Insight Into the Circadian Clock Within Rat Colonic Epithelial Cells

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Background & Aims: **The gastrointestinal tract exhibits diurnal rhythms in many physiologic functions. These rhythms are driven by food intake but are also preserved during food deprivation, suggesting the presence of endogenous circadian rhythmicity. The aim of the study was to provide insight into the circadian core clock mechanism within the rat colon. Moreover, the potency of a restricted feeding regime to shift the circadian clock in the colon was tested. The question of whether the colonic clock drives circadian expression in *NHE3*, an electroneutral Na^+/H^+ exchanger, was also addressed.** ***Methods:*** **Daily profiles in expression of clock genes *Per1*, *Per2*, *Cry1*, *Bmal1*, *Clock*, and *Rev-erbα*, and the *NHE3* transporter were examined by reverse transcriptase–polymerase chain reaction and their mRNA levels, as well as PER1 and BMAL1 protein levels, were localized in the colonic epithelium by in situ hybridization and immunocytochemistry, respectively.** ***Results:*** **Expression of *Per1*, *Per2*, *Cry1*, *Bmal1*, *Clock*, *Rev-erbα*, and *NHE3*, as well as PER1 and BMAL1 protein levels, exhibited circadian rhythmicity in the colon. The rhythms were in phase with those in the liver but phase-delayed relative to the master clock in the suprachiasmatic nucleus. Restricted feeding entrained the clock in the colon, because rhythms in clock genes as well as in *NHE3* expression were phase-advanced similarly to the clock in the liver.** ***Conclusions:*** **The rat colon harbors a circadian clock. The colonic clock is likely to drive rhythmic *NHE3* expression. Restricted feeding resets the colonic clock similarly to the clock in the liver.**

Diurnal rhythms in physiologic functions are observed in various organs and peripheral tissues, including the gastrointestinal tract (GIT).[1] The GIT exhibits daily rhythms in gut motility,[2] mucosal enzyme activities,[3] mucosal transporters,[4–7] and proliferation rates.[8,9] Although food intake can reset these rhythms, they may persist for several days independent of feeding.[3,10–13] Food deprivation for 2 days inhibits the diurnal rhythm of Na^+/glucose cotransporter (SGLT1) protein production but does not affect rhythmic SGLT1 mRNA expression.[6,14] Diurnal transcription of SGLT1 is thus mediated by factors other than food intake. These findings suggest that, potentially, 2 separate pathways might represent cues for diurnal variation in GIT rhythms: one pathway using cyclically available gut luminal signals such as nutrients, the other being the daily anticipatory mechanism that prepares the intestine for the expected variation in such signals before their exposure to luminal content.[5] Moreover, it is possible that the cyclical availability of luminal signals may serve as entraining cues for the daily anticipatory mechanism.

Circadian rhythms are generated by a self-sustained endogenous circadian clock that is located in the suprachiasmatic nuclei (SCN) of the hypothalamus.[15] The clock is directly reset by external time cues, mostly by daily alternations in the light–dark regime. This mechanism allows organisms to anticipate predictable daily changes in their external environment. Within the organism, the circadian clock times and synchronizes multiple metabolic processes so that they occur at appropriate times of day. Recent studies have shown that the circadian clock is present not only in the central nervous system but also in numerous peripheral organs, such as the liver, lungs, kidney, heart,[16–20] and oral mucosa.[21] Individual cells, in such peripheral tissues, are able to generate self-sustained circadian oscillations even in the absence of the master clock in the SCN.[22] Through humoral and neuronal output, rhythmic signals from the SCN inform the peripheral clocks about time of day and adjust their phase accordingly.[23]

The molecular core clock mechanism of the central and peripheral clocks is based on transcriptional-translational feedback loops that involve E-box–mediated transcriptional activation of a set of clock genes, namely of *Per1, Per2, Cry1*, *Cry2, Rev-erbα*, and *Rora*, by a het-

Abbreviations used in this paper: ANOVA, analysis of variance; FR, feeding regime; GIT, gastrointestinal tract; ISH, in situ hybridization; NHE3, Na^+/H^+ exchanger; OD, optical density; RT-PCR, reverse transcriptase–polymerase chain reaction; SCN, suprachiasmatic nuclei; SEM, standard error of the mean; SGLT1, Na^+/glucose cotransporter.

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erodimer composed of 2 clock gene protein products: CLOCK and BMAL1. After translation, repressor proteins PER1, PER2, CRY1, and CRY2 undergo post-translation modification, form heterodimers, and, after shuttling into the nucleus, repress the CLOCK-BMAL1–dependent transcription of their own genes. Proteins REV-ERBα and RORA repress or activate transcription of the *Bmal1* gene, respectively. These interdependent feedback loops are believed to generate the approximately 24-hour period of the molecular oscillator.[24] Although the critical components of the core clock mechanism are conserved between the SCN and peripheral tissues, their relative importance may vary. A relatively small number of first-order clock-controlled genes are directly regulated by CLOCK-BMAL1 heterodimers that bind to CACGTG E-box enhancers in their promoters.[25,26] Transcription of these clock-controlled genes is thus under circadian control and subsequently drives rhythmic transcription of subordinate genes serving as a critical mediator of circadian control over diverse physiologic events in different peripheral tissues. Circadian control over extensive and divergent portions of the transcriptome[27,28] and proteome[29] has been shown.

The phase of rhythmic clock gene expression in peripheral clocks may be delayed by 3–9 hours, compared with their expression in the SCN, depending on the tissue.[17] In addition to SCN signals, peripheral clocks may use feeding regime (FR) as an important timing cue that can, under certain circumstances, uncouple the peripheral clock from the SCN signals. Spontaneous feeding of nocturnal animals correlates well with the SCN-driven locomotor activity. However, when access to food is limited only to the light part of a light–dark cycle (ie, during the rest period of nocturnal rodents), clock gene expression in the liver, pancreas, heart, and other tissues becomes phase-advanced relative to that in animals fed ad libitum.[18,19] The rate of this advance is tissue specific (eg, lung responds slower than the liver). The reversed FR, however, does not affect the phase of clock gene expression within the SCN.[18,19,30] These studies suggest that circadian rhythms in the GIT, although usually entrained by the SCN master clock,[31,32] may be sufficient to maintain circadian time-keeping within individual peripheral clocks, through the same transcriptional-translational feedback mechanisms. This is supported by the recent identification of clock gene expression in the GIT.[33–36]

The current study was undertaken to obtain an insight into the rat intestinal peripheral clock. The main goal was to identify molecular core clock machinery and to localize the oscillatory cells within the rat colon. Daily profiles in expression of selected clock genes, namely of *Per1*, *Per2*, *Cry1*, *Bmal1*, *Clock*, and *Rev-erb*α, were determined by reverse transcriptase–polymerase chain reaction (RT-PCR), and their expression was localized within intestinal sections by in situ hybridization (ISH). Moreover, the levels of clock gene products PER1 and BMAL1 were detected within the intestinal sections by immunocytochemistry. The phases of oscillations in clock gene expression in the colon were compared with those in a well-characterized peripheral clock, the rat liver, and also with those in the SCN master clock. Moreover, the daily expression profile of the colonic electroneutral Na^+/H^+ exchanger (NHE3) was studied. NHE3 was chosen because it represents one of the major transporters in the colonic epithelium and because its promoter contains the E-box sequence,[37] through which CLOCK-BMAL1 heterodimers may control circadian expression of the *NHE3* gene. To elucidate whether the food intake may entrain the intestinal clock independently of the SCN, the profiles of several core clock genes and *NHE3* expression were studied in the rat colon under conditions of a reversed, restricted FR.

Materials and Methods

Experimental Animals

Two-month-old male Wistar rats (Bio Test, Konarovice, Czech Republic) were maintained for at least 4 weeks in a temperature of 23°C ± 2°C under light–dark cycle with 12 hours of light and 12 hours of darkness per day. Light was provided by overhead 40-W fluorescent tubes, and illumination was between 50 and 300 lux, depending on cage position in the animal room. Animals had free access to food and water. On the day of the experiment, animals were divided into 2 groups. The control group was fed ad libitum as previously, the light was not turned on at the time of usual dark-to-light transition, designated as circadian time 0 (CT0), and the animals were released into constant darkness. Starting from the CT0 (or from CT4), 3 animals per each time point were sampled every 4 (occasionally every 2) hours throughout the whole circadian cycle. The experimental group was subjected to a restricted FR, and access to food was permitted only for 6 hours during the light period (ie, between CT3 and CT9). Access to drinking water was not limited. The food restriction began with the removal of food pellets at CT9 and continued for the next 14 days. Thereafter, the animals were released into constant darkness and sampled as described in the control group. Food was not provided on the day of sampling. Rats of both groups were killed after deep anesthesia (thiopental 50 mg/kg intraperitoneally) by decapitation. All experiments were conducted under license no. A5228-01 with the US National Institutes of Health and in accordance with Animal Protection Law of the Czech Republic (license no. 42084/2003-1020).

Tissue Sampling

For in situ hybridization, the brains and transversal sections of the distal colon were removed, colons were rinsed in phosphate buffer, and both tissues were immediately frozen on dry ice and stored at −80°C. For RNA