Circadian regulation of uroguanylin and guanylin in the rat intestine

LAWRENCE A. SCHEVING AND WEN-HUI JIN
Division of Gastroenterology and Nutrition, Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Scheving, Lawrence A., and Wen-Hui Jin. Circadian regulation of uroguanylin and guanylin in the rat intestine. Am. J. Physiol. 277 (Cell Physiol. 46): C1177–C1183, 1999.—Uroguanylin (UGN) and guanylin (GN) are the endogenous intestinal ligands for guanylyl cyclase C (GC-C). We examined the circadian expression of UGN, GN, and GC-C in the jejunum, ileum, and proximal colon of young adult rats by Northern assays. These assays revealed that UGN is more abundant in the proximal small intestine, whereas GN and GC-C are more abundant in the proximal colon. mRNA levels showed significant circadian variation for UGN (3- to 18-fold peak/trough difference), GN (2.1- to 2.8-fold peak/trough difference), and GC-C (3- to 5-fold peak/trough difference). The maximal abundance occurred in the dark period for all three mRNAs, although peak UGN and GN expression occurred later in the dark period in the jejunum relative to the ileum and colon. Immunoblot analyses using monospecific polyclonal antibodies against UGN and GN prohormones confirmed the regional and circadian variation detected by Northern assays. Thus the expression of these genes is regulated not only by histological position but also by circadian time.

diurnal; guanylyl cyclase C; ileum; jejunum; colon

THE HEAT-STABLE ENTEROTOXIN (STa) is a small peptide, produced by some strains of enterotoxigenic bacteria, which binds and activates a receptor guanylyl cyclase, known as guanylyl cyclase C (GC-C) (22, 27). GC-C activation by STa during bacterial infection increases the intracellular levels of cGMP in enterocytes, causing a secretory diarrhea (7, 8). GC-C is normally regulated by the endogenous peptide ligands, uroguanylin (UGN) and guanylin (GN) (4, 10). cDNAs have been obtained for each of these peptides, which were originally isolated and sequenced from intestinal mucosa and urine.

Very little is known about the physiological regulation of UGN, GN, and GC-C. Colonic GN is downregulated in rats fed a low-salt diet, suggesting that GC-C activation by this peptide may regulate net dietary salt absorption (15). Zinc-deficient rats upregulate UGN mRNA, suggesting that the hypersecretory intestinal state associated with this condition may be related to inappropriate GC-C activation by UGN (1). Interestingly, intravenously administered UGN causes a diuresis, natriuresis, and kaliuresis, such that UGN may play a role in the neuroendocrine mechanism by which diet modulates renal function (9). Although UGN and GN are primarily expressed in the intestine, differences in their extraintestinal and intestinal patterns of expression have been reported (6, 19, 29). Whereas UGN is primarily expressed in differentiated enterocytes of mouse small intestinal villi, GN mRNA is elevated in intestinal and colonic crypts, as well as in differentiated villus enterocytes or surface colonocytes (5, 6, 17, 19, 20, 29). GC-C mRNA is expressed throughout the small and large intestines but appears to be more abundant in the colon, even though the binding of radiolabeled STa is higher in the small intestine (14).

In this paper, we examined the regional and circadian regulation of UGN, GN, and GC-C. GC-C may play a role in the hydration of mucus (17). Because intestinal secretory activity and mucus formation in the rat increase at night (18), coincident with nocturnal feeding, we hypothesized that the expression of GC-C and its regulatory ligands would increase at this time (26). To examine this hypothesis, we analyzed their mRNA and protein expression at various intestinal and colonic sites of animals at different circadian phases. We asked the general question whether time of day influenced their mRNA levels. We also raised monospecific antibodies against the UGN and GN precursor proteins to examine the regional and circadian expression by immunoblot.

MATERIALS AND METHODS

Animals. Animals were housed in wire-bottom cages in an American Association for Accreditation of Laboratory Animal Care accredited animal facility under approved research protocols. The room was kept at 22°C with a 12-h light period (0600–1800 h lights on) alternating with a 12-h dark period (1800–0600 h lights off). Six-week-old male Sprague-Dawley rats (Harlan, Indianapolis, IN) were adapted to the animal facility for 2 wk before the study. The animals were allowed food and water ad libitum. They were fed a standard pelleted laboratory chow (AIN-93G; Dyets, Bethlehem, PA).

Nucleic acid probes. UGN DNA sequence was a 455-bp RT-PCR product. The primer sequences were gcgcatgtcaggagcggtgt (forward) and ccaaatatgccgctgctac (reverse). RT-PCR was carried out with the Access RT-PCR system using rat jejunal RNA for first-strand synthesis (Promega, Madison, WI). The PCR amplification cycle was 1 cycle at 94°C for 3 min, 35 cycles at 94°C for 45 s, 63°C for 45 s, 72°C for 1 min, and, finally 1 cycle at 72°C for 10 min. The UGN fragment was gel purified and directly sequenced, confirming its identity to rat UGN. A GN fragment was generated by double digestion of the plasmid 553018 obtained from the mouse I.M.A.G.E. Consortium with restriction enzymes Srf I and Not I and was then gel purified before labeling. GC-C cDNA was provided by Drs. David L Garbers and Stephanie Schulz. A 5’ Sac I fragment of GC-C (nucleotides 1–868 correlating to the GC-C-specific extracellular domain) was prepared for Northern assay. The 185 ribosomal fragment was a 1.2-kb fragment of the mouse 18S RNA gene purchased from Ambion.
Northern blot analysis. The RNA from intestinal tissues was isolated by the use of the Tri Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer’s instructions (3). Intestinal pieces were quickly removed from a eutanized animal, flushed with ice-cold saline, and rapidly frozen in liquid nitrogen for later RNA extraction. The RNA was electrophoresed on a 1% formaldehyde denaturing agarose gel (20 µg/ lane), transferred to a positively charged nylon membrane (Ambion Northern Max Kit), and cross-linked to the membrane using ultraviolet light (Stratalinker; Stratagene). Prehybridization and hybridization were carried out at 42°C using buffers provided by the kit. After hybridization, the membranes were washed with low- and then high-stringency washing buffers as directed by the manufacturer’s instructions. The blots were then exposed to Kodak XAR-5 film at ~80°C with double intensifying screens for a minimum of 4 h for UGN and GN and 12 h for GC-C. The probes were stripped by exposing the filters to 0.1% SDS. Densitometry was performed using Gel-Pro software (Media Cybernetics, Silver Spring, MD). Several exposures were done initially to ensure linearity of signal.

Immunoblot analysis. We used a well-characterized affinity-purified antibody against the COOH-terminal peptide of GC-C (CNNSDHDSTYF) (22, 24). Antisera were also generated against peptide segments in the GN and UGN prohormones. For GN, the peptide VQDGDLSFPLESVKC (residues 40–56 of rat UGN linked at the NH2 terminus to the COOH-terminal cysteine residue) was coupled to keyhole limpet antigen (KLH) at the COOH-terminal cysteine residue. This peptide represents a 14-mer sequence that attached the coupling end of the 14-mer peptide that attached the peptide to either the KLH or the activated affinity matrix. For UGN, the peptide CNELEEKQMSDPQQQKSG (residues 38–44 linked to a COOH-terminal cysteine) was used. This 17-mer segment is unique to UGN and has no homology to any known protein, including GN. Rabbits were intradermally injected with these peptides in Freund’s complete adjuvant. They were reinjected with Freund’s incomplete adjuvant at 3- to 4-wk intervals. After the third boost, the antisera had high titers against the peptides, as determined by ELISA. The UGN antisera were not further purified.

For immunoblots, homogenate samples were prepared, and the proteins were resolved by 12% SDS-PAGE and blotted as previously described, except that the peptide was transferred to polyvinylidene difluoride as opposed to nitrocellulose (24, 25). We used a chemiluminescent detection strategy using GC-C and GN IgG at a concentration of 1.0 µg/ml and UGN antiserum at a 1:1,000 dilution. In preliminary experiments, parallel blots with antiserum preincubated with the antigenic peptide (0.5 mg/ml) for 1 h at room temperature were done to assess specificity. The bands shown in this paper were specific by this criteria and had appropriate molecular masses and regional distributions.

RESULTS

Regional distribution of UGN, GN, and GC-C mRNA. Figure 1 shows the regional expression of UGN, GN, and GC-C in the jejunum (second fifth of the small intestine), ileum (terminal fifth of the small intestine), and proximal colon (1 cm distal to the cecum). Each band displays a signal detected in RNA obtained from a single mouse (lane 1 of each panel contains RNA obtained from animal 1, lane 2 from animal 2, and so on). The animals in this experiment were all killed near the middle of the light phase at 1200 h. Single and appropriately sized mRNA species of ~0.75, 0.6, and 3.8 kb were seen for UGN, GN, and GC-C, respectively. No other bands were detected. To demonstrate equal RNA loading, the blot was also reprobed with an oligonucleotide that recognizes 18S RNA. Each message was detected at all intestinal sites, although this required a long autoradiographic exposure for UGN in the proximal colon (data not shown). Each mRNA had a tissue distribution similar to that recently reported for the rat (6) and mouse (29). UGN was strongly expressed in the jejunum and ileum but very weakly expressed in the proximal colon. In contrast, GN expression increased as one moved distally along the length of the intestine, peaking in the proximal colon. GC-C also appeared to be expressed at a higher level as one moved from the jejunum to the ileum to the proximal colon.

Regional distribution of UGN, GN, and GC-C protein. To further examine the regional variation of UGN, GN, and GC-C expression, we prepared homogenates from 12 different sites of the small and large intestines, with site 1 representing a sample obtained from a 1-cm region adjacent to the stomach and site 12 from a 1-cm segment adjacent to the rectum (Fig. 2). Again, the animals were killed near the middle of the light phase at 1200 h. The intervening sites represented samples obtained from the regions between these ends as identi-
We carried out three separate but parallel immunoblots with antibodies that recognized the midregion of the UGN precursor, the NH$_2$-terminal region of the GN precursor, and the COOH-terminal end of the GC-C protein. These immunoblots represent tissue obtained from a single representative animal. With the use of these antibodies, we observed for UGN and GN appropriately sized bands between 9 and 12 kDa that had a tissue distribution consistent with the Northern blot results. As previously described, immunoreactive GC-C on this 7% SDS-PAGE immunoblot resolved into three isoforms of 140, 130, and 85 kDa that represent a mature isoform, an immaturely glycosylated isoform, and a brush-border proteolytically cleaved isoform, respectively (22). About 25–30% of the total GC-C signal was concentrated in the 85-kDa isoform in small intestinal fractions 2–6 compared with 5–10% for fractions 1 and 7–10. This form was barely detected in fractions 11–12. The GC-C signal remained strong in the distal colon adjacent to the rectum, despite low GN and UGN expression at this site.

UGN, GC-C, and, to a lesser extent, GN mRNA display a circadian rhythm in mRNA. To determine whether the mRNA levels of either UGN, GN, or GC-C were regulated in a circadian manner, we harvested intestine, whereas GN was greatest in the terminal ileum, cecum, and proximal small intestine. A densitometric analysis revealed a >20-fold range in UGN or GN expression at the different sites.

GC-C was abundantly expressed throughout the small and large intestines, with the greatest signal intensity occurring in the proximal colon. About 2.5 times as much GC-C signal was detected in the cecum and proximal colon, compared with fractions 6 and 7 representing distal jejunum and proximal ileum, consistent with the Northern blot results. As previously described, immunoreactive GC-C on this 7% SDS-PAGE immunoblot resolved into three isoforms of 140, 130, and 85 kDa that represent a mature isoform, an immaturely glycosylated isoform, and a brush-border proteolytically cleaved isoform, respectively (22). About 25–30% of the total GC-C signal was concentrated in the 85-kDa isoform in small intestinal fractions 2–6 compared with 5–10% for fractions 1 and 7–10. This form was barely detected in fractions 11–12. The GC-C signal remained strong in the distal colon adjacent to the rectum, despite low GN and UGN expression at this site.

UGN, GC-C, and, to a lesser extent, GN mRNA display a circadian rhythm in mRNA. To determine whether the mRNA levels of either UGN, GN, or GC-C were regulated in a circadian manner, we harvested
tissues from rats at six different circadian periods and prepared Northern blots.

Figure 3 shows the circadian variation in the UGN and GN levels. Each panel contains the samples from two representative animals of a larger group (n = 4 or a total of 24 animals for the entire experiment). The P/T ratio represents the ratio between the means of the peak and trough groups and gives a general estimate of the variation over a 24-h period. Both UGN and GN exhibited a similar increased expression during the dark photoperiod when the animals were feeding; however, the UGN variation was greater and more consistent than that of the GN. For both transcripts, the maximal change occurred in the ileum compared with the jejunum or colon. For UGN, the greatest increase in the jejunum occurred in the latter half of the dark phase, when mRNA was ~5.5-fold greater than during the late light phase. In the ileum, the greatest increase in UGN occurred early in the dark phase when the mRNA level was ~18 times that which was observed in the light phase. For GN, the timing of peak expression was similar for both the jejunum and ileum, but the increase was no more than two to three times that observed in the light phase. GN expression in the colon showed even more interanimal variation. Although GN expression at this site was somewhat higher at night, it began to rise earlier in the light phase. Quantitation of the relative signal intensities for UGN and GN is shown in Fig. 4.

To further evaluate the circadian expression of the GC-C effector system, we evaluated the circadian expression of GC-C itself. This receptor guanylyl cyclase also exhibited significant circadian variation in mRNA abundance in the different tissues, with the peaks occurring throughout the dark phase and troughs occurring throughout the light phase. Figure 5 shows

**Fig. 4.** Quantitation of relative signal intensities for UGN and GN mRNA in rat intestine. All quantitation was done with Gel-Pro software. Relative signal intensities were normalized to 18S rRNA signal intensity. For each graph, the signal intensities were adjusted to the lowest (trough) point. Thus the relative abundance represents the fold increase at any time relative to the trough. Data are means ± SE of 4 separate determinations. In each data set, statistical significance was achieved at the P < 0.05 level comparing peak and trough levels. LD, light: dark.
GC-C expression in the jejunum, ileum, and proximal colon in four animals at 1900 h and four animals at 0700 h. For each tissue, similar increased expression persisted late in the dark period at 0300 h, whereas decreased expression persisted late in the light period at 1500 h (data not shown).

The UGN precursor protein displays a circadian rhythm. To determine whether UGN or GN precursor protein demonstrated circadian variation, we prepared jejunal or colonic protein at 0700 h and 1900 h. These represent the tissues where UGN (jejunum) and GN (proximal colon) were most abundantly expressed, as well as the times when the trough (0700 h; n = 5) and peak (1900 h; n = 5) mRNA expression were observed. Homogenates (50 µg protein/lane) were subjected to 12% SDS-PAGE and immunoblotting. Figure 6, top, shows that the amount of jejunal UGN precursor was five- to sixfold higher at 1900 h compared with 0700 h. In contrast, the GN signal in the proximal colon was only ~1.4-fold higher at 1900 h compared with 0700 h, although a greater difference (~2- to 3-fold) was observed in GN expression in the ileum at 1100 and 2300 h (data not shown). These results are consistent with the initial observation that UGN displays more circadian variation at the mRNA level in the jejunum than GN displays in the proximal colon.

DISCUSSION

In this paper, we examined the regional localization and circadian variation of three proteins that play roles in generating cGMP in the intestinal mucosa: UGN, GN, and GC-C. Our work confirms the results of several previous studies on the localization of these proteins along the alimentary canal (Figs. 1 and 2), including Northern blots for rat or mouse GN (12, 16, 17, 19, 29, 30) and UGN (16, 19, 29) and Western blots for rat GN (17, 20) and UGN (20). Although we did not examine the stomach, we showed that UGN is primarily expressed in the proximal intestine, whereas GN is primarily expressed in the ileum, cecum, and proximal large intestine. GC-C, in contrast, was expressed at high levels throughout the small and large intestines and showed much less variation in abundance, even proximal to the rectum where both UGN and GN were barely detectable (Fig. 2). The regulation of GC-C at this site may involve a yet to be determined UGN/GN-like peptide, circulating UGN or GN, peristaltic delivery of proximally secreted luminal UGN or GN, or a more sharply defined local regulation of GN or UGN expression. For example, we have not evaluated circadian variation at this site. In addition to detecting regional variations in the levels of GC-C, we also defined differences in the regional processing of GC-C. We found that the processing of mature 140-kDa GC-C to the 85-kDa brush-border isoform was accentuated in the proximal small intestine, except in the 1-cm duodenal segment immediately distal to the stomach, where it was lower. This is consistent with a role for pancreatic proteases in the processing of GC-C from its full-length form to the cleaved cell surface form.

The main contribution of this paper is the demonstration of appreciable circadian regulation of the members of the GC-C effector system (Figs. 3–6). The GC-C receptor, as well as its UGN and GN ligands, showed significant circadian variation at the mRNA level, with
the variation being particularly striking for UGN and GC-C but less so for GN. We found similar variation in UGN precursor levels (Fig. 6). Circadian rhythms have been described for many different enzyme activities, including maltase, lactase, and sucrase, which peak at night (26); however, previous biochemical work suggested that temporal variation in these activities was regulated posttranslationally by luminal factors, including pancreatic serine proteases (13). Yet several investigators have recently shown that a number of intestinal proteins display circadian rhythms in their mRNA abundance. Changes comparable to those reported for UGN and GC-C have been reported for proteins involved in carbohydrate breakdown and assimilation, such as GLUT-5 (2), sucrase, and the Na\textsuperscript{+}-glucose cotransporter 1 (SGLT1)(21). Some mRNAs, such as glyceraldehyde phosphate dehydrogenase and α-tubulin, however, were constant, indicating that the circadian changes were not due to temporal variation in the ratio of mRNA to the total RNA pool (21).

The existence of high-amplitude circadian rhythms in mRNA expression in the intestine and colon has several implications not only for the GC-C effector system but also for molecular physiological studies involving intestinal gene expression. Most researchers report the photoperiodic conditions under which their experimental animals are raised (usually a light-to-dark 12:12-h photoperiod, although this information is rarely provided by companies that now sell RNA blots). However, the kill and treatment times are rarely defined in relation to photoperiod or feeding. Without these times, information on the photoperiod has little meaning. Most researchers kill their animals early in the light phase at the beginning of the work day, which is the postprandial phase for the intestine, but this might vary from lab to lab or even within a lab or for a single individual. When high-amplitude circadian rhythms in gene expression are present, the simple but often overlooked variable of time of day can affect experimental results and lead to discrepancies, particularly if parallel control samples are not harvested for each experimental time. For example, it is customary for researchers to express RNA ratios for different tissues. When this is done for UGN and GN mRNA at different circadian phases, the ratio increases several-fold in the dark phase due to the disproportionate increase in the UGN mRNA level relative to that of GN.

More work is required to define the mechanisms of circadian regulation of UGN and GN and to determine whether the changes described here are reflected by circadian differences in the luminal or systemic secretion of these peptides or in prehormone processing to the smaller active peptide forms. The nocturnal increase may contribute to the increased mucus production that occurs at night (18), since hydration of mucus is a proposed function of the GC-C effector system (17). We do not know the relative contribution of feeding and photoperiod in the generation of such rhythms; however, our preliminary data indicate that feeding plays an important role in the regulation of these rhythms. We also do not yet know to what extent the circadian changes are due to transcriptional controls, such as circadian differences in transcriptional rates, or to posttranscriptional factors, such as mRNA stability. We have previously reported a circadian rhythm in the synthesis and phosphorylation of the cAMP response element binding protein transcriptional factor in the esophagus (23). Along this line, parallel mechanisms involving the circadian regulation of intestine-specific transcription factors by neuroendocrine or cellular factors may underlie the circadian variation described here. To this end, a recently published study exemplifies how this variation can arise. SGLT1 gene transcription varies in a circadian manner (21). Rhoads et al. (21) showed that an element for hepatocyte nuclear factor-1 (HNF-1) existed in the SGLT1 promoter that formed different complexes with small intestinal nuclear extracts, depending on the time when the animal was killed. Serological tests revealed that HNF-1α was present in complexes throughout the day, whereas HNF-1β binding exhibited circadian periodicity. Rhoads et al. (21) proposed that exchange of HNF-1 dimerization partners contributes to circadian changes in SGLT1 transcription. As the transcriptional factors that regulate GC-C, UGN, and GN become known, an understanding of the circadian regulation of GC-C and its ligands may require knowledge of the circadian regulation of these underlying transcriptional factors, which to date include HNF-1α (but not HNF-1β) for GN (11) and HNF-4 for GC-C (28).

The technical assistance of Jiji Thomas is appreciated.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-45925.

Address for reprint requests and other correspondence: L. A. Scheving, Dept. of Pediatrics; Div. of Gastroenterology and Nutrition, Vanderbilt Univ. School of Medicine, 21st and Garland Av., Nashville, TN 37232-2576 (E-mail: larry.scheving@mcmail.vanderbilt.edu).

Received 26 May 1998; accepted in final form 20 July 1999.

REFERENCES


Downloaded from http://ajpcell.physiology.org/ by 10.220.33.1 on September 30, 2017