Circadian expression of the Na^+/H^+ exchanger NHE3 in the mouse renal medulla

Hiromi NISHINAGA^{1,2}, Rie KOMATSU¹, Masao DOI¹, Jean-Michel FUSTIN¹, Hiroyuki YAMADA¹, Ryusuke OKURA², Yoshiaki YAMAGUCHI¹, Masahiro MATSUO¹, Noriaki EMOTO³ and Hitoshi OKAMURA^{1,2}

¹Department of Systems Biology, Graduate School of Pharmaceutical Science, Kyoto University, Kyoto 606-8501, Japan; ²Division of Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan; and ³Clinical Pharmacy, Kobe Pharmaceutical University, Kobe, Japan

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ABSTRACT

Renal tubular NHE3, the Na^+/H^+ exchanger, is a critical enzyme for electrolyte and acid-base homeostasis in the kidney. We previously demonstrated that the expression of this gene in the kidney followed a circadian rhythm directly regulated by clock genes acting on E-box elements present on its promoter region. In the present study, we further characterize the circadian expression of NHE3 in the mice kidney by *in situ* hybridization, and refine quantification of gene expression using real-time PCR combined with laser capture micro-dissection. We show NHE3 mRNA was strongly expressed in the inner stripe of the outer medulla and weakly in the cortex. Further real-time PCR data from dissected medullary nephron demonstrated clear circadian oscillations in the thick ascending limbs and the thin descending limbs, but not in the collecting ducts. The circadian changes of this molecule in the renal medulla may partially contribute to the circadian change of urinary electrolyte secretion.

In the kidney, Na^+/H^+ exchangers (NHEs) are transmembrane proteins that mediate the electroneutral exchange of Na^+ and H^+ across plasma membranes of most segments of the nephron (16). Thus far, NHE1, 2, 3, 4, 6, 7, 8 and 9 were identified in the kidney among ten mammalian isoforms (NHE1–10) (10, 17). NHE3 gene knockout studies suggest that NHE3, located along the apical membranes of proximal convoluted tubules, thin descending limbs of Henle's loop (TL) and thick ascending limbs of Henle's loop (TAL) (2), plays a major role for Na^+/H^+ transporting activity in the kidney (24, 25). Since NHE3 knockout mice showed decreased blood pressure as well as elevated plasma aldosterone and

renin mRNA in the kidney, this major renal transporter mediating Na^+ reabsorption also appears to play a major role in the long-term control of arterial blood pressure (3, 25). Renal NHE3 is known to be upregulated in metabolic acidosis (1, 27) and hypovolemia (11).

Interestingly, we previously demonstrated that this functionally important NHE isoform displays circadian rhythms at both the mRNA and protein levels in the rodent kidney (22). Moreover, the NHE3 is unique since it is so far the only known gene to be transcriptionally controlled by clock genes including *Per1/2*, *Cry1/2*, *Bmal1*, and *Clock* in the kidney (22). These observations were based on western blotting and northern blotting methods using whole-kidney homogenate, but analyses at the cellular level are so far lacking. In the present study, we further characterized the circadian expression profiles of NHE3 mRNA in the kidney by *in situ* hybridization and refined the anatomical resolution at the level of single segments of nephron using real-time PCR

Address correspondence to: Dr. Hitoshi Okamura
Department of Systems Biology, Graduate School of Pharmaceutical Science, Kyoto University, 46-29 Yoshida-Shimo-Adachi-cho, Sakyo-ku, Kyoto 606-8501, Japan
Tel: +81-75-753-9552, Fax: +81-75-753-9553
E-mail: okamurah@pharm.kyoto-u.ac.jp

combined with laser capture microdissection (LCM) technique.

MATERIALS AND METHODS

Animals. For examining the expression of NHE3 in the kidney, we purchased commercially available male C57Bl6 mice at their 3 months old (JAPS, Osaka, Japan). They were housed under standard 12 h : 12 h light-dark (LD: 300 lux fluorescent light) cycles at least for 2 weeks at $22 \pm 2^\circ\text{C}$ with freely provided diet and water. For the experiments in constant darkness, mice were placed in constant darkness for two days before sacrifice. For *in situ* hybridization ($n = 8$), the animals were sacrificed at various time points including CT4 ($n = 4$, circadian time 4 = 4 hours after the subjective dawn). For the laser microdissection study ($n = 18$), animals were sacrificed 4 hours intervals beginning at CT0 (circadian time 0 = time of subjective dawn) in the 2nd day of constant darkness. The Committees for Animal Research of Kyoto University approved the experimental protocol of this research.

Probes. For *in situ* hybridization, mouse NHE3 cDNA fragment (positions 1686 ~ 2261 of *mus musculus* Gene Bank accession number NM_001081060) containing vector was linearized with restriction enzymes and used as template for sense and antisense cRNA probes. Radiolabeled probes were made using ^{33}P -UTP (GE Healthcare Biolabs, Boston, MA) with a standard protocol for cRNA synthesis. By using the antisense probe, positive signals were expressed in many regions of the kidney, but the sense probe did not detect any signals.

For real-time PCR, mouse NHE3 cDNA fragment (positions 2073 ~ 2119 of *mus musculus* Gene Bank accession number NM_001081060), Synaptopodin (Synpo) cDNA fragment (positions 2441 ~ 2534 of *mus musculus* Gene Bank accession number XM_001479956), Aquaporin 1 (Aqp1) cDNA fragment (positions 701 ~ 877 of *mus musculus* Gene Bank accession number NM_007472), Tamm-Horsfall protein (Thp) cDNA fragment (positions 1041 ~ 1194 of *mus musculus* Gene Bank accession number NM_009470), Aquaporin 2 (Aqp2) cDNA fragment (positions 572 ~ 723 of *mus musculus* Gene Bank accession number NM_009699), and 36b4 cDNA fragment (positions 357 ~ 579 of *mus musculus* Gene Bank accession number AK010267) were synthesized with the following primers (sense and antisense, respectively): NHE3, 5'-aagctgtacaagagggagcg-3' and 5'-tgatggtgtagtgtgtgcc-3';

Synpo, 5'-ggaatgctgggatcgaggc-3' and 5'-tccaggctgctaggaggc-3'; Aqp1, 5'-tgccattggctgtgtgtg-3' and 5'-agtcataatgagcactgcc-3'; Thp, 5'-caatctggcttactgcaccg-3' and 5'-cagcctgtactccaattggg-3'; Aqp2, 5'-agccctgctctccattgg-3' and 5'-aggggtccgatccagagac-3'; 36b4, 5'-ctcactgagattcgggatag-3' and 5'-ctccacactgtctccagtc-3'.

In situ hybridization. Mice were deeply anesthetized with ether, and intracardially perfused with 10 mL of autoclaved ice cold saline, followed by 20 mL of a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). For the animals housed in darkness, anesthesia was performed under safe dark red light. The brains were removed, post-fixed in the same fixative for 24 h at 4°C and placed in 0.1 M PB containing 20% sucrose for 48 h. These brains were frozen using dry ice and stored at -80°C until use. Mouse brain sections were made 40 μm in thickness by a cryostat (Leica, Wetzlar, Germany). To minimize technical variations throughout the hybridization procedure, sections from different experimental conditions were gathered into one group and processed simultaneously. *In situ* hybridization histochemistry was performed as described previously (17). Briefly, tissue sections were processed with 1 $\mu\text{g}/\text{mL}$ proteinase K (0.1 M Tris buffer, pH 8.0, 50 mM EDTA, 10 min) at 37°C and 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were then incubated in the hybridization buffer (60% formamide, 10% dextran sulphate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.6 M NaCl, 0.2% N-laurylsarcosine, 500 mg/mL transfer RNA, 1 $\times$ Denhardt's solution and 0.25% sodium dodecyl sulphate) containing the ^{33}P -UTP-labeled antisense cRNA probes for 16 h at 60°C . After hybridization, these sections were rinsed in $2 \times \text{SSC}/50\%$ formamide for 45 min at 60°C , and rinsed in $2 \times \text{SSC}/50\%$ formamide for 15 min at 60°C and the sections were treated with a solution containing 10 $\mu\text{g}/\text{mL}$ RNase A (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 M NaCl) for 30 min at 37°C . The sections were further rinsed in $2 \times \text{SSC}/50\%$ formamide for 15 min at 60°C and in $0.4 \times \text{SSC}$ for 30 min at 60°C . Sections for free floating *in situ* hybridization were mounted onto gelatin-coated microscope slides, air-dried, and dehydrated. These sections together with ^{14}C -acrylic standards (Amersham, Buckinghamshire, U.K.) were exposed to Imaging plate in BAS5000 system (Fuji Film, Tokyo, Japan), and then to Biomax film (Kodak, Rochester, NY) for 2 days and subjected to the image analysis process. After this imaging process,

sections were counterstained with 0.1% Cresyl Echt Violet and 0.1% Hematoxyllin.

Microdissection of tubules and real time PCR. Mice were decapitated, and the kidneys were immediately taken and frozen in crushed dry ice, and stored at -80°C . Frozen tissue sections were cut at $5\text{ }\mu\text{m}$ in thickness by a cryostat and placed on slides. The specimens were fixed in acetic acid : ethanol (1 : 19), and stained with 0.1% Cresyl Echt Violet. Then, each glomerular and tubular regions were dissected out by a laser capture microdissection instrument (LMD6000; Leica, Wetzlar, Germany). RNA was extracted and purified using TRIzol reagent (Invitrogen Japan) and RNeasy Micro Kit (QIAGEN, Valencia, CA).

For real-time PCR, cDNA synthesis was performed with SuperScript III First-Strand Synthesis SuperMix (Invitrogen Japan, Tokyo, Japan). Real-time PCR was conducted with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen Japan) and analyzed on a StepOnePlus unit (Applied Biosystems, Foster City, CA). The amounts obtained by real-time PCR were normalized against the amounts of 36b4 (encodes acidic ribosomal phosphoprotein P0) mRNA. The 24-hour mRNA rhythms were statistically tested by one-way ANOVA, followed by Scheffe's multiple comparisons.

Immunohistochemistry. Mice were deeply anesthetized with ether and perfused through the left ventricle with 20 mL of phosphate-buffered saline followed by 50 mL of 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4). The kidneys were fixed in the same fixative and embedded in paraffin according to standard procedures. Paraffin sections were cut to a thickness $5\text{ }\mu\text{m}$, mounted on 3-aminopropyltrimethoxysilane (APS)-coated glass slides, deparaffinized in xylene, hydrated in a series of alcohol, and placed in phosphate buffered saline (PBS) plus 0.25% Triton X-100 for 25 min at room temperature. The specimens were then microwaved to enhance their antigenic reactivity and incubated with rabbit anti-Synaptopodin antibody (1 : 400 in dilution; Santa Cruz Biotech., Santa Cruz, CA) and visualized using FITC conjugated anti-rabbit IgG antibody (1 : 1000 in dilution). To visualize DNA, 100 ng/mL 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) was used. The fluorescent images were obtained with a laser scanning confocal microscope (Carl-Zeiss, Oberkochen, Germany).

RESULTS

Expression of NHE3 in the kidney by in situ hybridization

First, we examined the expression of NHE3 in the mice kidney by *in situ* hybridization. NHE3 mRNA was expressed in many regions of the kidney, but the expression level was strikingly different (Fig. 1). NHE3 mRNA signals were very high in the inner stripe of outer medulla (ISOM), particularly in the bordering area adjacent to outer stripe of outer medulla (OSOM). Weak to moderate level of signals were found in the cortex, but the density was not even, suggesting differential expression among nephron segments. OSOM mainly consisted of straight portion of proximal tubules and straight portion of distal tubules, showing very low level of expression. Mice ISOM is featured by the bundle of blood vessels with thin descending limbs of Henle's loop

Fig. 1 Distribution of NHE3 mRNA in the kidney. (A) Image of BAS5000 Imaging plate detected by *in situ* hybridization. (B) Original section counterstained with Cresyl Echt Violet (left) and the corresponding X-ray film image (right). This figure corresponds to the squared area of (A). Note the interbundle regions (asterisks) show the high levels of signals in the inner strips of outer medulla (ISOM) adjacent to the outer stripe of outer medulla (OSOM). Cx, renal cortex; IM, inner medulla. Bars = 1 mm.

(HL) of cortical nephron and the interbundle region rich in thick ascending limbs of Henle's loop (TAL) (9). The highest levels of signals were found in the interbundle region (Fig. 1). The inner medulla (IM) showed only background level of expression. Although above data were obtained in kidneys at CT12, the distribution characteristics were virtually the same in samples at other time points (data not shown). Thus, these findings demonstrate that the majority of NHE3 mRNA in the kidney is expressed in ISOM.

Real time PCR combined with laser capture microdissection technique in mice kidney

Next we sought to define which nephron segments showed NHE3 mRNA, and to characterize the NHE3 circadian expression profile in ISOM. ISOM is composed of thin descending limbs of Henle's loop (TL), TAL, and collecting duct (CD). Since quantitative *in situ* hybridization using radioisotope probe does not allow discrimination between these segments, we performed real-time PCR coupled with laser capture microdissection technique (LCM).

To assess the anatomical precision of this method, we first applied LCM and real-time PCR to well characterized markers. Synaptopodin (Synpo) is an actin-associated protein that may play a role in actin-based cell shape and motility, and is specific to epithelial cells in the renal glomerulus, as shown in Fig. 2A. We applied LCM to frozen section, lightly fixed with acetic acid and ethanol, then counterstained with Cresyl Echt Violet (Fig. 2B). Nephron segments can be discriminated in this section: glomeruli are identified by the accumulation of cells with chromatin-dense nuclei, proximal tubules are lined with cuboidal cells with blurred cell boundary, and distal tubules are lined with smaller cells with clearer cytoplasm. By applying LCM to this section, we demonstrated that Synpo mRNA is only expressed in the glomerulus, while showing 10% of relative expression in other segments (Fig. 2C). This finding demonstrates that real-time PCR combined with LCM can be safely applied to the quantification of mRNA level in a nephron segment specific manner.

In the present study, LCM technique was used to study the ISOM in the renal medulla. For confirming whether we can excise the appropriate segment, we used marker proteins specific to TL, TAL and CD. We choose Aqp1 (6), Thp (26), and Aqp2 (6) as markers of TL, TAL and CD, respectively (Fig. 2D, E, F). After confirming the specific expression of these markers, we examined the circadian expression profiles of NHE3 in the renal medulla.

Fig. 2 Application of the laser capture microdissection (LCM) technique to mice kidney. (A) Immunohistochemistry of Synaptopodin protein (SYNPO) and DAPI nuclear staining in the glomerulus. (B) A frozen section stained with Cresyl Echt Violet in the renal cortex. Note characteristic profiles of glomerulus (G), proximal tubule (PT) and distal tubule (DT) in this staining. LCM is applied to this section. Bar = 50 μ m. (C) Real-time PCR of Synpo mRNA in samples of nephron segments dissected by LCM. Note the positive signals only confined to the glomerulus with the high selectivity. (D, E, F) Real-time PCR of Aqp1 (D), Thp (E) and Aqp2 (F), which are the markers in thin descending limbs of Henle's loop (TL), thick ascending limbs of Henle's loop (TAL), and collecting duct (CD), respectively. RRA, relative RNA abundance.

Circadian expression of NHE3 in the renal medulla of mice kidney

Circadian change of the expression of NHE3 mRNA in the TAL, TL and CD located in the inner stripe of outer medulla (ISOM) in constant darkness was measured by real-time PCR coupled with LCM. The abundance of NHE3 mRNA showed a circadian rhythm (ANOVA; F [5,17] = 11.21, P = 0.0005) in the TAL with a peak at CT12, and the trough at CT4 (Fig. 3). In contrast, there were virtually no expressions of this gene in the CD. In the TL, daily expression of NHE3 mRNA showed circadian

Fig. 3 The expression profile of NHE3 mRNA (black-filled circles) in the thick ascending limbs of Henle's loop (TAL) by real-time PCR. For reference, the expression profile of NHE3 mRNA in collecting duct is shown (open circles). Mice were sampled in constant dark conditions. The relative RNA abundance was determined with the peak of NHE3 mRNA value in TAL adjusted to 100%. Values are expressed as means $\pm$ SEM ($n = 3$).

Fig. 4 The expression profile of NHE3 mRNA (black-filled circles) in the thin descending limbs of Henle's loop (TL) by real-time PCR. NHE3 mRNA in collecting duct is shown as open circles. Mice were sampled in constant dark conditions. The relative RNA abundance was determined with the peak of NHE3 mRNA value in TL adjusted to 100%. Values are expressed as means $\pm$ SEM ($n = 3$).

rhythm with a peak at CT12 (ANOVA; $F [5,17] = 4.69$, $P = 0.010$) (Fig. 4). These findings demonstrate that TAL and TL, expressed NHE3 mRNA abundantly, both show a circadian rhythm of NHE3 mRNA with the similar peak-trough profiles.

DISCUSSION

In the present study, we characterized NHE3 expression profiles in the mice kidney by the *in situ* hybridization, and further quantified its circadian rhythm by the real-time PCR combined with laser microdissection technique (LCM). NHE3 mRNA was strongly expressed in the inner stripe of the outer medulla (ISOM) of the kidney. Real-time PCR data in each nephron segment in the medulla region demonstrated clear circadian oscillations of NHE3 gene expression in TAL and TL. The circadian change of this gene in TAL of renal medulla may partially contribute to the circadian change of urinary electrolyte secretion.

Previously, we provided several lines of molecular evidence showing that NHE3 is a clock controlled gene directly regulated by the circadian oscillator in the kidney (22). First, NHE3 transcripts exhibited an apparent circadian oscillation in the kidney. Second, the rhythmic expression of NHE3 mRNA was blunted in the $Cry1^{-/-}Cry2^{-/-}$ mice lacking circadian oscillator. Third, luciferase-based transcription assays demonstrated that the NHE3 gene was transactivated by CLOCK:BMAL1 heterodimers and repressed by PER2 and CRY1 proteins. Fourth, gel shift assay revealed that CLOCK:BMAL1 heterodimers transactivated NHE3 through direct binding to the E-box in the promoter region. Considering the fact that NHE3 is a membrane transporter, NHE3 should qualify as an output gene that can be directly linked to the kidney clockwork.

For the localization of NHE3 in the renal nephron, previous studies were done mostly by immunohistochemistry (2, 4). These experiments clearly demonstrated the intense staining of the apical membrane of proximal convoluted tubules, and in the apical membrane of TAL. Weak staining was observed in TL. NHE3 staining was absent in the remainder of the nephron. In the present experiment, the signal from the cortical structure was markedly lower than that in the medullary structure. Considering the discrepancy between the results obtained by immunocytochemistry and *in situ* hybridization, it is possible that the different post-transcriptional regulation of NHE3 expression in proximal convoluted tubules leads to increased protein levels compared to that in TAL and/or TL in medullary segment. The application of more quantitative methods to measure mRNAs and proteins abundance in these nephron segments will address this question.

LCM is based on the adherence of visually selected area to a thermoplastic membrane, which overlies

the dehydrated tissue section and is focally melted by firing of a low-energy infrared laser pulse. The melted membrane forms a composite with the selected tissue area, which can then be easily removed by simple lifting of the membrane. By this technique, good quality DNA, mRNA, and proteins can be extracted successfully from captured tissue fragments, down to the single cell level. Previously, a few studies successfully adopted LCM in combination with real-time PCR for analyzing levels of mRNA in each nephron segment (7, 23). In this paper, we used marker genes to discriminate each nephron segments, from which NHE3 mRNA was measured. The application of markers improves the reliability of LCM, and should be more widely used for analyzing gene expression in nephron segments.

In many organisms, most physiological events including urinary secretion display daily oscillations, generated by an internal self-sustained molecular oscillator referred to as the biological clock (12). It is now known that this rhythm is generated at the gene expression level, referred to as the molecular oscillator, composed of interacting positive and negative transcription/translation feedback loops of clock genes, including *Clock* and *Bmal1* as positive factors, and *Per1/Per2*, *Cry1/Cry2* as negative factors (20). This oscillatory system localizes not only in the master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus, but also in most cells of the body (15, 28). Thus, it is speculated that the mammalian circadian system is composed of multioscillatory systems, governed by the SCN (14, 20). Kidney is not exceptional, since clear circadian clock gene oscillations were observed in rodent kidney (22).

Renal Na^+ excretion are known to display a significant diurnal variation in both animals and humans (13, 21), believed to be derived from the diurnal change of the renal blood flow. In contrast, daily changes in renal cellular functions have not been investigated yet, and particularly, the underlying molecular mechanisms remain unclear. Although several circadian molecular databases were recently assembled for the brain, blood vessels and liver (5, 18), circadian kidney molecular data are astonishingly rare (8). One reason is that the kidney is composed of complex mixture of highly differentiated, specialized cells and structures. The use of LCM to discriminate between segments solves in part of this problem, here successfully applied for the segment-specific measurements of circadian expression profiles of NHE3. The present data strongly suggest that the clear circadian changes of NHE3 expression

in the TAL and TL contribute, at least in part, to the daily change of urinary concentration of Na^+ and urinary volume (19, 21).

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