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Circadian clock genes directly regulate expression of the Na^+/H^+ exchanger NHE3 in the kidney

Mohammad Saifur Rohman,[1] Noriaki Emoto, Hidemi Nonaka, Ryusuke Okura, Masataka Nishimura, Kazuhiro Yagita, Gijsbertus T.J. van der Horst, Masafumi Matsuo, Hitoshi Okamura, and Mitsuhiro Yokoyama

Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School, Kobe, Japan; Division of Molecular Brain Science, Department of Brain Sciences, Kobe University Graduate School, Kobe, Japan; Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan; and MGC, Department of Cell Biology and Genetics, Erasmus MC, Rotterdam, The Netherlands

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Background. Daily rhythms in mammalian physiology are generated by a transcription/translation feedback loop orchestrated by a set of clock genes. However, little is known about the molecular cascade from the clock gene oscillators to cellular function.

Methods. The mRNA expression profiles of *NHE3* and clock genes were examined in mice and rat kidneys. First, luciferase assays followed by a site directed mutagenesis of an E-box sequence were used to assess the CLOCK:BMAL1-transactivated NHE3 promoter activity. A direct binding of CLOCK:BMAL1 heterodimers to an E-box sequences of NHE3 promoter was confirmed by electrophoretic mobility shift assay (EMSA).

Results. We present evidence that renal tubular *NHE3*, the Na^+/H^+ exchanger critical for systemic electrolyte and acid-base homeostasis, is a clock-controlled gene regulated directly by CLOCK:BMAL1 heterodimers in kidneys. *NHE3* mRNA level in rat kidney displayed circadian kinetics, and this circadian expression was severely blunted in homozygous *CRY1/2* double-deficient mice, suggesting that the transcriptional machinery of peripheral clocks in renal tubular cells directly regulates the circadian expression of *NHE3*. By analyzing the 5′ upstream region of the *NHE3* gene, we found an E box critical for the transcription of *NHE3* via the CLOCK:BMAL1-driven circadian oscillator. The circadian expression of *NHE3* mRNA was reflected by oscillating protein levels in the proximal tubules of the rat kidney.

Conclusion. NHE3 should represent an output gene of the peripheral oscillators in kidney, which is regulated directly by CLOCK:BMAL1 heterodimers.

[1]The current address for Dr. Rohman is Faculty of Medicine, Brawijaya University, Malang, Indonesia.

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Circadian rhythmicity is observed in many aspects of cellular function, including membrane excitation, energy metabolism, and cell division [1–3]. In mammals, the clock system is composed of a central clock in the suprachiasmatic nucleus which generates the standard time of the body at systemic levels, and peripheral clocks which perform the effector processes of the circadian rhythms with the aid of systemic suprachiasmatic nucleus signals in the variety of organs [4, 5].

In both central and peripheral clock systems, circadian rhythmicity is generated at the cellular level by the circadian core oscillator composed of an autoregulatory transcription-(post)translation-based feedback loop involving a set of clock genes. In this molecular loop, two transcriptional activator genes, CLOCK and BMAL1, regulate gene expression by binding to specific enhancer elements, termed E boxes (CTCGTG). Target genes of these activators include several repressor proteins, including PER1, PER2, PER3, CRY1, and CRY2, which function to inhibit the CLOCK:BMAL1 complex, thus generating a circadian oscillation in their own transcription [6–8].

In the kidney, plasma Na^+ concentration and renal Na^+ excretion are known to display a significant diurnal variation in both animals and humans [9–13]. The mechanisms involving these diurnal changes are related to the regulation of renal blood flow and renal cellular functions, though the underlying molecular mechanism remains unclear. Since a number of genes in the kidney show diurnal variation, we searched for genes likely involved in these processes. NHE3 is one of the Na^+/H^+ exchangers (NHEs) that catalyze the electroneutral exchange of one extracellular Na^+ for one intracellular H^+ across the plasma membrane, to mediate bulk reabsorption of filtered Na^+ in the proximal convoluted tubule [14–16]. Gene knockout experiments in mice have been suggested that NHE3 is required for maintenance of

normal set point for Na^+ fluid volume balance. In addition, NHE3 knockout mice shown a decreased blood pressure, an elevated plasma aldosterone and renin mRNA in the kidney [17], thus supporting the view that the major renal transporter mediating Na^+ reabsorption plays a central role in long-term control of arterial blood pressure [18, 19].

In the present study, we investigated whether the expression of NHE3 has diurnal variations, and whether it is regulated by the circadian clock system. We demonstrate here that NHE3 mRNA and protein display circadian expression in the rodent kidney. Circadian expression of *NHE3* is mediated by the direct control of CLOCK:BMAL1 heterodimers through an E box in the promoter of the *NHE3* gene. This conclusion is further supported by genetic studies. In homozygous *CRY1/2* double-deficient mice, circadian expression of *NHE3* mRNA is significantly reduced. Immunohistochemistry experiments clearly showed the colocalization of NHE3 and PER2 in the proximal tubule cells in kidney. Taken together, these results strongly suggest that the transcriptional regulation of *NHE3* is controlled by the core oscillator, and that *NHE3* represents a clock-controlled gene (*Ccg*) in the kidney.

METHODS

Materials

Enzymes used in molecular cloning were obtained from Roche Molecular Biochemicals or from New England Biolabs (Beverly, MA, USA).

Animals and procedures

Sprague-Dawley rats purchased 5 weeks postpartum were exposed to 2 weeks of 12-hour light (fluorescent light, 300 lux)/dark cycles and then kept in complete darkness (dark/dark) for 2 days as a continuation of the dark phase of the last cycle. Three- to 5-week-old Balb-c and *mCry1*$^{-/-}$ *mCry2*$^{-/-}$ mice [20] were housed under the same conditions as Sprague-Dawley rats. The mRNA expression profiles of *NHE3* and clock genes were examined in the second dark-dark cycle every 4 hours, starting at the beginning of the light cycle. The care and use of the animals strictly followed the guidelines of the Animal Research Committee of Kobe University Graduate School of Medicine.

Northern blot analysis

Ten micrograms of total RNA was electrophoresed in a 1.2% denaturing agarose gel containing formaldehyde and transferred onto nylon membranes. The blots were hybridized in QuickHyb hybridization solution (Stratagene, La Jolla, CA, USA) at 68°C or in a 50% formamide-containing hybridization solution at 42°C. To obtain a specific probe for rat *NHE3 (rNHE3)*, a fragment of the *rNHE3* cDNA was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers 5′-GGTCAATGTGGACTTCAGCAC-3′ and 5′-GGGGAGAACACAGGATTATCAAT-3′ (GeneBank, accession number M.85300.1). The clock gene probes were generated as previously described [21]. The probes were labeled with [α ^{32}P] deoxycytidimine triphosphate (dCTP) using random priming after sequence confirmations, and were exposed to the imaging plates of a Fuji-Bio Imaging Analyzer BAS 2000 (Fuji Photo Film, Kanagawa, Japan).

Construction of plasmids

The promoter of *rNHE3* was constructed by amplification of a 1.36 kb fragment of the 5′ flanking region of *rNHE3* gene from rat genomic DNA using the primers 5′-TCCAGTTCCTTACCCAGTCAGTCTC-3′ and 5′-GCTCCAGGAGCCGACACGCATAC-3′. The amplified fragment was digested with *Bam*HI followed by blunt end generation at one end and cut with *Kpn*I at the other. This fragment was directionally cloned into *Sma*I-*Kpn*I digested picagene basic vector (PGV-B) luciferase reporter (Tokyo, Japan), resulting in a −1360/+58 bp (relative to the transcriptional initiation sites) promoter construct. The constructs spanning −605/+58 bp, −484/+58 bp, −315/+58 bp, and −17/+58 bp were generated by digesting the 1.36 kb fragment of *Bam*HI-*Kpn*I 5′ flanking region of *rNHE3* with *Eco*RI, *Nco*I, *Sal*I, *and Sma*I, respectively. These fragments were subcloned into the *Sma*I/*Kpn*I sites of PGV-B and used after confirmation by enzymatic digestion. Deletion of −141/−18 bp fragment was done with *Bst*XI and *Sma*I to remove the putative promoter region, whereas removal of the −17/+58 bp fragment was accomplished by digestion of the −1360/+58 bp promoter construct with *Sma*I and *Kpn*I. Clock gene expression constructs were obtained by RT-PCR from the coding regions of *mPer2* (AF035830), *mCry1* (AB000777), *hBMAL1* (AB000813), and *hClock* (AB002332), as previously described [22, 23].

Mutagenesis of E box

Mutant construct was made by site directed mutagenesis [24] using the Muta-Gene Phagemid In Vitro Mutagenesis Kit, version 2 (Bio-Rad Laboratories, Hercules, CA, USA) as described by the manufacturer. All constructs were verified by sequencing the final plasmids.

Transcriptional assay

Opossum kidney (OK) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 2 mmol/L L-glutamine, Earle's balanced salt solution (BSS), and 0.1 mmol/L nonessential amino acids supplemented with 10% fetal bovine serum (FBS), 1.5 g/L sodium bicarbonate, and 1.0 mmol/L sodium pyruvate (Sigma Chemical Co., St. Louis, MO, USA). OK cells passages 4 to 10 were plated at 70% to 80% confluency in 60 mm dishes 24 hours before transfection. The cells were transfected with