybe the relative change in sodium levels that is critical; the sodium restriction model is at least a valuable approach to examine the endogenous stimulation of Ang II.

Although low salt is clearly of benefit in controlling volume-expanded hypertension, our data suggest that in conditions of circadian dysfunction, low salt hyperactivates the renin angiotensin system. Indeed, recent human data hints that sodium restriction may potentially exhibit condition-dependent detrimental effects4,8,13,42 albeit the raised study limitations.47 Aside from the effect on volume depletion, reduction of sodium in diet can also induce an array of changes as demonstrated in the human urinary metabolome, affecting pathways related to cardiovascular risk, nitric oxide production, oxidative stress, osmotic regulation, and metabolism.48 Studies exploring the effect of sodium restriction and its effect on rhythmic blood pressure are limited,49–51 and little is known with regard to how low-salt diets influence blood pressure during human circadian dysfunction and circadian decline, as in sleep disorders,52–55 shift work,56 and aging,57 given the prevalence of hypertension in these conditions.

In conclusion, the circadian clock may exert a significant restraint on the renin–angiotensin pathway. Infused Ang II or low salt–induced renin–angiotensin signaling synergize with a dysfunctional circadian clock to produce night-time hypertension and vascular hypertrophy. Ultimately, such studies may provide additional insight into the pathogenesis of hypertension, the complex effects of sodium restriction in blood pressure regulation, and the underlying contribution of the circadian clock.

**Perspectives**

Blood pressure control remains a major challenge in preventing cardiovascular disease, despite the numerous and effective antihypertensives available and the general knowledge about the benefits of lifestyle modifications. Aside from the difficulties in managing blood pressure, there is even still complexity identifying a uniformly indicated target blood pressure. For example, the 2014 JNC8 report suggested that blood pressure may not need be as aggressively lowered to standard

![Figure 3](http://hyper.ahajournals.org/)

A low-salt diet causes nondipping hypertension during circadian dysfunction. A, Blood pressure measured on a 0.3% salt diet (TD6604) diet (Teklad), which is the standard mouse diet, shown as blood pressure trace (left) and night/day quantification (right; n=5). B, Mice were then placed on a 0.01–0.02% low-salt diet (TD90228), which caused nondipping hypertension in Per-triple knockout (TKO) mice, but did cause an expected reduction in blood pressure in wild-type (WT) mice. C, WT and Per-TKO mice that were on low-salt diet were then administered the angiotensin 1 (AT1) receptor blocker losartan in drinking water (0.6 g/L). Losartan-lowered blood pressure, in both WT and Per-TKO mice, but also partially restored the circadian variation in blood pressure in Per-TKO mice. D, The standard light cycle conditions/LD cycle was shortened from 12:12 hours L:D to 4:4 hours L:D, whereas mice were maintained on a low-salt diet. By ~5 weeks (32 days), blood pressure rhythm was abolished in WT mice. E, Blood pressure analysis of telemetric data during conditions of shortened light cycle revealed that blood pressure rhythm was abolished in WT mice, whereas the ablation of rhythm persisted in Per-TKO mice on a chronic low salt under a 4:4 hours L:D cycle. F, Quantitative morphometry of the aorta and Van Giesen staining of aortic cross-sections isolated from WT and Per-TKO mice under conditions of chronic low salt, showed exacerbated medial thickening in Per-TKO mice compared with WT mice (n=5, P<0.05, Unpaired Student t test). MAP indicates mean arterial pressure.
normotensive ranges in elderly patients; however, more recent results from SPRINT (Systolic Blood Pressure Intervention Trial) suggest that aggressive blood pressure reduction to <120 mm Hg diastolic does offer increased health benefit in nondiabetic patients at increased cardiovascular risk. Although current standards are based on day-time blood pressure, standards for appropriate night-time blood pressure are lacking. Indeed, the current data, albeit in mice, underscores the significance of time of day variation in blood pressure, with even modest changes in dipping blood pressure profile causing pathological vascular remodeling. Greater implementation of ambulatory blood pressure readings in humans could help to establish more defined standards of healthy blood pressure according to time of day, and even may prove to identify more effective chronotherapeutic antihypertensive strategies.

Figure 4. Low salt alters the renin-angiotensin pathway in Per-triple knockout (TKO) mice. A. The level of renin expression in kidney was measured by quantitative polymerase chain reaction (qPCR). Kidney renin expression in Per-TKO was increased >2-fold compared with wild-type (WT) in conditions of low salt. B. Plasma renin concentration was determined with an ELISA kit (Mouse Renin 1 DuoSet, R&D Systems). Per-TKO mice display a significant elevation in plasma renin (*P<0.05, unpaired Student t test). C. In aorta, renin expression was determined by Western blotting, which also showed a significant increase in renin in low salt-treated Per-TKO mice. Shown as Western blot of 3 representative WT and 3 representative Per-TKO mice and densitometry (WT n=5, KO n=3, *P<0.05, unpaired Student t test). Aorta (D) and Kidneys (E) were harvested from WT and Per-TKO mice in standard light cycle conditions/LD at 6-h intervals for 42 hours under normal diet conditions. Aorta and kidney angiotensin 1 (AT1) receptor expression show a phase shift with higher day-time expression in Per-TKO versus WT mice. F. Relative mRNA expression by qPCR of control and Per2 with siRNA transfection (50 nmol). G. Western blotting shows Per2 knockdown decreases AT1 receptor expression in human aortic smooth muscle cells, and it is further decreased with 24 hours of angiotensin II treatment (100 nmol/L), quantified by densitometry (n=3 per group, *P<0.05, 1-way ANOVA).
Sources of Funding
This study was supported by grants from the National Institutes of Health/National Heart, Lung, and Blood Institute (RO1HL089576 to R.D. Rudic) and the American Heart Association (AHASE00078 to P. Pati).

Disclosures
None.

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**Novelty and Significance**

**What is New?**

- Circadian clock dysfunction can exacerbate angiotensin-induced hypertension.
- In conditions of low-salt diet, circadian arrhythmicity may also cause paradoxical nondipping blood pressure.

**What is Relevant?**

- This work is relevant to our understanding of high blood pressure in that it demonstrates an interaction of the circadian clock and circadian rhythm in renin–angiotensin system regulation, hypertension development, and resultant vascular remodeling.

**Summary**

Angiotensin II infusion causes nondipping hypertension in Period isofrom knockout mice. Endogenous stimulation of the angiotensin II system by low salt also causes a nondipping blood pressure profile during circadian dysfunction. The renin–angiotensin signaling pathway is hyperactivated in Per-triple knockout mice.
Low-Salt Diet and Circadian Dysfunction Synergize to Induce Angiotensin II–Dependent Hypertension in Mice


Hypertension. 2016;67:661-668; originally published online January 18, 2016; doi: 10.1161/HYPERTENSIONAHA.115.06194

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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A LOW SALT DIET AND CIRCADIAN DYSFUNCTION SYNERGIZE TO INDUCE ANGIOTENSIN II-DEPENDENT HYPERTENSION IN MICE


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Supplementary Methods

Blood pressure telemetry
To implant blood pressure telemetry devices, mice were anesthetized with an intraperitoneal injection of ketamine and xylazine and a neckline incision was made. Mice were instrumented with an arterial catheter connected to an implantable transducer (model TA11PA-C10; Data Sciences International, St. Paul, MN) to monitor arterial pressure and locomotor activity 24 hours a day by telemetry in unrestrained mice. The tip of the telemeter catheter was inserted in the aortic arch via the left common carotid artery and the transducer was implanted subcutaneously in the abdomen. The incision was closed with 5-0 nylon sutures. Following surgery, the mice were housed in individual cages and allowed at least 6 days for recovery. Mice were given ad libitum access to water and either standard rodent chow a (0.3% salt diet, Teklad, TD8604) or on a low salt diet (0.01-0.02% salt, TD90228). Baseline recordings of MAP, locomotor activity, and heart rate were then taken every 10 minutes with the DataQuest System (Data Sciences International, St. Paul, MN) continuously as indicated. Values were averaged over 3 hour intervals.

The effect of light cycle on blood pressure and locomotor activity
In WT and Per2-KO mice we assessed the influence of the standard 12:12-h light-dark schedule (LD) versus conditions of constant darkness (DD), or free running conditions, on cardiovascular rhythms and activity. Studies were performed on 9- to 12-week-old male Per2-KO and control mice. Radiotelemetric transmitters were implanted for blood pressure telemetry. Mice were allowed to recover for 6 to 15 days following surgery and kept on a standard 12h/12h LD cycle. Baseline recordings of MAP, locomotor activity, and heart rate were then taken for 5 continuous days. Mice were then transferred to constant darkness (DD) to determine the effect on locomotor activity as well as cardiovascular rhythms. Mice were given 6 days to acclimate in DD, and then 6 days of recording were taken.

Angiotensin II Infusion studies
Angiotensin II was used to induce hypertension in WT and Per2-KO mice for 4 weeks. The treated mice were implanted with mini-osmotic pumps in the intrascapular region (Alzet 2004) that delivered Ang II at a dose of 1000 ng/kg/min. After 1 week of Ang II infusion in standard LD conditions, the mice were transferred to DD for the remainder of the study. 4 days of recording were taken at the end of week 2 and then at the end of week 3. (Recording at the end of week 4 for 3 days was also taken.) In order to determine the effect of light cycle on blood pressure and locomotor activity, mice were transferred to constant darkness (DD). Mice were given 6 days to acclimate in DD, and then 6 days of recording were taken. After 1 week of angiotensin II infusion under standard LD conditions (12 hours of light and 12 hours of darkness), mice were transferred to DD (constant darkness) for the remaining 3 weeks of the study. Radiotelemetric recording of blood pressure was taken 7 days after implantation of osmotic mini-pumps.
**Morphometry**

After completion of Ang II blood pressure recordings, mice were anesthetized, exsanguinated, and perfused through the left ventricle with 0.9% saline. Aorta was harvested and processed for frozen sectioning. The abdominal aortas were quickly thawed, dissected in formalin, cut into 3 mm segments, and frozen in OCT. Aortae were cut into 5 μm sections for quantitative analysis of blood vessel structure. Aortic sections were stained with van Giesen stain. Wall thickness was measured directly as the distance between the external elastic lamina and the internal elastic lamina.

**The effect of low salt diet in Per-TKO mice**

Studies were performed on 3- to 5-month-old male littermate WT and Per1-Per2-Per3-Triple KO (Per-TKO) mice. Mice were housed in individual cages and allowed at least 1 week for recovery in standard LD conditions. Ad libitum access to water and a normal salt diet (8604 Teklad Rodent Diet, Harlan Laboratories Inc, Madison, Wisconsin) was provided for the mice. Baseline radiotelemetric recording was taken for 1 week. Values were averaged over 3 hour intervals. Mice were then placed on a 0.01% NaCl low salt diet (TD.90228, Harlan) and telemetric recording was started after 3 days. After 2 weeks of low salt diet, losartan (Sigma Aldrich) at a concentration of 0.6 g/L was administered via drinking water for 5 days. Losartan was then discontinued and low salt diet was maintained. For the study conducted in an accelerated light cycle to derange circadian rhythms in WT mice, the LD cycle was shortened from 12 hr:12 hr L:D to 4hr:4hr L:D while the WT mice were continued on low salt diet.

**Circadian oscillation studies in Per-TKO mice**

WT and Per-TKO mice (WT n=4, KO n=4 per timepoint) were sacrificed at 6 hour intervals for 42 hours by cervical dislocation at 6 hour intervals for 42 hours. Tissues were harvested, flash frozen in liquid nitrogen, and stored at -80°C for future QPCR analysis. Blood was collected and tissues were harvested, flash frozen in liquid nitrogen, and stored at -80°C for future QPCR analysis.

**Measurement of plasma renin**

Blood was collected from mice and plasma was isolated. 10 uL of each plasma sample was used as indicated in the Mouse Renin 1 DuoSet (R&D Systems), an ELISA development kit for the determination of renin concentration. For the pre- and post-low chronic low salt study, plasma renin concentration was measured by incubation of plasma samples with an excess of rat angiotensinogen in the presence of EDTA (0.02 M) for 30 min at 37°C. Radioimmunoassay using a commercially available kit (Cat# 1553; DiaSorin, MN, USA) was used to quantify Ang I generated in plasma samples.

**Real-time quantitative PCR**

Total RNA was isolated from mouse kidneys and aortae using the PureLink® RNA Mini Kit (Ambion). cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR was carried out using a SYBR Green Supermix (Bio-Rad) and relative gene expression was assessed with the following
primers: renin1 (forward primer, ACGCACCGCTACCTTTGAACGAA; reverse primer, CACGGGGGAGGTAAGATTGGTC), AT1 (forward primer, CTTCGGCCAGCAGTCTGTTC; reverse primer, GCCAAGCCAGCCCATCAGC). Data was quantified by the \( \Delta \Delta CT \) method and values were normalized with respect to GAPDH or 18S rRNA.

Western blot
Mouse aortae were dissected free of fat and crushed in liquid nitrogen. Protein was extracted and loaded onto 10% SDS-PAGE gels. Renin (Novus, catalog # 57986, 1:1000 dilution) expression was detected with anti-rabbit polyclonal antibodies (Cell Signaling), and detected with enhanced chemiluminescence (ECL kit, Amersham) by autoradiography. AT1 receptor expression was detected with an anti-goat polyclonal antibody (Novus, catalog # 57073). Image J software was used for densitometry.

Cell culture, siRNA and Angiotensin II treatment
Human aortic smooth muscle cells (HASMCs) were cultured in smooth muscle cell basal medium. Per2 siRNA (Silencer® Pre-designed siRNA, Ambion) sense (CCACCCAUACCAACAAGUtt), antisense (CAAUUUUGGUAGUAUGGGUGtgt). Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) was used for siRNA delivery. Cells were lysed and total RNA was isolated as described previously. Quantitative real-time PCR was used to confirm knockdown of Per2 with the following primers (forward primer, GCGCCTCTGACCCGTGATG; reverse primer, GTGAGCCGGAGCCCAGAG). Cells were treated for 24 hours with 100 nmol/L Angiotensin II (Sigma) and harvested for Western blot analysis.

Per2,3-KO study with exogenous Angiotensin II and light cycle disruption
Angiotensin II via mini-osmotic pump was used to induce hypertension in 11 week-old male WT and Per2,3-KO mice for 4 weeks. Mice were allowed to recover in standard LD conditions and then subjected to an altered light cycle protocol (mimicking shift work). After 1 week of AngII infusion under standard LD conditions, the mice were placed in DD for 1 week. Mice were returned to LD after one wk. After 6 days mice were placed in DD. Following 4 days in DD, mice were returned to LD for 4 days. Mice were then placed in DD for 1 week.

Exogenous AngII infusion in Per-TKO mice
Studies were performed on 16-20 week-old male Per1,2,3-KO (Per TKO) mice. The angiotensin II infused group of mice was kept on a light-12h dark cycle while the other group of mice was kept. The other group of mice were kept in DD for 4 weeks. The treated mice were implanted with mini-osmotic pumps in the intrascapular region (Alzet 2004) that delivered Ang II at a dose of 1000 ng/kg/min. Ang II was continuously administered to Per TKO mice for 4 weeks.

Morphometry
After completion of Ang II administration, mice were anesthetized, exsanguinated, and perfused through the left ventricle with 0.9% saline. Aorta was harvested and processed for frozen sectioning. Aortae were fixed in 4% phosphate-buffered
formaldehyde, frozen in OCT, and cut into 5 μm sections for quantitative analysis of blood vessel structure. Aortic sections were stained with van Giesen stain. Wall thickness was measured directly as the distance between the external elastic lamina and the internal elastic lamina.

**Spironolactone treatment**

Spironolactone (0.1g/L, Sigma Aldrich) was given via drinking water in conjunction with low salt for 12 days.

**Macrophage isolation**

1 mL of thioglycolate was injected into Per2-Luc mice prior to collection. Peritoneal cells were collected by lavage and red blood cells were lysed with hypotonic buffer. Peritoneal macrophages were isolated from Per2-Luc mice 3 days after injection with thioglycolate and isolated macrophages were cultured in complete RPMI medium.

**Real-time quantitative PCR**

Total RNA was isolated from mouse kidneys, hearts, and suprachiasmatic nuclei using the PureLink® RNA Mini Kit (Ambion). cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR was carried out using a SYBR Green Supermix (Bio-Rad) and relative gene expression was assessed with the following primers: renin1 (forward primer, ACGCACCCTACCTTTGAACGAA; reverse primer, CACGGGGGAGGTAAGATTGGTC), ANP (forward primer, TCACCCTGGGGCTTTCTCCTCGTCT; reverse primer, TGCGGCCCCTGCTTCCTCA), AT1 (forward primer, CTTCGGCCAGCGTCAGTTTC; reverse primer, GCCAAGCCAGCCATCAGC). Data was quantified by the ΔΔCT method and values were normalized with respect to GAPDH.
Supplementary Figures

Figure S1.
(A) Baseline locomotor activity is similar between WT and Per2-KO mice. (B) After 1 week of Angiotensin II administration in LD, rhythmic locomotor activity is decreased in Per2-KO mice compared to WT mice. (C) Locomotor activity rhythm is abolished in Per2-KO mice in after 2 weeks of AngII in DD conditions, yet WT mice are unaffected.
Angiotensin II infusion and light cycle shifting increases vascular hypertrophy in Per2,3-KO mice. Following chronic AngII infusion by osmotic minipump, morphometric analysis of aortic cross sections from WT and Per2,3-KO mice show an increase in wall thickness of Per2,3-KO mice as compared to WT. (n=4, *p<0.05, Unpaired Student t test)
Figure S3.

DD conditions and AngII administration independently increase wall thickness in Per-TKO mice. Angiotensin II infusion increases aortic wall thickness in Per-TKO mice while 4 weeks of DD leads to similar wall thickening, quantified by morphometry (n=5, *p<0.05, Unpaired Student t test).
Figure S4.

(A) WT mice show circadian variation in heart rate, yet Per-TKO mice lack rhythmicity under basal conditions. (B) On a low salt diet, heart rate decreases in WT mice and shows increased variation in Per-TKO mice (C) Locomotor activity is decreased in Per-TKO mice compared to WT mice under basal conditions. (D) Locomotor activity rhythm in Per-TKO mice is altered on a low salt diet.
Figure S5.

(A) 6 months of low salt increased plasma renin in Per-TKO mice. Plasma samples were incubated with an excess of partially purified rat angiotensinogen at 37°C. A commercially available kit was used to quantify Ang I generated in the plasma by radioimmunoassay. Analysis of samples were blinded by coding (WT n=7-10, KO n=7-9, *p<0.05, 1-way ANOVA). (B) Kidneys were harvested from WT and Per-TKO mice in LD at 6 hour intervals for 42 hours under normal diet conditions. Q-PCR analysis revealed similar relative mRNA renin expression in WT and Per-TKO mice on a normal salt diet.
Spironolactone does not fully restore circadian rhythm in blood pressure in Per-TKO mice on low salt diet. (A) After losartan administration was interrupted, mice were resumed on the standard water, and blood pressure returned to non-dipping in Per-TKO mice. (B) The mice were then administered the aldosterone (mineralocorticoid) antagonist spironolactone in drinking water (0.1 g/L), which lowered blood pressure, but did not fully restore rhythmic blood pressure.
Figure S7.

(A) In WT mice fed a low salt diet, the LD cycle was shortened from the standard 12hr:12hr L:D to 4hr:4hr L:D. A drastically shortened light cycle initially did not affect blood pressure rhythm in WT mice in days 1-18, and circadian rhythm in blood pressure persisted. (B) However, after 3 weeks of being subjected to 4hr:4hr L:D WT mice began to show a blunted rhythm with decreased peak-to-trough distance shown as the trace of mean arterial blood pressure (n=4).
(A) Per-TKO mice on a chronic low salt diet exhibit cardiac hypertrophy (n=4 per group, *p<0.05, Unpaired Student t test). (B) Body weight is decreased in both WT and Per-TKO mice on a chronic low salt diet (n=4 per group, *p<0.05, 1-way ANOVA).
Hearts from WT and Per-TKO mice in LD were harvested at 6 hour intervals for 42 hours under normal diet conditions. ANP expression dramatically peaks in Per-TKO mice at CT12 and while the peak in WT mice occurs at CT24.
Figure S10.

**Angiotensin II decreases Per2 promoter activity in macrophages.** Peritoneal macrophages were isolated from Per2-Luc mice, cultured, and treated with AngII (100 nmol/L, Sigma). Per2 promoter activity was measured over 48 hours by automatic bioluminescence monitoring. Per2-Luc cells treated with AngII exhibited a decreased amplitude of Per2 oscillation compared to control cells (n=8, *p*<0.001, 2-way ANOVA).