The underlying mechanism of the circadian expression of concentrative nucleoside transporter 2 in the intestinal epithelial cells

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The underlying mechanism of the circadian expression of concentrative nucleoside transporter 2 in the intestinal epithelial cells

(マウス消化管における核酸輸送トランスポーターCNT2 の概日リズム制御機構)

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[Background and objective]

Many biological processes are subject to daily oscillations, and some of these are generated by a self-sustained oscillation mechanism called “circadian clock”. Molecular studies of the circadian clock system have revealed that oscillations in the expression of specific clock genes and their products play central role in the generation of 24-hour rhythms in physiology and behavior. In mammals, the master circadian pace maker is located in the hypothalamic suprachiasmatic nucleus (SCN). The circadian oscillation in the SCN are entrained to a 24-hour period by daily light input from visual neural system and produce output signals for coordinating the phase of independent oscillator in peripheral tissues. The peripheral oscillators generate daily rhythms in output physiology through the periodic activation/repression of clock-controlled output genes.

Concentrative nucleoside transporter 1 (CNT1) and concentrative nucleoside transporter 2 (CNT2), encoded by the *Slc28a1* and *Slc28a2* gene respectively, are mainly expressed in the apical membrane of intestinal epithelial cells. These transporters exhibit sodium dependent uptake of nucleosides, nucleobases and some nucleoside-derived drugs from the intestinal lumen into the epithelial cells. In mammals, nucleosides metabolism is timely orchestrated in the liver. Several data already showed that the activities of de novo and salvage pathway enzymes exhibit a circadian oscillation and are under the control of the circadian clock. Furthermore, there was a time dependent variation of nucleosides and free bases abundance. These rhythms are not only influenced by the circadian clock system, but are under the control of other factor such as food intake. Therefore, day and night difference in food intake produces the circadian changes in the uptake of nucleoside from the small intestine. However, understanding of the role for CNTs in the circadian changes in the intestinal uptake of nucleosides is still limited.

The objective of the study is to explore whether CNTs are involved in the circadian regulation of intestinal uptake of nucleosides in mice. To achieve this, the underlying mechanism of the circadian expression of CNTs was investigated by focusing on its plasmalemmal localization process. The scaffold protein PDZK1 played an important role in the circadian changes in the plasmalemmal localization of CNT2 in the intestinal epithelial cells. Therefore, the influence of food intake on the expression of the scaffold protein and membrane expression of CNT2 were also investigated by manipulation of feeding schedule.

[Materials and methods]

Animals and treatment: Male *Clock* mutant mice (Clk/Clk) with an ICR background and wild type mice of the same strain were housed in a controlled room under a standardized light-dark cycle condition (lights on at a Zeitgeber time (ZT) 0 and lights off at ZT12) at a temperature of 24 ± 1 ºC and a humidity of 60 ± 5% with food and water available *ad libitum* during the 2 weeks of adaptation before the start of the experiment. All experimental procedures were performed under the approval and guidelines of Kyushu University.

Manipulation of feeding schedule: After the 2 week of adaptation, mice were divided into 2 groups: day time feeding group and night time feeding group, this feeding regimen lasted for 7 days. In brief fasting experiment, food was removed 24-hours then was reintroduced. In both manipulation of feeding schedule experiments, mice had freely access to water during all the experiments.

Cell culture: aMos7 cells (mouse small intestine epithelial cell lines) were firstly thawed with Dulbecco’s Modified Eagle Medium supplemented with 20% of fetal bovine serum (FBS) and 10 μg/ml of human-recombinant insulin then maintained in DMEM supplemented with 10% of FBS. Cells were kept at 37ºC under a humidified 5% CO2 atmosphere. Stable aMos7 cells expressing PDZK1 were also prepared by antibiotic selection.

Luciferase reporter assay: NIH3T3 cells were transfected with luciferase reporter vector under the control

of the mouse *Pdzk1* gene promoter. Cells were also co-transfected with clock genes expression vectors. Cells were harvested 24-hours after transfection and lysates were analyzed using the Dual-Luciferase reporter assay system.

Co-immunoprecipitation analysis: Transient aMos7 expressing Flag-tagged Pdzk1 were immunoprecipitated following the manufacturer's instructions. Immune precipitated protein were detected by Western blotting.

Western blotting: Membrane fraction were prepared from mice intestinal epithelial cells at 6 different time points. Fraction containing 20 or 40 μg of proteins were then resolved and probed with antibodies against CNT1, CNT2 and PDZK1. Specific antigen-antibody complexes were visualized using horseradish peroxidase conjugated secondary antibodies and chemiluminescence reagents. Negative gel staining was used as control

Assessment of nucleosides uptake into the small intestine: A segment of small intestine was sampled and filled with Krebs-Ringer buffer containing 50μM of [^{14}C]-adenosine. This segment was tied from both extremities and was incubated again in the Krebs-Ringer buffer at 37°C for 15 min. After washing and homogenization of the segment, radioactivity was assessed.

Quantitative RT-PCR and Real time PCR analysis: Total RNA was extracted from the intestinal epithelial cells of mice at 6 different time points. cDNA was synthesized and amplified by RT-PCR. A real-time PCR analysis was performed on diluted cDNA.

Statistical analysis: All values are expressed as mean with standard errors (S.E.) of the mean. The significance of the daily variation for each parameter was tested by analysis of variance (ANOVA). The statistical significance of differences between groups was analyzed by ANOVA followed by a Tukey-Kramer post hoc test or Dunnett's post hoc test. Values of $P<0.05$ were considered significant.

[Results]

Evaluation of the underlying mechanism of circadian expression of CNT2 in the intestinal epithelial cells of mice

Both nucleoside transporters CNT1 and CNT2 were detected in the cell lysate, but there were no circadian variation in the protein levels of these transporter. CNT1 and CNT2 are mainly expressed at the apical site of intestinal epithelial cells, so their protein levels in the plasm membrane fraction of the intestinal epithelial cells were also tested at in 6 time points. While CNT1 protein levels did not show significant circadian variation in the membrane fraction, CNT2 protein was expressed in a circadian time-dependent manner with a peak during the early dark phase ($P<0.05$, ANOVA). As supported by this finding, the uptake of adenosine, a typical substrate of CNT2, into intestinal epithelial cells also exhibited a significant time-dependent variation with higher uptake ability during the early dark phase.

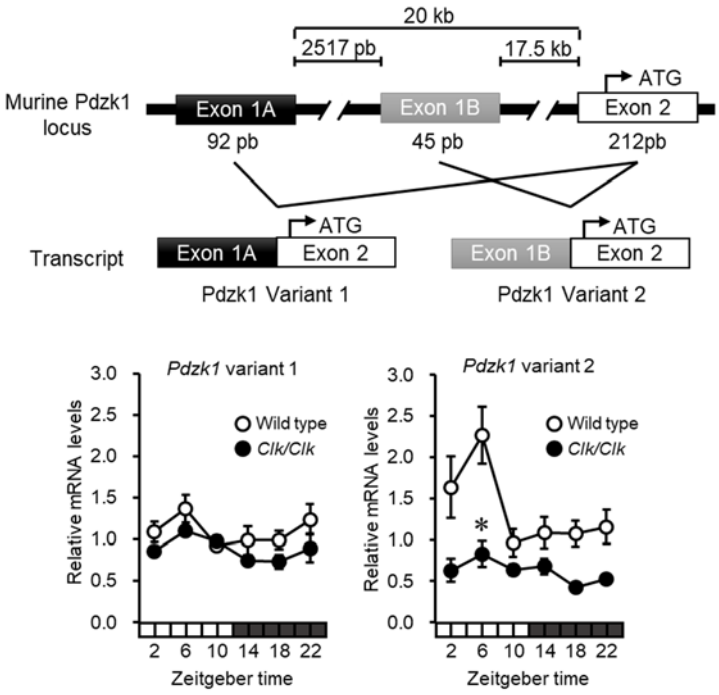

Figure 1. Circadian expression of *Pdzk1* transcript variant *2* in mouse small intestine. Upper panel shows genomic organization of the mouse *Pdzk1* gene. Lower panel show the temporal expression profiles of *Pdzk1* gene variants in the intestinal epithelial cells of wild type and *Clock* mutant (*Clk/Clk*) mice. The mRNA levels of *Pdzk1* variant 2 in wild type exhibit a significant circadian oscillation ($P<0.01$, ANOVA). *$P<0.05$ compared with the wild type group at the corresponding ZTs. Values show the means ± S.E. (N=3-6).

Since PDZK1 functions as a scaffold protein and is known to localize and support several membrane transporters in the intestine and in kidney. The protein levels of PDZK1 in the membrane fraction of intestinal epithelial cells also exhibited a circadian oscillation with a peak during dark phase and a trough during light phase. The rhythmic phase of PDZK1 protein expression was similar to those of CNT2. The results of immunoprecipitation experiment revealed that PDZK1 bound to CNT2 by

protein-protein interaction. Temporal accumulation of PDZK1 protein in the intestinal epithelial cells enhanced the membrane localization of CNT2 during certain times of the day. The enhancement of CNT2 membrane localization by PDZK1 also facilitated the uptake of adenosine from the intestinal lumen into the epithelial cells.

Comparison of the sequences tag of the mouse genomic DNA sequences revealed that the distances of the leader exons (termed as exon 1A and exon 1B) from the first coding exon (termed as exon 2) in genomic DNA was 20 kb for exon 1A and 17.5 kb for exon 1B (**Fig. 1**). Although the exon 1A-containing *Pdzk1* transcript (referred as variant 1) was expressed similarly in the liver, kidney and small intestine, exon 1B-conteining *Pdzk1* transcript (referred as variant 2) was highly expressed in the small intestine. In the small intestine in wild type mice the mRNA levels of *Pdzk1* transcript variant 2 but not of variant 1 showed a significant circadian oscillation with a peak during midlight phase ($P<0.01$, **Fig. 1**). The rhythmic oscillation in the *Pdzk1* transcript variant 2 was severely damped in *Clock* mutant (*Clk/Clk*) mice, suggesting that the intestinal expression of this transcript variant is under the control of molecular circadian clock.

The luciferase reporter constructs that contain the promoter region of the mouse *Pdzk1* variant 2 could be response to circadian orphan nuclear receptors, RORα and REV-ERBα. RORα enhanced the transcriptional activity of *Pdzk1* variant 2, but the transactivation effect was repressed by REV-ERBα. The positive and negative regulation by those orphan nuclear receptors ultimately generated the time-dependent changes in PDZK1 expression. These results suggest a novel molecular mechanism of circadian expression of cell surface transporters. The time-dependent accumulation of PDZK1 appeared to stabilize the plasmalemmal localization of CNT2 by constituting the fundamental building blocks of membrane protein complexes.

The influence of the feeding schedule on the plasmalemmal expression of CNT2 in the mouse small intestine

The mammalian circadian system is hierarchically organized by central and peripheral oscillators. An ensemble of coupled oscillators in the suprachiasmatic nucleus (SCN) of the hypothalamus is entrained to a 24-hour period by daily light input from the visual system. Neural and humoral output signals from the SCN coordinate the phase of independent circadian oscillators in peripheral tissues throughout the organism. The lighting cycle is the most powerful “Zeitgeber” for the SCN oscillators, but the act of feeding generates dominant cues for biological rhythms in the peripheral tissues so that under certain conditions, they can override the entraining signals coming from the SCN.

To investigate the influence of feeding regimen on the rhythmicity in the expression of clock genes and *Pdzk1*, mice were fed with their food during the dark phase or the light phase. The rhythmic phase in the expression of clock gens in the night-time restricted feeding group were similar to those observed under ad libitum feeding condition. By contrast, the rhythmic phase of clock gene expression was completely reversed by day-time restricted feeding. Similar food-entrainable effects were also observed in the mRNA expression of *Pdzk1* variant 2. The mRNA of *Pdzk1* variant 1 did not show a significant circadian variation under ad libitum feeding condition. However, the mRNA levels of this transcript variant exhibited a significant circadian variation under both night time- and day time-restricted feeding conditions. These results suggest that the mRNA expression of *Pdzk1* variant 1 is under the control of food-entrainable factors which are independent from molecular circadian clock.

Under 24-hour fasting condition, the rhythmic phase of mRNA expression of clock genes were comparable to those observed in mice under ad libitum feeding condition. However, the mRNA levels of *Pdzk1* variant 1 and variant 2 did not show significant circadian oscillation. Similar disruptive effect of fasting was also observed in the rhythms of the plasmalemmal expression of PDZK1 and CNT2 in intestinal epithelial cells. These results suggest a direct effect of feeding on the rhythmic expression of CNT2 as well as its scaffold protein PDZK1.

[Discussion]

In general it has been thought that the circadian changes in the amount of cell surface protein in intestinal epithelial cells are regulated at nearly any stage of the gene expression process as well as their degradation. In

addition to these processes, a recent study from our laboratory has revealed that subcellular localization plays an essential role for the circadian expression of transporters of plasma membrane of the mouse hepatic cells[1]. The present finding showed that PDZK1-mediated plasmalemmal localization process governed the circadian expression of CNT2 in the plasma membrane of intestinal epithelial cell. The rhythmic expression of PDZK1 was associated with the time-dependent transcription of *Pdzk1* variant 2, but not variant 1. Indeed the results of luciferase reporter assay revealed that the transcription of *Pdzk1* transcript variant 2 was regulated by retinoic orphan nuclear receptors RORα and REV-ERBα. Since the mRNA expression of *Rorα* ad *Rev-erbα* exhibited circadian oscillation in the intestinal epithelial cells, these orphan nuclear receptors consisted of a reciprocating mechanism in which RORα activated the transcription of the Pdzk1 gene, whereas REV-ERBα periodically suppressed transcription at the time of day when REV-ERBα was abundant (**Fig. 2**).

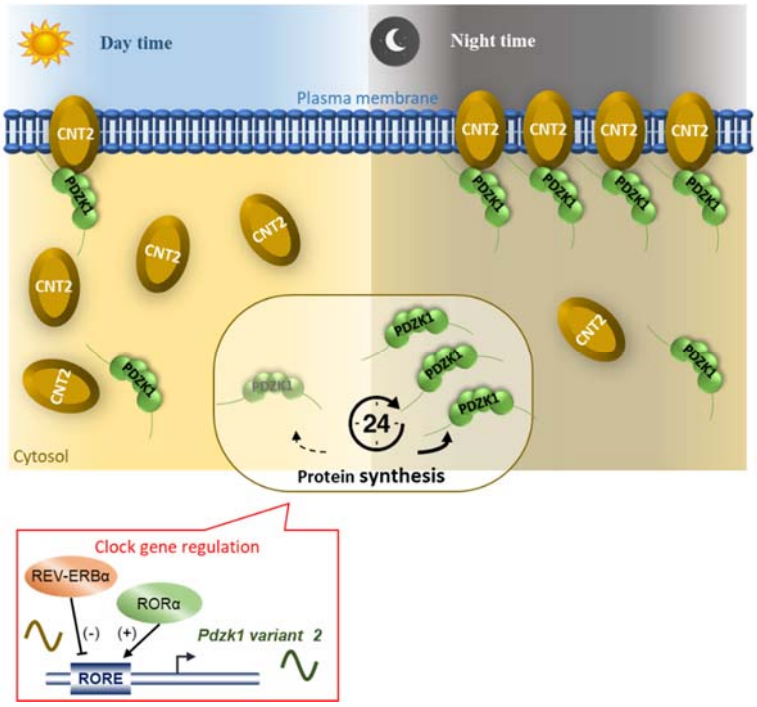

Figure 2 Schematic diagram indicating the PDZK1-regulated circadian localization of CNT2 in the plasma membrane of mouse intestinal epithelial cells. The expression of *Pdzk1* mRNA is under the control of molecular circadian clock. RORα activates the transcription of *Pdzk1* while the transactivation was periodically repressed by REV-ERBα. The positive and negative regulation of Pdzk1 transcription by RORα and REV-ERBα causes circadian changes in PDZK1 protein abundance. Temporal accumulation of PDZK1 in the intestinal epithelial cells enhances the membrane localization of CNT2 through direct interaction thereby facilitating the uptake of nucleosides and their analogs.

The manipulation of feeding schedule modulates the PDZK1-mediated localization of CNT2 in the plasma membrane by affecting the expression not only of *Pdzk1* variant 2 but also variant 1 transcript. Modulation of feeding schedule also affected the rhythmic phase of the clock genes in the intestinal epithelial cells. The rhythmicity in the expression of *Rorα* and *Rev-erbα* was also completely reversed by day time-restricted feeding. Changes in the expression of the orphan nuclear receptors seemed to cause the phase-shift of the rhythm in the expression of *Pdzk1* transcript variant 2. As compared with ad libitum feeding condition, the time-restricted feeding enhanced the contrast between fasting and feeding states. Such contrast appeared to generate a significant circadian oscillation in the mRNA levels of *Pdzk1* transcript variant 1. PPARα activates various genes expression during fasting condition[2,3] and positively regulates human *Pdzk1* gene. Since there are DNA sequences showing homology with PPREs in the up-stream region of *Pdzk1* variant 1. PPARα may be responsible for the food-entrainable rhythm of this variant. Further investigation is required for clarifying this point.

[References]

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