Published in final edited form as:

Can J Physiol Pharmacol. 2023 March 01; 101(3): 136–146. doi:10.1139/cjpp-2022-0134.

The Circadian Clock Protein PER1 is Important in Maintaining Endothelin Axis Regulation in Dahl Salt Sensitive Rats

Hannah M. Costello[1,2], **Alexandria Juffre**[1,2,3], **Kit-Yan Cheng**[1,2], **Phillip Bratanatawira**[2], **G. Ryan Crislip**[1,2], **Adrian Zietara**[4,5], **Denisha R. Spires**[4], **Alexander Staruschenko**[5,6], **Lauren G. Douma**[1,2,3], **Michelle L. Gumz**[1,2,3,7,*]

[1]Department of Physiology and Functional Genomics, University of Florida, Gainesville, FL 32610

[2]Department of Medicine, Division of Nephrology, Hypertension, and Renal Transplantation, University of Florida, Gainesville, FL 32610

[3]Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL 32610

[4]Department of Physiology, Medical College of Wisconsin, Milwaukee, WI 53226

[5]Department of Molecular Pharmacology and Physiology, University of South Florida, Tampa, FL 33602

[6]James A. Haley Veterans' Hospital, Tampa, FL 33612

[7]Center for Integrative Cardiovascular and Metabolic Diseases, University of Florida, Gainesville, FL 32610

Abstract

Endothelin-1 (ET-1) is a peptide hormone that acts on its receptors to regulate sodium handling in the kidney's collecting duct. Dysregulation of the endothelin axis is associated with various diseases, including salt-sensitive hypertension and chronic kidney disease. Previously, our lab has shown that the circadian clock gene PER1 regulates ET-1 levels in mice. However, the regulation of ET-1 by PER1 has never been investigated in rats. Therefore, we used a novel model where knockout of *Per1* was performed in Dahl salt-sensitive rat background ($SS^{Per1-/-}$) to test a hypothesis that PER1 regulates the ET-1 axis in this model. Here, we show increased renal ET-1 peptide levels and altered endothelin axis gene expression in several tissues, including the kidney, adrenal glands, and liver in $SS^{Per1-/-}$ compared with control SS rats. *Edn1* antisense lncRNA *Edn1-AS,* which has previously been suggested to be regulated by PER1, was also altered in $SS^{Per1-/-}$ rats compared with control SS rats. These data further support the hypothesis that PER1 is a negative regulator of *Edn1* and is important in the regulation of the endothelin axis in a tissue-specific manner.

*To whom correspondence should be addressed: 1345 Center Drive, Box 100274, Gainesville, FL 32610 Michelle.Gumz@medicine.ufl.edu.

Competing Interests

The authors declare there are no competing interests.

Disclosures

Dr. Gumz is a guest editor of this special issue but was not involved and did not have access to information regarding the peer-review process or final disposition of this article. An alternate editor oversaw the peer-review and decision-making process for this article.

Keywords

Endothelin-1; ET-1; Period1; Kidney; Adrenal glands; salt-sensitive hypertension

Introduction

Many physiological functions exhibit circadian rhythms, which are coordinated by intrinsic circadian clocks, specifically the central clock, which resides in the suprachiasmatic nucleus (SCN) of the hypothalamus and peripheral clocks throughout the body. Both the central and peripheral clocks share the same core molecular clock present in nearly every cell, which comprises transcription factors BMAL1, CLOCK, CRYPTOCHROME (CRY), and PERIOD (PER). The molecular clock acts as a transcription-translation feedback loop to regulate its own expression as well as the transcription of nearly half of all genes (R. Zhang et al., 2014). Specifically, BMAL1 and CLOCK heterodimerize and bind to enhancer box (E-box) promoter response elements to activate the transcription of target genes, including the genes encoding PER and CRY. PER and CRY heterodimerize and act as a negative feedback loop to inhibit the actions of BMAL1 and CLOCK, and, therefore, decrease their own transcription (Partch et al., 2014). The cycling between these sets of transcription factors creates the 24-hour oscillations observed not only in gene expression but in physiological functions, like blood pressure (Crislip et al., 2020; Douma & Gumz, 2018; D. Zhang et al., 2020). While the role of the circadian clock in the maintenance of these rhythms is critical for overall health, the molecular clock also is important in responding to cues, such as increased salt intake, to preserve homeostasis.

The *Edn1* gene exhibits a circadian rhythm of expression in mice, rats, and humans ((Dhaun et al., 2014; Hill et al., 2021) and reviewed in (Douma, Barral, et al., 2020)). *Edn1* encodes for endothelin-1 (ET-1), a peptide hormone that has a variety of tissue-specific functions. For example, in the vasculature, ET-1 can induce vasoconstriction through its interactions with the endothelin A receptor (ET_A) (Kostov, 2021). The highest production of ET-1 in the body is within the kidney, and both the ET_A and endothelin B receptor (ET_B) are expressed in kidney cells (Kohan et al., 2011). In a healthy kidney, ET-1 in the collecting duct regulates sodium handling, via inhibition of sodium transport and promoting natriuresis, through interactions with its receptors. Dysregulation of ET-1 signaling is associated with various diseases (de Miguel et al., 2016; Speed & Pollock, 2013). In humans, increased plasma and urinary ET-1 levels are observed in patients with chronic kidney disease (CKD). Alteration in ET-1 expression rhythms and/or metabolism may also contribute to salt-sensitive and essential hypertension (Hwang et al., 1998; Zoccali et al., 1995). In mice, overexpression of ET-1 results in increased blood pressure and chronic renal failure (Berillo et al., 2021; Grenda et al., 2007). Pharmacological interventions targeting the endothelin receptors have been associated with negative side effects in patients (Kohan et al., 2012; Smeijer et al., 2021; Waijer et al., 2021). Further understanding of how the endothelin axis is regulated, especially in a pathophysiological state, may assist in the development of successful ET-1 signaling inhibition strategies.

Transcription of the *Edn1* gene is heavily regulated, but *Edn1* mRNA is also regulated post-transcriptionally through microRNA (miRNA) functions (Houde et al., 2016; Jacobs et al., 2013a; Stow et al., 2011). Our lab has recently identified a long non-coding RNA (lncRNA), *EDN1-AS*, present in human kidney cells that modulates ET-1 production (Douma, Solocinski, et al., 2020). Like *Edn1*, transcription of *EDN1-AS* exhibits a circadian rhythm. Additionally, our lab has demonstrated that the circadian clock protein PER1, a PER homolog, mediates the regulation of *Edn1* transcription (Richards et al., 2014; Stow et al., 2012). The regulation of ET-1 production by PER1 seems to be important not only for maintaining rhythms of ET-1 production, but also for proper transcriptional responses to cellular signals, like the mineralocorticoid aldosterone (Douma, Crislip, et al., 2020; Douma et al., 2022). In fact, both *Edn1* and *Per1* are significantly upregulated in mouse inner medullary collecting duct cells in response to short-term exposure to aldosterone (Gumz et al., 2003).

For the first time, a *Per1* knockout (KO) rat on the Dahl salt-sensitive background ($SS^{Per1-/-}$) has been generated in order to determine the role of PER1 in a model of salt-sensitive hypertension (Zietara et al., 2022). Compared to control Dahl salt-sensitive rats (SS), male $SS^{Per1-/-}$ rats have significantly increased blood pressure after 3 weeks of a high salt diet, coupled with loss of circadian synchrony of blood pressure and a decline in renal function. The regulation of ET-1 by PER1 has never been investigated in rats. As our lab has previously shown that PER1 modulates *Edn1* transcription, the objective of the current study was to determine if the endothelin axis is dysregulated in $SS^{Per1-/-}$ rats, contributing to the reported renal phenotypes. Indeed, $SS^{Per1-/-}$ rats exhibited increased renal ET-1 peptide levels and altered endothelin axis gene expression in several tissues, including the kidney, adrenal gland, and liver. In these tissues, *Per1* KO had differing effects on clock gene expression. Together, these data further support the hypothesis that the endothelin axis might be regulated by PER1 in a tissue-specific manner.

Methods

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Animals.

All animal experiments adhered to the National Institute of Health Guide for the Care and Use of Laboratory Animals and all protocols were reviewed and approved by the Medical College of Wisconsin IACUC. The $SS^{Per1-/-}$ rat was created on the Dahl salt-sensitive rat background at the Medical College of Wisconsin Gene Editing Rat Resource Center using CRISPR/Cas9. One base pair of the *Per1* gene, depicted as “g”, was deleted in exon 1 (CTCCTCCAGGACAAAAAGGTTCTCCGGgCCTGGGGTCTCCTCCCCCATCAGCCCCT), resulting in a truncated protein consisting of a predicted 109 amino acids. This deletion has been confirmed by genomic DNA and mRNA sequencing as well as Western blot (Zietara et al., 2022). Rats were housed in 12:12-hour light-dark cycled rooms, weaned at 3 weeks of age and placed on a 0.4% NaCl diet (normal salt; Dyets, Inc.; D113755). At around 10 weeks of age, male rats were switched to a 4% NaCl diet (high

salt; Dyets, Inc.; D113756). Only male rats were used in these studies as previous studies in global *Per1* KO mice showed females were protected from changes in the endothelin axis, unlike males (Douma, Crislip, et al., 2020).

RNA isolation.

Male $SS^{Per1-/-}$ and SS control rats were were anesthetized following either a normal salt diet (0.4% NaCl, # D113755; Dyets Inc.) or 3 weeks on the high salt diet (4.0% NaCl, #D113756; Dyets, Inc.), and their kidneys were flushed (3 mL/min/kidney until blanched) with PBS via aortic catheterization between 1:00-4:00 PM, during the rat inactive period, as previously described (Golosova et al., 2020; Klemens et al., 2021). Kidneys were collected along with adrenal glands and liver, and tissues were snap-frozen in liquid nitrogen. Total RNA was isolated using Trizol (Invitrogen) and treated with DNaseI (Ambion).

Real-time quantitative RT-PCR.

cDNA from kidney, adrenal, and liver RNA samples was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan probes (Applied Biosystems) (Table 1) were used for gene expression analysis. Cycle threshold (Ct) values were normalized against β-actin (*Actb*), and relative quantification was performed using the ΔΔCt (Livak & Schmittgen, 2001). Gene expression levels were relativized to SS control normal salt expression levels.

Strand-Specific RT-PCR for EDN1-AS Relative Quantification.

Rat strand-specific *EDN1-AS* primers were designed to base-pair within the intron between exons 4 and 5, homologous to the position of the equivalent primer (SS4) used in (Douma, Solocinski, et al., 2020). Strand-specific kidney *EDN1-AS* cDNA was generated by using primer rSS4 along with Reverse Transcriptase (RT) from ThermoFisher per manufacturer instructions (Table 2). For each cDNA sample, a corresponding reaction with no reverse transcriptase was used as a negative control to ensure no genomic DNA was present in the samples. Additionally, for each sample, a reaction with random hexamer primers was also performed. The cDNA *EDN1-AS* products (+/− RT) were amplified using PCR primers rPCR1 and rPCR2 (35 amplification cycles) (Table 2). PCR of GAPDH with cDNA generated with random hexamer primers was used for normalization (25 amplification cycles). Gel band pixels were measured using ImageJ. Gene expression levels were relativized to SS control normal salt expression levels.

Protein isolation and ET-1 ELISA.

Protein samples from whole kidney tissue were isolated using T-PER Tissue Protein Extraction Reagent supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific) and quantified using Pierce BCA Protein Assay Kit (Thermo Scientific). Renal ET-1 peptide was measured by ELISA from R&D Systems (Endothelin-1 QuantiGlo ELISA kit). This ELISA kit detects both full-length ET-1 and processed ET-1. The cross-reactivity for this kit is 51% for ET-2, 0.01% for full-length ET-2, and 9% for ET-3. The detectable range is from 0.064-250 pg/mL. ELISA was performed according to the manufacturer's instructions.

Statistics:

Graphpad Prism was used to perform Student's unpaired *t*-test to determine genotype effects and 2-way ANOVA to determine the effect of diet (normal salt, high salt), genotype, and its interaction. Data are presented as mean ± SE.

Results

Kidney Endothelin Axis

To determine if the endothelin axis is altered as a result of *Per1* KO in Dahl SS rats, qPCR was performed using total kidney cDNA from male SS$^{Per1-/-}$ and SS control rats on normal and high salt diets to measure the expression of the endothelin gene, *Edn1*, and the endothelin receptors (*Ednra* and *Ednrb*). *Edn1* expression was significantly increased in SS$^{Per1-/-}$ compared to SS control rats ($P_{genotype}$=0.0358) (Figure 1A). There was no diet effect on *Edn1* expression. Moreover, there were no diet or genotype effects on mRNA levels of endothelin receptors (*Ednra* and *Ednrb*) expression (Figures 1B-C). To further test ET-1 pathway, ET-1 peptide levels from SS$^{Per1-/-}$ and SS rat kidneys following a high salt diet were measured by ELISA to determine if the changes in mRNA expression were observed at the protein level. Indeed, SS$^{Per1-/-}$ rats have significantly elevated ET-1 peptide levels compared to SS control rats following a high salt diet (P=0.0011) (Figure 1D).

Previously, our lab demonstrated that *Edn1* expression is positively regulated by the lncRNA *EDN1-AS* present in human proximal tubule cells (Douma, Solocinski, et al., 2020). Strand-specific PCR was used to determine if *EDN1-AS* is present in rat whole kidney RNA samples. Similar to what we reported in human cells, *EDN1-AS* expression was detected in SS$^{Per1-/-}$ and control rats (Figure 2A). Since SS$^{Per1-/-}$ rats have increased *Edn1* and ET-1 levels, *EDN1-AS* expression levels on normal or high salt diets were measured by strand-specific PCR to determine if *EDN1-AS* also exhibited changes in expression. GAPDH expression was used to normalize *EDN1-AS* expression for each sample. Expression levels were relativized to control SS normal salt values. Interestingly, there was a significant genotype effect of *EDN1-AS* expression in SS$^{Per1-/-}$ rats compared to controls ($P_{genotype}$=0.0455) (Figure 2B).

Adrenal and Liver Endothelin Axis

Since SS$^{Per1-/-}$ rats have *Per1* KO in every cell of their body, expression of the endothelin axis genes was measured in extra-renal tissues to determine if there were any changes. Expression of endothelin-related genes was measured in the adrenals of SS$^{Per1-/-}$ and SS control rats on normal or high salt diets. There was no significant difference in *Edn1* expression between SS$^{Per1-/-}$ and SS control rats (Figure 3A). Interestingly, *Edn1* expression significantly changed in response to a high salt diet (P_{diet}=0.0105). There were no significant genotype or diet effects for *Ednra* expression (Figure 3B). *Ednrb* expression was significantly different between SS$^{Per1-/-}$ and SS control rats ($P_{genotype}$=0.0118) (Figure 3C). Additionally, there was a significant increase in *Ednrb* expression between diets (P_{diet}=0.0290).

Expression of endothelin-related genes was also measured in liver mRNA samples of $SS^{Per1-/-}$ and SS control rats on normal or high salt diets. There were no significant differences in *Edn1* and *Ednra* expression between $SS^{Per1-/-}$ and SS control rats (Figures 3D-E). Although, there was a significant interaction between genotype and diet effect in *Ednrb* expression ($P_{interaction}$=0.0023).

Clock gene peripheral tissue profile in $SS^{Per1-/-}$ rats

PER1 is a core circadian clock protein used in the transcription-translation feedback loop to regulate the expression of thousands of genes. Expression of other core circadian clock genes was measured in whole kidney mRNA samples of $SS^{Per1-/-}$ and SS control rats on either normal or high salt diets. *Bmal1* expression was not significantly different between genotypes or diets (Figure 4A). Interestingly, *Clock* kidney expression was significantly decreased in $SS^{Per1-/-}$ rats compared to SS control rats ($P_{genotype}$=0.0288) (Figure 4B). There were no significant differences in expression of the *Period* homolog, *Per2* (Figure 4C). *Cry1* expression was significantly increased in $SS^{Per1-/-}$ rats ($P_{genotype}$=0.0469) and following a high salt diet (P_{diet}=0.0214) (Figure 4D). There were no significant genotype or diet effects on *Cry2* expression (Figure 4E).

Expression of circadian clock genes was also measured in the adrenal glands and liver samples of $SS^{Per1-/-}$ and SS control rats on either normal salt or high salt diets. Adrenal *Bmal1*, *Clock,* and *Per2* gene expression were not altered between genotypes or following a high salt diet (Figures 5A-C). *Cry1* expression was significantly different between diets (P_{diet}=0.0336) (Figure 5D). Interestingly, adrenal *Cry2* expression was significantly reduced in $SS^{Per1-/-}$ rats compared with SS control rats ($P_{genotype}$=0.0064) (Figure 5E). Like the adrenal gland, there was no significant differences between genotypes or diets in liver *Bmal1*, or *Clock* expression (Figures 6A-B). However, *Per2* and *Cry1* expression had a significant diet effect (P_{diet} =0.0390 and P_{diet} =0.0091, respectively), but there was no significant difference between genotypes (Figures 6C-D). There were no significant genotype or diet effects on liver *Cry2* expression (Figure 6E).

Discussion

In the present study, we show PER1-mediated regulation of the endothelin axis in a tissue-specific manner in the Dahl salt-sensitive rat model. Furthermore, lack of PER1 had differing effects on clock gene expression within peripheral tissues tested, specifically kidney, adrenal gland, and liver. Overall, these data provide further evidence for a role of PER1 in maintaining endothelin axis regulation.

Previously, our lab has shown that PER1 is a negative regulator of *Edn1* in cell culture and mouse models (Gumz et al., 2003; Stow et al., 2012). Indeed, global *Per1* KO in 129/sv and C57BL/6 mice resulted in increased ET-1 peptide levels in the kidney (Douma, Crislip, et al., 2020; Stow et al., 2012). Recently, we demonstrated that ET-1 peptide levels were also increased in the kidneys of distal nephron and collecting duct (kidney-specific) KS-*Per1* KO mice (Douma et al., 2022). Consistent with these previous findings, here we show for the first time that $SS^{Per1-/-}$ rats exhibited increased renal ET-1 peptide levels. Additionally, *Edn1* mRNA levels were increased in the kidneys of $SS^{Per1-/-}$ rats and decreased in the

adrenal glands of both groups on a high salt diet. With PER1 shown to regulate ET-1 in cell culture models, mice, and now rats, these findings further support the connection between PER1 and ET-1. As well, our findings are in line with those of Pollock and colleagues, who have consistently demonstrated a link between circadian rhythms and the action, as well as the regulation of ET-1 (Johnston et al., 2016; Speed, Hyndman, Kasztan, et al., 2018; Speed, Hyndman, Roth, et al., 2018a).

Previous work in the SS$^{Per1-/-}$ rats showed that lack of PER1 caused a decline in renal function, as there was reduced creatinine clearance following high salt diet when compared with control SS rats (Zietara et al., 2022). Elevated ET-1 levels have been associated with renal inflammation and fibrosis (reviewed by (Dhaun et al., 2012)). With elevated renal ET-1 peptide levels in SS$^{Per1-/-}$ rats, it is tempting to speculate that this could be a potential mechanism behind the decline in renal function seen. However, further work will need to be performed to determine the link between elevated renal ET-1 and worsened renal function, and whether this could be ET_A or ET_B receptor-mediated. This could be informative as to whether the changes in the ET system are maladaptive or compensatory as ET_A is linked with prohypertensive and antinatriutic effects while, ET_B is linked with antihypertensive and natiruiretic effects. Although, recent findings suggest a role for ET_A in this pro-natriuretic effect (reviewed in (de Miguel et al., 2016)). Furthermore, ET-1 was measured in whole kidneys so whether this increase is present in cortex and/or medulla is unknown. This is important as ET_A is primarly present in the cortex, with ET_B in the medulla. A previous study by Speed et al. showed that the renal medullary ET system is impaired in Dahl SS rats and appears to contribute to salt-sensitivity (Speed et al., 2011). Future work will look to address the contribution of PER1 in this impairment, and whether this is a potential mechanism behind the exacerbated hypertension and loss of circadian synchrony of blood pressure in SS$^{Per1-/-}$ rats.

Edn1 antisense lncRNA *Edn1-AS* was also increased in SS$^{Per1-/-}$ rats compared with control SS rats. *Edn1-AS* expression has previously been suggested to be regulated by PER1 (Douma, Solocinski, et al., 2020). A focus for future studies will be to understand the role of PER1 in the regulation of ET-1, potentially via *Edn1-AS*. Potential mechanisms for *Edn1-AS* regulating ET-1 at the mRNA level could be that *Edn1-AS* works as a miRNA sponge, sequestering miRNA that would inhibit *Edn1* translation. Furthermore, it has been reported that *Edn1* is subject to regulation by mir-709, a miRNA found to bind in the *Edn1* 3' UTR (Jacobs et al., 2013b). *Edn1-AS* could also bind the sense DNA and form an R-loop, which would keep the chromatin open and accessible to transcriptional machinery (Yao et al., 2019).

The adrenal endothelin axis is an understudied area. However, the current study shows changes in mRNA of adrenal *Edn1* in response to salt and *Ednrb* in response to *Per1* KO in SS rats. Conflicting evidence is available regarding a relationship between the endothelin axis and the adrenal hormone aldosterone. Interestingly, SS$^{Per1-/-}$ rats exhibit higher plasma aldosterone levels (Zietara et al., 2022). Previous work has suggested that ET-1 via ET_B promotes both aldosterone secretion *in vitro* and *in vivo* and proliferation of adrenal cells (Belloni et al., 1996; Cozza et al., 1989; Delarue et al., 2004; Rossi et al., 2000; Zeng et al., 1992). Furthermore, *Edn1* is a target gene of aldosterone in both the kidney and

colon (Gumz et al., 2003; Wong et al., 2007), but whether it has a local effect on adrenal *Edn1* is unknown. In patients with primary aldosteronism, significant upregulation of the endothelin axis was not found in adrenal gland tissue of these patients compared with healthy individuals (Morello et al., 2009). Nevertheless, it would be of interest to further explore the relationship between the endothelin axis and aldosterone, and whether PER1 plays a role.

PER1 has previously been suggested to regulate the liver endothelin axis, as expression of the endothelin axis genes were altered in a time-dependent manner in the liver of *Per1* heterozygous mice (Richards et al., 2014). However, our data only shows significant changes in hepatic *Ednrb* mRNA levels. Again, limited studies have assessed the role of the endothelin axis in the liver. ET-1 has been shown to promote activation of hepatic stellate cells (HSCs), resulting in elevated cell proliferation and contraction, and ultimately leading to liver fibrosis and injury. HSCs express both ET_A and ET_B receptors, but whether the fibrosis and injury is ET_A and/or ET_B-mediated remains in question (reviewed in (Ezhilarasan, 2020)). With a significant difference in the interaction between genotype and diet in *Ednrb* expression, future studies could assess whether $SS^{Per1-/-}$ rats have increased incidence of liver fibrosis. Liver ET_B has also been suggested to promote nitric oxide release to increase expression of bile secretory genes and regulate the secretion of bile into the gallbladder (Rodriguez et al., 2013). A study in a double KO *Per1/Per2* mouse model demonstrated dysregulation of bile acid homeostasis which led to hepatic cholestasis (Ma et al., 2009). Interestingly, bile acids have been suggested to regulate blood pressure and could play a role in the development of salt-sensitive hypertension (reviewed in (Ishimwe et al., 2022)). Therefore, it would be interesting to assess bile secretory genes in $SS^{Per1-/-}$ rats to determine if PER1, via liver endothelin axis, plays a role in bile acid homeostasis, worsening salt-sensitive hypertension.

KO of one clock gene raises the question of what happens to the rest of the clock machinery. Interestingly, loss of PER1 had different effects on clock gene expression in the kidney, adrenal gland, and liver. Although, whether this was a direct effect of lack of PER1, changes in tissue-specific endothelin axis expression, and/or the high salt diet remains in question. Our work in kidney specific-*Per1* KO mice revealed altered circadian clock gene expression in the adrenal gland (Douma et al., 2022). Furthermore, mice overexpressing ET-1 showed decreased *Cry2* expression in the adrenal gland, as well as increased plasma aldosterone levels. Interestingly, this increased plasma aldosterone was reversed with the ET_A receptor antagonist atrasentan (Berillo et al., 2021). Knocking out *Cry2*, as well as *Cry1*, in a double KO mouse model also showed elevated plasma aldosterone levels, accompanied with salt-sensitive hypertension (Okamura et al., 2016). In these rats, compensation in response to *Per1* KO and increased ET-1 could alter clock gene expression and play a role in the worsened salt-sensitive phenotype and/or desynchrony in blood pressure rhythms previously reported (Zietara et al., 2022). With differing effects on circadian clock gene expression in tissues lacking PER1, diverse animal models are crucial for understanding the implications this has on physiological and pathophysiological function.

Given that PER1 is a transcription factor, the role of PER1 on transcription of the endothelin axis genes was a focus of this study. Renal ET-1 peptide levels were assessed, but a

limitation of this study is that protein expression of its receptors was not measured. Furthermore, tissues were only collected at a time range during their inactive period. Previous work in rats has shown that high salt causes either shift or suppression of clock gene expression in renal inner medulla primarily during the active phase and causes region-specific dysschronization of renal clock gene rhythms (Speed, Hyndman, Roth, et al., 2018b). Although, we only found a diet effect on *Cry1* expression, but with only at one time point, we could only capture a snapshot. There is also compelling data on the circadian rhythm of *Edn1* mRNA levels peaking during the active period in mice (reviewed in (Douma, Barral, et al., 2020)). Together, this highlights the need for future studies to investigate ET-1 production, endothelin axis expression, and clock gene expression throughout the 24-hour period in kidneys of SS$^{Per1-/-}$ rats at baseline and following a high salt diet. Another limitation of these studies is that this work was only carried out in male rats. Previous work has demonstrated sex differences in the ratio of ET_A to ET_B, with a higher ratio in male Sprague-Dawley rats (Jin et al., 2013). Therefore, future studies should also focus on investigating the endothelin axis in female SS$^{Per1-/-}$ rats. With increases in renal ET-1, future studies looking at the impact of blocking its receptors with antagonists would be of interest to determine if this prevented the worsening of salt-sensitive phenotype and/or renal function. In the SONAR trial, the ET_A receptor antagonist, atrasentan, reduced the risk of renal events in patients with diabetes and CKD (Heerspink et al., 2019). Therefore, this raises a question for future studies: is ET_A receptor activation involved in worsening of renal function in SS$^{Per1-/-}$ rats?

For the first time, we have shown that PER1 is important for regulation of ET-1 in Dahl SS rats. A potential mechanism could be via PER1 regulation of *Edn1* antisense lncRNA *Edn1-AS*, a regulatory mechanism of ET-1. This work supports the idea that the PER1 is not only important in maintaining rhythmicity of physiological functions, but is also important for adapting to changes in environmental cues, like increased dietary salt, to maintain homeostasis.

Funding

This work was supported by the National Institutes of Health grants (R35 HL135749 (to AS)), (R01 DK109570 (to MLG and AS)), (R56DK128271 (to M.L.G.)), and Department of Veteran Affairs grants (I01 BX004024 (to AS)).

Data Availability

Data generated or analyzed during this study are provided in full within the published article.

References

Belloni AS, Rossi GP, Andreis PG, Neri G, Albertin G, Pessina AC, & Nussdorfer GG (1996). Endothelin adrenocortical secretagogue effect is mediated by the B receptor in rats. Hypertension (Dallas, Tex. : 1979), 27(5), 1153–1159. 10.1161/01.hyp.27.5.1153 [PubMed: 8621210]

Berillo O, Coelho SC, Mahjoub N, Offermanns S, Paradis P, & Schiffrin EL (2021). Aldosterone contributes to hypertension in male mice inducibly overexpressing human endothelin-1 in endothelium. Journal of Hypertension, 39(9), 1908–1917. 10.1097/HJH.0000000000002880 [PubMed: 34039912]

Cozza EN, Gomez-Sanchez CE, Foecking MF, & Chiou S (1989). Endothelin binding to cultured calf adrenal zona glomerulosa cells and stimulation of aldosterone secretion. The Journal of Clinical Investigation, 84(3), 1032–1035. 10.1172/JCI114226 [PubMed: 2547837]

Crislip GR, Douma LG, Masten SH, Cheng KY, Lynch IJ, Johnston JG, Barral D, Glasford KB, Holzworth MR, Verlander XJW, Wingo CS, & Gumz ML (2020). Differences in renal BMAL1 contribution to Na+homeostasis and blood pressure control in male and female mice. Am J Physiol Renal Physiol, 318(6), F1463–F1477. 10.1152/AJPRENAL.00014.2020/ASSET/IMAGES/LARGE/ZH20062091030009.JPEG [PubMed: 32338037]

Delarue C, Conlon JM, Remy-Jouet I, Fournier A, & Vaudry H (2004). Endothelins as local activators of adrenocortical cells. Journal of Molecular Endocrinology, 32(1), 1–7. 10.1677/jme.0.0320001 [PubMed: 14765988]

de Miguel C, Speed JS, Kasztan M, Gohar EY, & Pollock DM (2016). Endothelin-1 and the kidney: new perspectives and recent findings. Current Opinion in Nephrology and Hypertension, 25(1), 35–41. 10.1097/MNH.0000000000000185 [PubMed: 26625864]

Dhaun N, Moorhouse R, MacIntyre IM, Melville V, Oosthuyzen W, Kimmitt RA, Brown KE, Kennedy ED, Goddard J, & Webb DJ (2014). Diurnal variation in blood pressure and arterial stiffness in chronic kidney disease: the role of endothelin-1. Hypertension (Dallas, Tex. : 1979), 64(2), 296–304. 10.1161/HYPERTENSIONAHA.114.03533 [PubMed: 24890823]

Dhaun N, Webb DJ, & Kluth DC (2012). Endothelin-1 and the kidney--beyond BP. British Journal of Pharmacology, 167(4), 720–731. 10.1111/j.1476-5381.2012.02070.x [PubMed: 22670597]

Douma LG, Barral D, & Gumz ML (2020). Interplay of the circadian clock and endothelin system. Physiology, 36(1), 35–43. 10.1152/physiol.00021.2020

Douma LG, Costello HM, Crislip GR, Cheng K-Y, Lynch IJ, Juffre A, Barral D, Masten SH, Roig E, Beguiristain K, Li W, Bratanatawira P, Wingo CS, & Gumz ML (2022). Kidney-Specific KO of the Circadian Clock Protein PER1 Alters Renal Sodium Handling, Aldosterone Levels, and Kidney/Adrenal Gene Expression. American Journal of Physiology. Renal Physiology. 10.1152/ajprenal.00385.2021

Douma LG, Crislip GR, Cheng K-Y, Barral D, Masten S, Holzworth M, Roig E, Glasford K, Beguiristain K, Li W, Bratanatawira P, Lynch IJ, Cain BD, Wingo CS, & Gumz ML (2020). Knockout of the circadian clock protein PER1 results in sex-dependent alterations of ET-1 production in mice in response to a high-salt diet plus mineralocorticoid treatment. Canadian Journal of Physiology and Pharmacology, 98(9), 579–586. 10.1139/cjpp-2019-0688 [PubMed: 32437627]

Douma LG, & Gumz ML (2018). Circadian clock-mediated regulation of blood pressure. In Free Radical Biology and Medicine (Vol. 119, pp. 108–114). Elsevier Inc. 10.1016/j.freeradbiomed.2017.11.024 [PubMed: 29198725]

Douma LG, Solocinski K, Masten SH, Barral DH, Barilovits SJ, Jeffers LA, Alder KD, Patel R, Wingo CS, Brown KD, Cain BD, & Gumz ML (2020). EDN1-AS, A Novel Long Non-coding RNA Regulating Endothelin-1 in Human Proximal Tubule Cells. Frontiers in Physiology, 11, 209. 10.3389/fphys.2020.00209 [PubMed: 32231591]

Ezhilarasan D (2020). Endothelin-1 in portal hypertension: The intricate role of hepatic stellate cells. Experimental Biology and Medicine (Maywood, N.J.), 245(16), 1504–1512. 10.1177/1535370220949148

Golosova D, Palygin O, Bohovyk R, Klemens CA, Levchenko V, Spires DR, Isaeva E, El-Meanawy A, & Staruschenko A (2020). Role of opioid signaling in kidney damage during the development of salt-induced hypertension. Life Science Alliance, 3(12). 10.26508/lsa.202000853

Grenda R, Wühl E, Litwin M, Janas R, Śladowska J, Arbeiter K, Berg U, Caldas-Afonso A, Fischbach M, Mehls O, Sallay P, & Schaefer F (2007). Urinary excretion of endothelin-1 (ET-1), transforming growth factor-β1 (TGF-β1) and vascular endothelial growth factor (VEGF 165) in paediatric chronic kidney diseases: Results of the ESCAPE trial. Nephrology Dialysis Transplantation, 22(12), 3487–3494. 10.1093/ndt/gfm300

Gumz ML, Popp MP, Wingo CS, & Cain BD (2003). Early transcriptional effects of aldosterone in a mouse inner medullary collecting duct cell line. American Journal of Physiology. Renal Physiology, 285(4), F664–73. 10.1152/ajprenal.00353.2002 [PubMed: 12770840]

Heerspink HJL, Parving H-H, Andress DL, Bakris G, Correa-Rotter R, Hou F-F, Kitzman DW, Kohan D, Makino H, McMurray J. J. v, Melnick JZ, Miller MG, Pergola PE, Perkovic V, Tobe S, Yi T, Wigderson M, de Zeeuw D, Elbert A, … Karim S (2019). Atrasentan and renal events in patients with type 2 diabetes and chronic kidney disease (SONAR): a double-blind, randomised, placebo-controlled trial. The Lancet, 393(10184), 1937–1947. 10.1016/S0140-6736(19)30772-X

Hill AM, Crislip GR, Stowie A, Ellis I, Ramsey A, Castanon-Cervantes O, Gumz ML, & Davidson AJ (2021). Environmental circadian disruption suppresses rhythms in kidney function and accelerates excretion of renal injury markers in urine of male hypertensive rats. American Journal of Physiology. Renal Physiology, 320(2), F224–F233. 10.1152/ajprenal.00421.2020 [PubMed: 33356955]

Houde M, Desbiens L, & D'Orléans-Juste P (2016). Endothelin-1: Biosynthesis, Signaling and Vasoreactivity. Advances in Pharmacology (San Diego, Calif.), 77, 143–175. 10.1016/bs.apha.2016.05.002 [PubMed: 27451097]

Hwang YS, Hsieh TJ, Lee YJ, & Tsai JH (1998). Circadian rhythm of urinary endothelin-1 excretion in mild hypertensive patients. American Journal of Hypertension, 11(11 Pt 1), 1344–1351. 10.1016/s0895-7061(98)00170-8 [PubMed: 9832178]

Ishimwe JA, Dola T, Ertuglu LA, & Kirabo A (2022). Bile Acids and Salt-sensitive Hypertension: A Role of the Gut-liver Axis. American Journal of Physiology-Heart and Circulatory Physiology. 10.1152/ajpheart.00027.2022

Jacobs ME, Wingo CS, & Cain BD (2013a). An emerging role for microRNA in the regulation of endothelin-1. Frontiers in Physiology, 4, 22. 10.3389/fphys.2013.00022 [PubMed: 23424003]

Jacobs ME, Wingo CS, & Cain BD (2013b). An emerging role for microRNA in the regulation of endothelin-1. Frontiers in Physiology, 4, 22. 10.3389/fphys.2013.00022 [PubMed: 23424003]

Jin C, Speed JS, Hyndman KA, O'Connor PM, & Pollock DM (2013). Sex differences in ET-1 receptor expression and Ca2+ signaling in the IMCD. American Journal of Physiology. Renal Physiology, 305(8), F1099–104. 10.1152/ajprenal.00400.2013 [PubMed: 23946290]

Johnston JG, Speed JS, Jin C, & Pollock DM (2016). Loss of endothelin B receptor function impairs sodium excretion in a time- and sex-dependent manner. American Journal of Physiology. Renal Physiology, 311(5), F991–F998. 10.1152/ajprenal.00103.2016 [PubMed: 27582096]

Klemens CA, Chulkov EG, Wu J, Hye Khan MA, Levchenko V, Flister MJ, Imig JD, Kriegel AJ, Palygin O, & Staruschenko A (2021). Loss of Chloride Channel 6 (CLC-6) Affects Vascular Smooth Muscle Contractility and Arterial Stiffness via Alterations to Golgi Calcium Stores. Hypertension (Dallas, Tex. : 1979), 77(2), 582–593. 10.1161/HYPERTENSIONAHA.120.16589 [PubMed: 33390052]

Kohan DE, Cleland JG, Rubin LJ, Theodorescu D, & Barton M (2012). Clinical trials with endothelin receptor antagonists: what went wrong and where can we improve? Life Sciences, 91(13–14), 528–539. 10.1016/j.lfs.2012.07.034 [PubMed: 22967485]

Kohan DE, Rossi NF, Inscho EW, & Pollock DM (2011). Regulation of blood pressure and salt homeostasis by endothelin. In Physiological Reviews (Vol. 91, Issue 1, pp. 1–77). 10.1152/physrev.00060.2009 [PubMed: 21248162]

Kostov K (2021). The causal relationship between endothelin-1 and hypertension: Focusing on endothelial dysfunction, arterial stiffness, vascular remodeling, and blood pressure regulation. In Life (Vol. 11, Issue 9). MDPI. 10.3390/life11090986

Livak KJ, & Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.), 25(4), 402–408. 10.1006/meth.2001.1262 [PubMed: 11846609]

Ma K, Xiao R, Tseng H-T, Shan L, Fu L, & Moore DD (2009). Circadian dysregulation disrupts bile acid homeostasis. PloS One, 4(8), e6843. 10.1371/journal.pone.0006843 [PubMed: 19718444]

Morello F, Schiavone D, Mengozzi G, Bertello C, Liew CC, Bisbocci D, Mulatero P, & Veglio F (2009). Adrenal endothelin-1 levels are not associated with aldosterone secretion in primary aldosteronism. European Journal of Endocrinology, 160(3), 453–458. 10.1530/EJE-08-0828 [PubMed: 19073831]

Okamura H, Doi M, Goto K, & Kojima R (2016). Clock genes and salt-sensitive hypertension: a new type of aldosterone-synthesizing enzyme controlled by the circadian clock and angiotensin

II. Hypertension Research : Official Journal of the Japanese Society of Hypertension, 39(10), 681–687. 10.1038/hr.2016.91 [PubMed: 27439492]

Partch CL, Green CB, & Takahashi JS (2014). Molecular architecture of the mammalian circadian clock. In Trends in Cell Biology (Vol. 24, Issue 2, pp. 90–99). 10.1016/j.tcb.2013.07.002 [PubMed: 23916625]

Richards J, Welch AK, Barilovits SJ, All S, Cheng K-Y, Wingo CS, Cain BD, & Gumz ML (2014). Tissue-specific and time-dependent regulation of the endothelin axis by the circadian clock protein Per1. Life Sciences, 118(2), 255–262. 10.1016/j.lfs.2014.03.028 [PubMed: 24721511]

Rodriguez MR, Soria LR, Ventimiglia MS, Najenson AC, di María A, Dabas P, Fellet A, Marinelli RA, Vatta MS, & Bianciotti LG (2013). Endothelin-1 and −3 induce choleresis in the rat through ETB receptors coupled to nitric oxide and vagovagal reflexes. Clinical Science (London, England : 1979), 125(11), 521–532. 10.1042/CS20120633 [PubMed: 23642207]

Rossi GP, Belloni AS, Nussdorfer GG, & Pessina AC (2000). Endothelin-1 and the adrenal gland. Journal of Cardiovascular Pharmacology, 35(4 Suppl 2), S17–20. 10.1097/00005344-200000002-00005

Smeijer JD, Kohan DE, Webb DJ, Dhaun N, & Heerspink HJL (2021). Endothelin receptor antagonists for the treatment of diabetic and nondiabetic chronic kidney disease. Current Opinion in Nephrology and Hypertension, 30(4), 456–465. 10.1097/MNH.0000000000000716 [PubMed: 33990507]

Speed JS, Hyndman KA, Kasztan M, Johnston JG, Roth KJ, Titze JM, & Pollock DM (2018). Diurnal pattern in skin Na+ and water content is associated with salt-sensitive hypertension in ETB receptor-deficient rats. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 314(4), R544–R551. 10.1152/ajpregu.00312.2017 [PubMed: 29351432]

Speed JS, Hyndman KA, Roth K, Heimlich JB, Kasztan M, Fox BM, Johnston JG, Becker BK, Jin C, Gamble KL, Young ME, Pollock JS, & Pollock DM (2018a). High dietary sodium causes dyssynchrony of the renal molecular clock in rats. American Journal of Physiology. Renal Physiology, 314(1), F89–F98. 10.1152/ajprenal.00028.2017 [PubMed: 28971988]

Speed JS, Hyndman KA, Roth K, Heimlich JB, Kasztan M, Fox BM, Johnston JG, Becker BK, Jin C, Gamble KL, Young ME, Pollock JS, & Pollock DM (2018b). High dietary sodium causes dyssynchrony of the renal molecular clock in rats. American Journal of Physiology-Renal Physiology, 314(1), F89–F98. 10.1152/ajprenal.00028.2017 [PubMed: 28971988]

Speed JS, LaMarca B, Berry H, Cockrell K, George EM, & Granger JP (2011). Renal medullary endothelin-1 is decreased in Dahl salt-sensitive rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 301(2), R519–R523. 10.1152/ajpregu.00207.2011 [PubMed: 21613578]

Speed JS, & Pollock DM (2013). Endothelin, kidney disease, and hypertension. Hypertension, 61(6), 1142–1145. 10.1161/HYPERTENSIONAHA.113.00595 [PubMed: 23608655]

Stow LR, Jacobs ME, Wingo CS, & Cain BD (2011). Endothelin-1 gene regulation. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 25(1), 16–28. 10.1096/fj.10-161612 [PubMed: 20837776]

Stow LR, Richards J, Cheng K-Y, Lynch IJ, Jeffers LA, Greenlee MM, Cain BD, Wingo CS, & Gumz ML (2012). The circadian protein period 1 contributes to blood pressure control and coordinately regulates renal sodium transport genes. Hypertension (Dallas, Tex. : 1979), 59(6), 1151–1156. 10.1161/HYPERTENSIONAHA.112.190892 [PubMed: 22526258]

Waijer SW, Gansevoort RT, Bakris GL, Correa-Rotter R, Hou F-F, Kohan DE, Kitzman DW, Makino H, McMurray J. J. v, Perkovic V, Tobe S, Parving H-H, de Zeeuw D, & Heerspink HJL (2021). The Effect of Atrasentan on Kidney and Heart Failure Outcomes by Baseline Albuminuria and Kidney Function: A Post Hoc Analysis of the SONAR Randomized Trial. Clinical Journal of the American Society of Nephrology: CJASN. 10.2215/CJN.07340521

Wong S, Brennan FE, Young MJ, Fuller PJ, & Cole TJ (2007). A direct effect of aldosterone on endothelin-1 gene expression in vivo. Endocrinology, 148(4), 1511–1517. 10.1210/en.2006-0965 [PubMed: 17218419]

Yao R-W, Wang Y, & Chen L-L (2019). Cellular functions of long noncoding RNAs. Nature Cell Biology, 21(5), 542–551. 10.1038/s41556-019-0311-8 [PubMed: 31048766]

Zeng ZP, Naruse M, Guan BJ, Naruse K, Sun ML, Zang MF, Demura H, & Shi YF (1992). Endothelin stimulates aldosterone secretion in vitro from normal adrenocortical tissue, but not adenoma tissue, in primary aldosteronism. The Journal of Clinical Endocrinology and Metabolism, 74(4), 874–878. 10.1210/jcem.74.4.1548353 [PubMed: 1548353]

Zhang D, Jin C, Obi IE, Rhoads MK, Soliman RH, Sedaka RS, Miller Allan J, Tao B, Speed JS, Pollock JS, & David Pollock XM (2020). Loss of circadian gene Bmal1 in the collecting duct lowers blood pressure in male, but not female, mice. Am J Physiol Renal Physiol, 318(3), F710–F719. 10.1152/ajprenal [PubMed: 31904281]

Zhang R, Lahens NF, Ballance HI, Hughes ME, & Hogenesch JB (2014). A circadian gene expression atlas in mammals: Implications for biology and medicine. Proceedings of the National Academy of Sciences of the United States of America, 111(45), 16219–16224. 10.1073/pnas.1408886111 [PubMed: 25349387]

Zietara A, Spires DR, Juffre A, Costello HM, Crislip GR, Douma LG, Levchenko V, Dissanayake L. v., Klemens CA, Nikolaienko O, Geurts AM, Gumz ML, & Staruschenko A (2022). Knockout of the circadian clock protein PER1 exacerbates hypertension and increases kidney injury in Dahl salt-sensitive rats. Hypertension , *In Press*.

Zoccali C, Leonardis D, Parlongo S, Mallamaci F, & Postorino M (1995). Urinary and plasma endothelin 1 in essential hypertension and in hypertension secondary to renoparenchymal disease. Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association, 10(8), 1320–1323. [PubMed: 8538921]

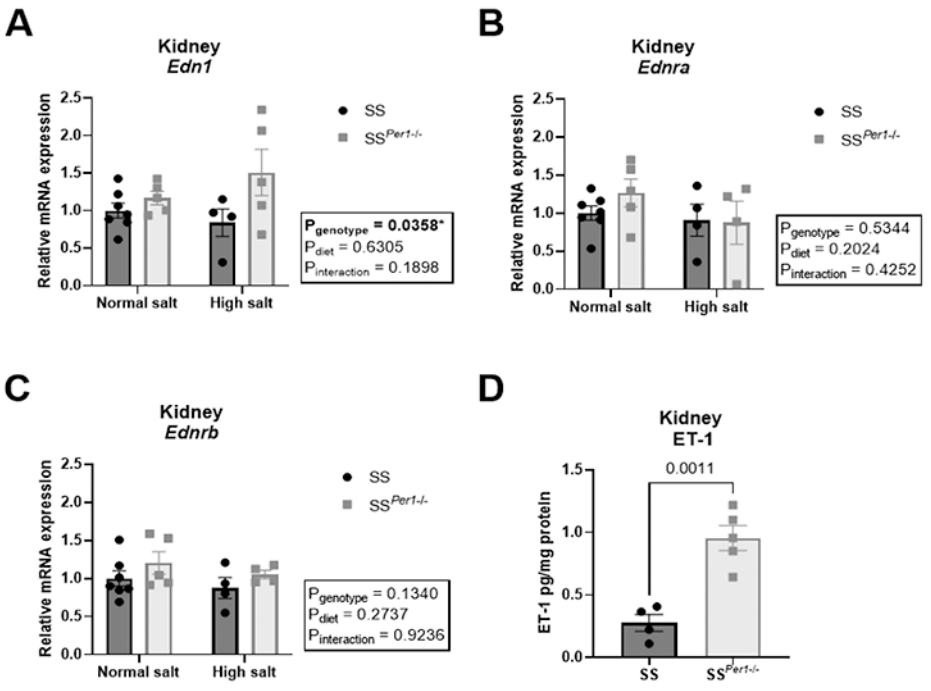

Figure 1. Endothelin axis gene expression in the kidney of male SS$^{Per1-/-}$ rats.

SS$^{Per1-/-}$ (grey squares) and SS (black circles) kidneys were collected between 1:00-4:00 PM on either a normal or high salt diet. Total RNA was isolated, cDNA generated, and relative mRNA expression measured using TaqMan assay. Relative mRNA expression of **A.** endothelin-1 gene (*Edn1*), **B.** endothelin receptor A gene (*Ednra*), and **C.** endothelin receptor B gene (*Ednrb*) in SS$^{Per1-/-}$ and SS kidneys. β-actin was used as the reference gene and expression normalized to SS normal salt data. **D.** Kidney endothelin-1 (ET-1) peptide levels were measured following 3 weeks high salt diet by ELISA. Data are mean ± SE. Genotype, diet, and genotype*diet interaction effects were determined by 2-way ANOVA. *P<0.05, n=4-7 rats per group.

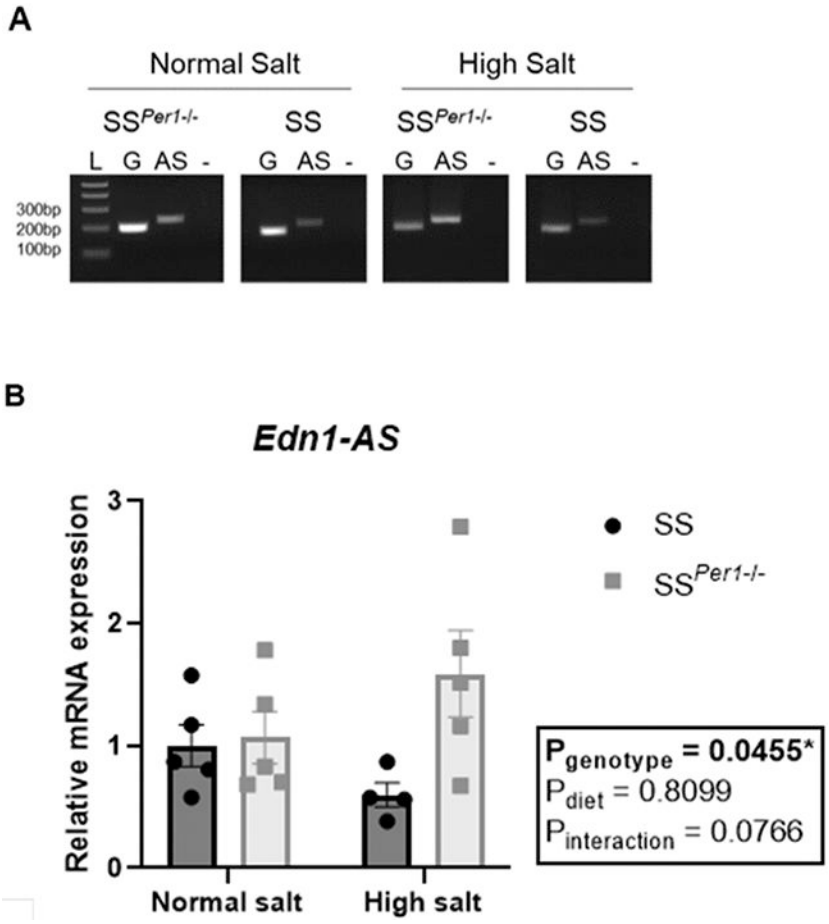

Figure 2. *EDN1-AS* detection and expression level in the kidney of male SS$^{Per1-/-}$ rats.

Strand-specific RT-PCR was used to measure the relative level of *EDN1-AS* in whole kidney samples from SS$^{Per1-/-}$ and SS control rats on normal salt or high salt diet. **A.** Strand-specific RT primer rSS4 (AS) was used to generate *EDN1-AS* cDNA samples using whole kidney RNA. For each AS reaction, an equivalent RT reaction was performed using no RT (−) to ensure no genomic DNA contamination. Random hexamer RT primers were used to generate cDNA samples for GAPDH measurement (G). For each reaction, 20 ng of cDNA was used in PCR reactions to amplify *EDN1-AS* or GAPDH. Representative gel images are shown for each group. Gel ladder (L) molecular weights are listed for reference. **B.** Gel band pixels were measured using ImageJ. AS band intensity was normalized to the respective GAPDH band. *EDN1-AS* expression levels were relativized to SS control normal salt expression levels. Data are mean ± SE. Genotype, diet, and genotype*diet interaction effects were determined by 2-way ANOVA. *P<0.05, n=4-5 rats per group.

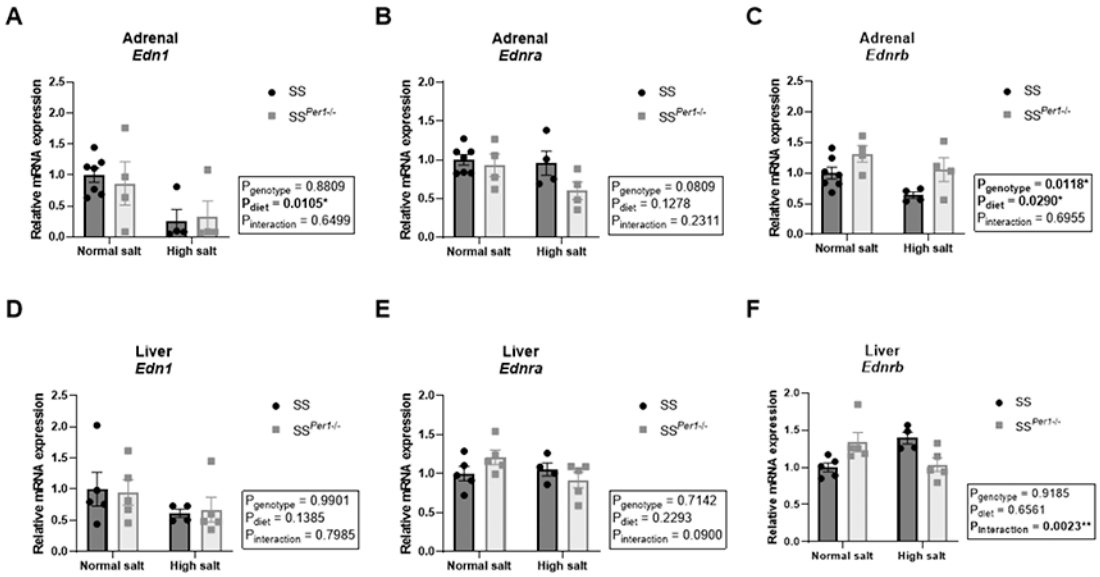

Figure 3. Endothelin axis gene expression in the adrenal and liver of male SS$^{Per1-/-}$ rats.

SS$^{Per1-/-}$ (grey squares) and SS (black circles) adrenal glands and liver were collected between 1:00-4:00 PM on either a normal or high salt diet. Total RNA was isolated, cDNA generated, and relative mRNA expression measured using TaqMan assay. Relative mRNA expression of endothelin-1 gene (*Edn1*), endothelin receptor A gene (*Ednra*), and endothelin receptor B gene (*Ednrb*) in the adrenal glands (**A**-**C**) and livers (**D**-**F**) of SS$^{Per1-/-}$ and SS rats . β-actin was used as the reference gene and expression normalized to SS normal salt data. Data are mean ± SE. Genotype, diet, and genotype*diet interaction effects were determined by 2-way ANOVA. *P<0.05, **P<0.01, n=4-7 rats per group.

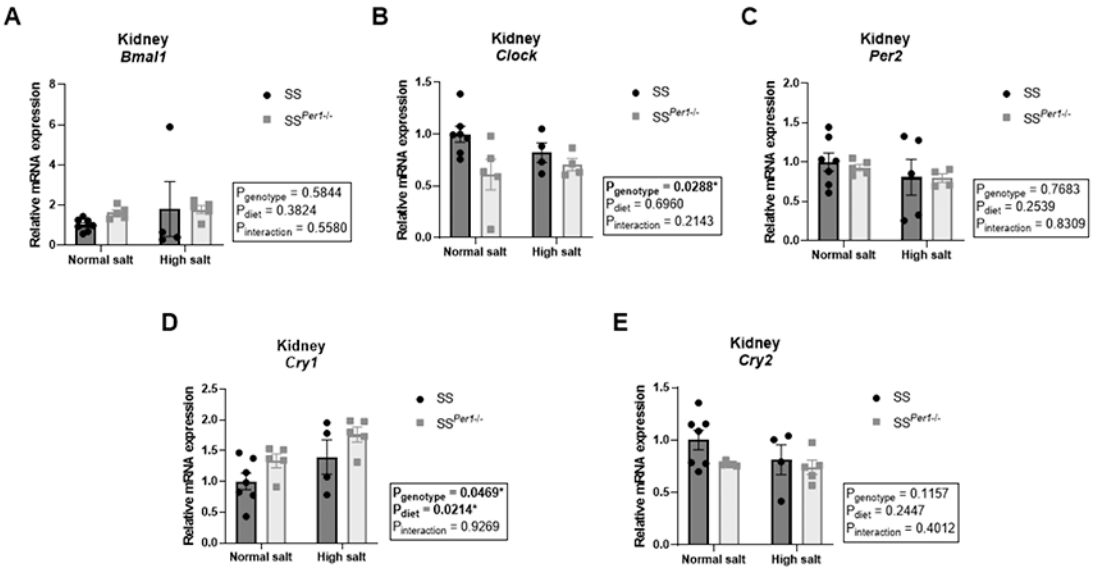

Figure 4. Circadian clock gene expression in the kidney of male SS$^{Per1-/-}$ rats.

SS$^{Per1-/-}$ (grey squares) and SS (black circles) kidneys were collected between 1:00-4:00 PM on either a normal or high salt diet. Total RNA was isolated, cDNA generated, and relative mRNA expression measured using TaqMan assay. Relative mRNA expression of **A.** *Bmal1*, **B.** *Clock*, **C.** *Per2*, **D.** *Cry1*, and **E.** *Cry2* in SS$^{Per1-/-}$ and SS kidneys. β-actin was used as the reference gene and expression normalized to SS normal salt data. Data are mean ± SE. Genotype, diet, and genotype*diet interaction effects were determined by 2-way ANOVA. *P<0.05, n=4-7 rats per group.

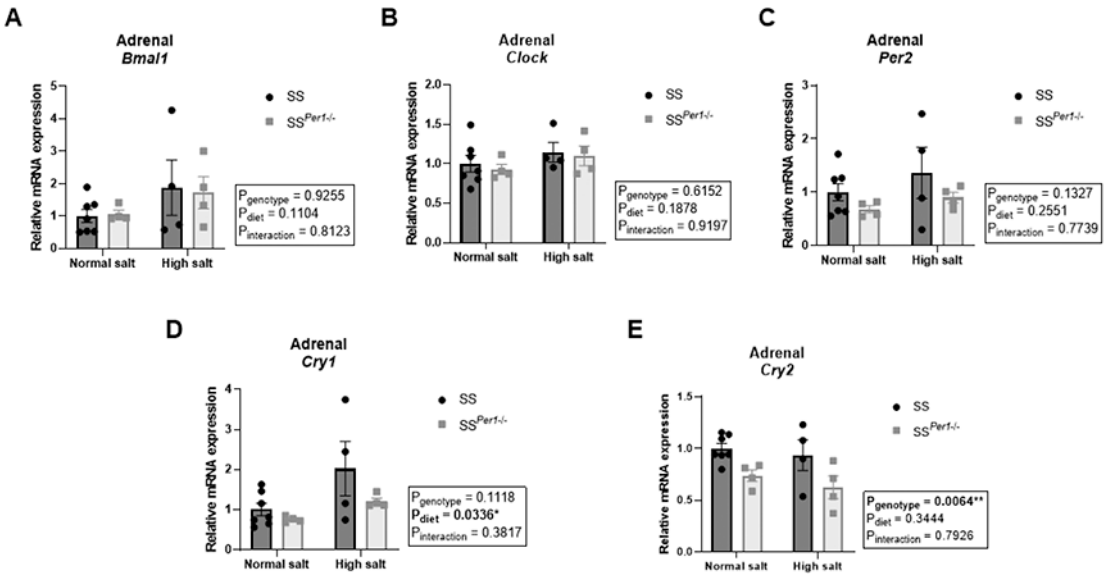

Figure 5. Circadian clock gene expression in the adrenal gland of male SS$^{Per1-/-}$ rats.

SS$^{Per1-/-}$ (grey squares) and SS (black circles) adrenal glands were collected between 1:00-4:00 PM on either a normal or high salt diet. Total RNA was isolated, cDNA generated, and relative mRNA expression measured using TaqMan assay. Relative mRNA expression of **A.** *Bmal1*, **B.** *Clock*, **C.** *Per2*, **D.** *Cry1*, and **E.** *Cry2* in SS$^{Per1-/-}$ and SS adrenal glands. β-actin was used as the reference gene and expression normalized to SS normal salt data. Data are mean ± SE. Genotype, diet, and genotype*diet interaction effects were determined by 2-way ANOVA. *P<0.05, **P<0.01, n=4–7 rats per group.

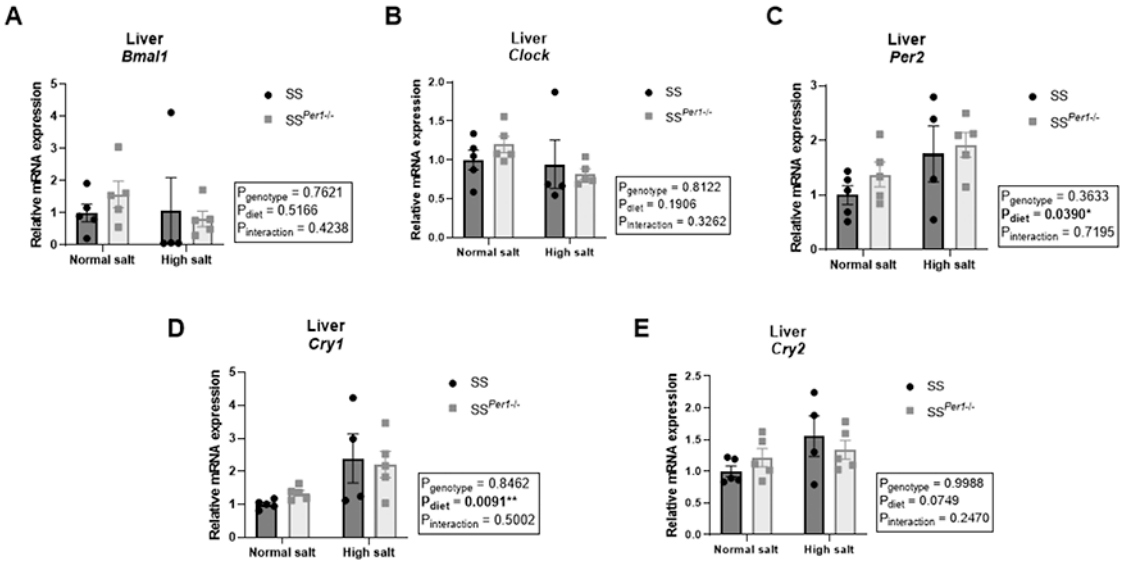

Figure 6. Circadian clock gene expression in the liver of male SS$^{Per1-/-}$ rats.

SS$^{Per1-/-}$ (grey squares) and SS (black circles) liver samples were collected between 1:00-4:00 PM on either a normal or high salt diet. Total RNA was isolated, cDNA generated, and relative mRNA expression measured using TaqMan assay. Relative mRNA expression of **A.** *Bmal1*, **B.** *Clock*, **C.** *Per2*, **D.** *Cry1*, and **E.** *Cry2* in SS$^{Per1-/-}$ and SS livers. β-actin was used as the reference gene and expression normalized to SS normal salt data. Data are mean ± SE. Genotype, diet, and genotype*diet interaction effects were determined by 2-way ANOVA. *P<0.05, **P<0.01, n=4-5 rats per group.

Table 1.

TaqMan rat probe sequences

Quantitative PCR probes		
Gene	**Species**	**TaqMan Assay Identification**
Actb	Rat	Rn00667869_m1
Bmal1	Rat	Rn00577590_m1
Clock	Rat	Rn00573120_m1
Per2	Rat	Rn01427704_m1
Cry1	Rat	Rn01503063_m1
Cry2	Rat	Rn01485701_m1
Edn1	Rat	Rn00561129_m1
Ednra	Rat	Rn00561137_m1
Ednrb	Rat	Rn00569139_m1

Actb, β-actin; *Bmal1*, brain and muscle ARNT-like 1; *Clock*, circadian locomotor output cycles kaput; *Per2*, period 2; *Cry1* and *Cry2*, cryptochrome 1 and 2; *Edn1*, endothelin-1; *Ednra* and *Ednrb*, endothelin receptors A and B.

Table 2

EDN1-AS strand-specific RT-PCR rat primers and sequences

EDN1-AS Strand Specific Rat RT Primer		
Name	**Sequence (5’-3’)**	
rSS4	CCACAGCACCAAACAGCATAGACAG	
PCT Rat Primers		
Name	**Sequence (5’-3’)**	**Product Size**
rPCRI	CAGCAACAGCATCAAGACCTCCTTT	235 bp
rPCR2	GGTCCTCTGCCAGTCTGAACAAGAA	
rGAPDH Fwd	CCCAACTAACTCGCCTATTTCTTGC	199 bp
rGAPDH Rev	CTTCCCATTCTCAGCCTTGACTGT	