Diurnal changes in intestinal apolipoprotein A-IV and its relation to food intake and corticosterone in rats

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Shen, Ling, Li-yun Ma, Xiao-fa Qin, Ronald Jandacek, Randall Sakai, and Min Liu. Diurnal changes in intestinal apolipoprotein A-IV and its relation to food intake and corticosterone in rats. Am J Physiol Gastrointest Liver Physiol 288: G48–G53, 2005. First published August 26, 2004; doi:10.1152/ajpgi.00064.2004.—To further investigate the role of intestinal apolipoprotein A-IV (apo A-IV) in the management of daily food intake, we examined the diurnal patterns in apo A-IV gene and protein expression in freely feeding (FF) and food-restricted (FR; food provided 4 h daily for 4 wk) rats that were killed at 3-h intervals throughout the 24-h diurnal cycle. In FF rats, the intestinal apo A-IV mRNA and protein levels showed a circadian rhythm concomitant with the feeding pattern. The daily pattern of fluctuation of apo A-IV, however, was altered in FR rats, which had a marked increase in intestinal apo A-IV levels during the 4-h feeding period of light phase. In both FF and FR rats, increased plasma corticosterone (Cort) levels temporally coincided with the phase shift of intestinal apo A-IV mRNA and protein expression. Depletion of Cort by adrenalectomy abolished the diurnal rhythm by decreasing the apo A-IV expression during the dark period but did not change the feeding rhythm. Exposure of adrenalectomized rats to consistent Cort level (50-mg continuous release Cort pellet) resulted in fixed apo A-IV levels throughout the day. These results indicate that intestinal apo A-IV exhibits a diurnal rhythm, which can be regulated by endogenous Cort independently of the light-dark cue. The fact that intestinal apo A-IV levels were consistent with the food intake during the normal diurnal cycle as well as during the cycle of 4-h feeding each day suggests that intestinal apo A-IV is involved in the regulation of daily food intake.

circadian rhythm; intestine

APOLIPROTEIN A-IV (apo A-IV) is a major protein component of intestinal triacylglycerol-rich lipoproteins such as chylomicrons and very low-density lipoproteins (3). In the rat, apo A-IV is synthesized in the intestine and the liver, with the intestine accounting for the majority of the circulating apo A-IV (30). It has been demonstrated that apo A-IV production by the small intestine is markedly stimulated as a result of fat absorption and appears to be mediated by the formation of chylomicrons (3, 15). Apo A-IV has been reported to regulate food intake (20, 28). Intravenous administration of apo A-IV significantly inhibits food intake in a dose-dependent manner without eliciting signs of toxicity (9, 10). Thus it would seem reasonable to speculate that under physiological conditions, feeding behavior may reflect levels of apo A-IV in the intestine.

It has been demonstrated in a previous study (11) that serum apo A-IV in ad libitum-fed rats exhibited a circadian rhythm. With ad libitum feeding, serum apo A-IV concentration showed a rhythmic change concomitant with the feeding pattern. Whether intestinal apo A-IV gene and protein expression, however, would fluctuate across the 24-h light-dark cycle in association with spontaneous food intake is unknown. Also, the interaction of intestinal apo A-IV with daily food intake has not been well defined. Therefore, in the present study, we determined whether the gene and protein expression of apo A-IV in the intestine exhibited a circadian rhythm in ad libitum-fed rats.

A number of studies (16, 24) showed that animals on a food-restricted (FR) regimen (food availability for 4 h during light phase) consumed ~25–30% less food and maintained a steady body weight in contrast to rats maintained on a free-feeding (FF) regimen with progressing weight gain. In the present study, a food-restriction paradigm (17), which alters feeding behavior while leaving the light-dark cues unchanged, was used to determine whether there is a direct relationship between intestinal apo A-IV and feeding.

It has been demonstrated that adrenal glucocorticoids play a role in the regulation of feeding and the development of obesity. The peak and nadir of glucocorticoid diurnal secretion over 24 h coincide with the initiation and termination, respectively, of the active feeding period (1, 2). Central administration of corticosterone (Cort) or its analogs stimulates food intake and promotes obesity (26, 27). Most obese rodent models are hypercorticosteronemic, and adrenalectomy (ADX) decreases food consumption and prevents the development of obesity (13, 31). At present, there are no studies that have examined the effects of ADX on apo A-IV gene and protein expression in the intestine. Thus one of the goals of the present study was to examine the diurnal profiles of intestinal apo A-IV gene and protein expression and their relationship to circulating Cort.

MATERIALS AND METHODS

Subjects and housing conditions. Adult male Sprague-Dawley rats (250–275 g, purchased from Harlan, Indianapolis, IN) were housed individually in a light- and temperature-controlled room (lights on 0600–1800, 21°C). Food (pelleted chow, Teklad Rodent Chow, Harlan) and water were available ad libitum. Animals were allowed to acclimate to these housing conditions for 1 wk before experiments were started. All procedures were performed in accordance with institutional guidelines of the Institutional Animal Care and Use Committees at the University of Cincinnati.

Experimental protocol. Two groups of rats were used: one group was allowed to feed freely (FF), and the other was food restricted (FR).
with food available from 1000–1400 for 4 wk. Water was available ad libitum to all rats. Body weight, 24-h food intake for FF rats, and 4-h food intake for FR rats were recorded daily. The animals were killed by decapitation at 3-h intervals (5–6 FR rats/time point and 4 FR rats/time point) throughout the 24-h period. The intestine was rapidly removed from each rat, immediately frozen in liquid nitrogen, and then stored at −80°C until mRNA and protein extraction. Trunk blood was collected, centrifuged, and plasma was stored at −80°C. Separate aliquots of plasma were taken for assay of apo A-IV measured by ELISA, and Cort was determined by radioimmunoassay.

To determine the effects of Cort on the diurnal variation in intestinal apo A-IV gene and protein expression, another three groups of rats were used. Forty-six rats were sham operated (n = 15) or adrenalectomized (n = 31) by a dorsolateral approach to remove the adrenal glands bilaterally (7). Initial body weight and daily presurgery food intake were balanced between sham-operated and ADX groups. In addition, a placebo or a 50-mg Cort pellet (21-day release; Innovative Research of America, Toledo, OH) was implanted subcutaneously under the dorsal neck to produce constant Cort levels in ADX rats. Drinking water for all ADX rats was replaced with 0.9% saline. Body weight changes and food intake were recorded daily after surgery. All of the animals were killed by decapitation at 0900 and 2100, 7 days after surgery. The reason we chose the time points at 0900 and 2100 was because the intestinal apo A-IV gene and protein levels changed dramatically at these time points. Trunk blood was collected, and the intestine was quickly removed as described above. Only ADX rats with plasma Cort levels >1 μg/dl at the time of death were regarded as indications of the completeness in ADX and were included in the results.

Relative RT-PCR for apo A-IV mRNA. Intestinal total RNA was isolated with Tri Reagent according to the manufacturer’s instructions (Molecular Research Center, Cincinnati, OH), and total RNA concentration was determined spectrophotometrically at 260 nm. The sequences of the primers for apo A-IV were determined as previously described (6) and are depicted in Table 1. To ensure uniform and reproducible reaction conditions, both RT and PCR reaction regents were prepared as master mixes and aliquoted into individual tubes before using them for each batch of reactions. Ten nanograms of total RNA were reverse transcribed to first-strand cDNA using random hexamers and Moloney murine leukemia virus (MMLV) RT according to manufacturer instruction (Amersham Pharmacia Biotech, Piscataway, NJ). The amplification was performed using 1 cycle at 95°C for 5 min followed by 25 cycles at 92°C for 30 s, 58°C for 30 s, 72°C for 45 s, and then final extension at 72°C for 5 min. PCR products were separated on 1.5% agarose gel and stained with GelStar. The amounts of the bands were quantified using a PhosphorImager (Molecular Dynamics, Amersham Biosciences). To determine whether equal amounts of total RNA had been added to the RT-PCR reaction, the housekeeping gene for GAPDH (primers in Table 1) was used as an internal control. The levels of amplified apo A-IV mRNA in the intestine were expressed as a ratio of GAPDH mRNA amplified in the same RT-PCR assay.

The following control experiments were performed to ensure specificity of the RT-PCR reaction. First, the in vitro transcribed RNAs (target and standard) were amplified without primer [pd(N)6] for reverse transcription to ensure that the amplification products obtained were derived only from the RNAs and not from remaining DNA sequences caused by incomplete digestion of the templates. Second, a no-template negative control reaction was carried out to control for contamination of the PCR components. As determined by GelStar staining, no PCR product was detected in either case (data not shown).

Western blot analysis for apo A-IV protein. The intestinal A-IV protein level was assessed by semiquantitative Western blot analysis. Antiserum against rat apo A-IV was raised from goat as described previously (15). The tissues were homogenized, and the supernatant (containing 10-μg intestinal protein) was separated by 12% polyacrylamide gel electrophoresis, transferred onto nitrocellulose sheets, and probed with apo A-IV antibody (1:3,000 dilution). The amount of immunocomplexes was quantitated using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The blots were stripped and reincubated with monoclonal antibody against actin (Boehringer-Mannheim, Mannheim, Germany; 1:3,000 dilution). The reacted membranes were exposed to X-ray film (Kodak Scientific imaging film, Rochester, NY). Film density, measured as transmittance, was expressed as volume-adjusted optical density. The amount of apo A-IV protein was normalized to the respective individual density values reflecting actin protein levels and was expressed as a ratio.

Measurement of plasma Cort. Total plasma Cort was measured by RIA using rabbit antiserum raised against Cort (B3–163) obtained from Endocrine Sciences (Calabasas Hills, CA). Briefly, 20-μl duplicate samples of plasma were heated at 60°C for 2 h to denature Cort binding globulin and were incubated overnight with Cort antibody. [3H]Corticosterone (New England Nuclear Life Sciences, Boston, MA) was used as a radioactive tracer. Free and bound Cort were separated by charcoal.

Statistical analysis. Daily body weight changes are presented as percent of initial body weight. Data from different time points were analyzed by repeated-measures ANOVA followed by Tukey’s multiple comparison. Data from ADX, ADX plus Cort, and Sham-operated rats were compared by one-way ANOVA. Results were expressed as the means ± SE, and P < 0.05 was considered statistically significant.

RESULTS

Diurnal expression of intestinal apo A-IV mRNA and protein relative to food intake in FF rats. Food intake was monitored every 3 h throughout the 24-h light-dark cycle in FF rats. Under ad libitum feeding conditions, rats consumed very little food during the light phase. A dramatic increase in food intake was observed after the onset of the dark cycle (1800), and active food intake was maintained from 1800–0300. Thereafter, the amount of food ingestion declined gradually until 0600. Thus food intake showed a strong nocturnal feeding pattern (Fig. 1A).

In FF rats, intestinal apo A-IV mRNA levels and food intake were positively correlated with each other during the diurnal cycle. Figure 1B shows the diurnal expression of intestinal apo A-IV mRNA at eight time points across the light-dark cycle. The apo A-IV mRNA level decreased from 0600 onward and

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Apo A-IV, apolipoprotein A-IV.
was sustained until 1500. An increase in apo A-IV mRNA level began at 1800 during the dark phase, and the higher level was sustained throughout the dark phase (Fig. 1B). The expression of apo A-IV protein closely followed the changes in apo A-IV mRNA levels (Fig. 1C).

Alterations in daily fluctuations of apo A-IV mRNA and protein expression in FR rats. In the present study, FF rats steadily gained body weight during the 4 wk of observation (data not shown). Food restriction (4 h/day, from 1000 to 1400) initially resulted in loss of animal body weight during the first 3 days. Thereafter, rats slowly regained their initial body weight by day 9 and then gained weight at a steady but slower rate than the FF rats. At the end of the 4 wk, the body weight of FR rats was 12% below that of FF rats. The lower rate of body weight gain in FR rats reflected lower average food consumption. FR rats, for example, consumed 20.8 ± 0.8 g compared with 28.4 ± 0.7 g for FF rats at day 26.

The daily patterns of fluctuation in intestinal apo A-IV mRNA and protein levels were altered in FR rats when compared with the FF rats. As shown in Fig. 2A, FR rats had

![Fig. 1](image1.png)

**Fig. 1.** A: 3-h interval food intake (g) under ad libitum-feeding and -drinking conditions in rats. B and C: daily profiles of dynamic changes in intestinal mRNA (B) and protein (C) levels in freely feeding (FF) rats. Values are expressed as means ± SE, n = approximately 5–6. Dissimilar letters denote statistically significant differences (P < 0.05). Shaded bands represent the light-off phase (1800–0600). apo A-IV, apolipoprotein A-IV.

was sustained until 1500. An increase in apo A-IV mRNA level began at 1800 during the dark phase, and the higher level was sustained throughout the dark phase (Fig. 1B). The average expression of apo A-IV mRNA in the light cycle (0600–1800) was significantly lower than that in the dark cycle (1800–0600; P < 0.01). Statistical analysis indicated significant effects of time of day on apo A-IV mRNA expression in the intestine (P < 0.05). Post hoc comparisons indicated that the

![Fig. 2](image2.png)

**Fig. 2.** Comparison of dynamic changes in intestinal apo A-IV mRNA (A) and protein (B) levels between the FF and food-restricted (FR) rats. Values are expressed as means ± SE, n = 4. *P < 0.05, compared with levels of apo A-IV mRNA or protein at the same time point. Shaded bands represent the light-off phase (1800–0600).
increases in intestinal apo A-IV mRNA level, which occurred during the 4-h feeding period in the light phase. When we compared the pattern of intestinal apo A-IV mRNA levels between the FF and the FR rats (Fig. 2B), both groups of animals showed totally different patterns. With the exception of an overlap at 0600, the pattern observed in the FR rats is exactly opposite that of the FF rats. The difference between the two groups at 0900, 1200, and 1500 is statistically significant ($P < 0.05$). The fluctuation of apo A-IV protein level in FR rats showed a similar pattern as mRNA level (Fig. 2C).

**Daily fluctuations in plasma Cort levels in FF and FR rats.** In FF rats, circulating Cort showed a well-defined diurnal rhythm (Fig. 3A). Plasma Cort levels were low in the morning (from 0600 to 1200), and then they tended to increase at 3 h before the dark phase and dramatically rise to reach a peak at the light-dark transition phase (1800; 18.5 ± 0.67 μg/dl), right before the higher apo A-IV mRNA and protein levels in the intestine of the FF rats. Subsequently, plasma Cort levels declined to 10.5 ± 1.57 μg/dl at 2100 and 3.4 ± 0.58 μg/dl 3 h before returning to the nadir at 0600. The change in feeding regimen in the FR rats significantly shifted the daily peak of plasma Cort levels to 0900, again peaking before the highest level of intestinal apo A-IV level (Fig. 3B). Thus, in both the FF and FR rats, the sharp rise in Cort preceded the increasing phase of apo A-IV protein expression in the intestine of both FF and FR rats. The peak of apo A-IV protein expression lagged ~3–4 h behind the peak of plasma Cort (Fig. 3, A and B).

**Effect of ADX and constant Cort levels on the body weight, the diurnal feeding, and apo A-IV levels in the intestine.** In sham-operated rats, plasma Cort showed a normal diurnal variation, i.e., low at 0900 (1.4 ± 0.67 μg/dl) and high at 2100 (10.15 ± 2.39 μg/dl). Cort was undetectable in ADX with placebo pellet. ADX rats given 50-mg Cort pellets had plasma Cort levels elevated over the sham control values at 0900, but they were constant throughout the day. Levels of circulating Cort for rats given the 50-mg Cort pellet were 10.3 ± 1.31 μg/dl at 0900 and 11.4 ± 0.66 μg/dl at 2100.

Daily food intake and body weight were measured for 8 days starting from the day before surgery. ADX resulted in a reduction in body weight (~11.1 ± 0.9 g from the presurgery initial body wt, $n = 12$), whereas sham controls gained 22.5 ± 1.4 g from their presurgery initial body weight in 7 days ($n = 14$, $P < 0.001$ vs. ADX rats). ADX rats ate significantly less than sham controls. ADX rats, for example, consumed 16.7 ± 0.9 g compared with 24.9 ± 1.2 g for sham-operated rats at day 7 ($P < 0.01$). As demonstrated in previous studies (4, 12), despite the association of Cort with feeding behavior, ADX did not alter rat normal feeding rhythm (data not shown). The effects of ADX on food intake and body weight can be reversed partially, but not significantly, by Cort (50 mg) replacement (data not shown), as reported previously (5, 14).

The effects of Cort on the diurnal expression of apo A-IV gene and protein in the intestine were examined in sham-adrenalectomized or adrenalectomized rats given placebo or 50-mg Cort pellets. Sham controls maintained the diurnal rhythm of apo A-IV mRNA and protein expression as observed in intact rats and protein, with significantly higher levels of apo A-IV in the evening (2100) than in the morning (0900; $P < 0.01$, Fig. 4, A and B). Removal of endogenous glucocorticoids by ADX had no significant effect on apo A-IV expression at 0900; however, ADX significantly downregulated the 2100 expression by ~33% (mRNA) and 55% (protein) of apo A-IV levels found in sham controls, thereby eliminating the diurnal rhythm of apo A-IV expression ($P < 0.05$, Fig. 4, A and B). The diurnal variation in apo A-IV expression was also abolished in rats with constant Cort levels (50-mg pellet), which restored apo A-IV mRNA and protein expression with a significant increase in the morning compared with sham controls, resulting in flat levels of apo A-IV expression throughout the day (Fig. 4, A and B).

**DISCUSSION**

In this study, we demonstrated that the intestinal gene and protein expression of apo A-IV in ad libitum-fed rats varied in response to time of day. We showed that the circadian rhythm of the intestinal apo A-IV mRNA and protein levels displayed are positively correlated with the diurnal cycle food intake. Both of these parameters were significantly higher during the dark phase than during the light phase, suggesting that the consumption of food during the dark period greatly enhances
and C were provided with food at the time point of 1000 (Fig. 2, point worth noting is the observation that intestinal apo A-IV in gene and protein expression of intestinal apo A-IV. One option of food is necessary for the generation of the daily patterns period. These findings indicate that availability and consumption of food availability limited to the lights-on small intestine were altered in FR rats compared with the FF rats. Despite an unchanged light-dark cycle, the peak of the nocturnal increase in the intestinal apo A-IV gene and protein expression demonstrated that the peak diurnal pattern of apo A-IV protein levels, the surge in the diurnal peak (at 2100), and Cort replacement restored the decrease in apo A-IV levels corresponding to the sham control rats at 2100. 

To determine whether the changes in apo A-IV mRNA and protein levels are caused by consumption of food or the circadian rhythm, we observed the intestinal apo A-IV gene and protein expression in FR rats (food provided 4 h daily between 1000 and 1400). We found that the daily patterns of the fluctuation of apo A-IV mRNA and protein levels in the small intestine were altered in FR rats compared with the FF rats. Despite an unchanged light-dark cycle, the peak of the gene and protein expression of apo A-IV in the intestine was shifted in time of food availability limited to the lights-on period. These findings indicate that availability and consumption of food is necessary for the generation of the daily patterns in gene and protein expression of intestinal apo A-IV. One point worth noting is the observation that intestinal apo A-IV gene and protein expression tended to increase before the rats were provided with food at the time point of 1000 (Fig. 2, A and C). Thus it is unlikely that the increase in intestinal apo A-IV level is regulated only by the presence of food in the lumen. It is likely that the anticipation of food triggers this increase, although we do not know the signal involved.

The fact that intestinal apo A-IV increased during the dark phase, positively related to the most active feeding period of the rat, is potentially of physiological significance. Several lines of evidence suggest that apo A-IV plays an important role in the regulation of feeding behavior (9, 10, 19). If apo A-IV were a physiological regulator of food intake, the intestinal apo A-IV levels would be predicted to be increased when animals were eating. Through its satiety role, apo A-IV may regulate the upper gastrointestinal function, such as gastric emptying and gastric acid secretion (21, 22) and thereby control over-eating in the animal. Rodriguez, et al. (23) have demonstrated that the increase in circulating apo A-IV increases rapidly after the ingestion of a meal and so there is ample time for apo A-IV to play a role in the regulation of food intake during the dark phase. Our current observations lend support to the concept that the physiological role of apo A-IV may be as a regulator of daily food intake.

The mechanism by which intestinal apo A-IV mRNA and protein expression fluctuates diurnally is not clear; however, the increase of apo A-IV before the onset of the dark cycle in FF rats may suggest that the diurnal rhythm of apo A-IV levels is unlikely to be light entrained. Analysis of diurnal events that precede the increasing phases of apo A-IV expression may help us to identify the regulatory factor/s of diurnal expression of apo A-IV mRNA and protein. In a carefully conducted study described by Wilkinson et al. (29), peak Cort levels occurred just before feeding, making Cort a potential regulator of intestinal apo A-IV. Consistent with this report, we observed that a single peak in plasma Cort levels occurred just before the active feeding period (the dark cycle). The trough of secretion was observed in the early light cycle, indicating very low levels of Cort. Interestingly, plasma Cort levels in FR rats rose 1 h before the onset of feeding and decreased to a basal range at the end of 4 h of feeding, again supporting the notion that there is a tight relationship with the onset of feeding behavior (8). Analysis of the temporal relationship between Cort and apo A-IV gene and protein expression demonstrated that the peak in plasma Cort and the highest intestinal apo A-IV mRNA and protein levels coincide in both FF and FR rats. Compared with the diurnal pattern of apo A-IV protein levels, the surge in plasma Cort preceded the increase in apo A-IV, suggesting that Cort may act as a signal for increased apo A-IV expression.

This suggestion is supported by the observations that both ADX and constant level of Cort abolished the diurnal rhythm of apo A-IV mRNA and protein expression. ADX significantly lowered intestinal apo A-IV levels corresponding to the sham diurnal peak (at 2100), and Cort replacement restored the decrease in apo A-IV levels in ADX animals (Fig. 4). Whereas it is tempting to conclude that circulating Cort was the factor regulating the intestinal apo A-IV gene and protein expression, we must be cautious and point out that the data are highly suggestive but by no means conclusive. It is possible that other humoral factors, such as leptin and insulin, may also play a role in regulating apo A-IV mRNA and protein levels or mediate the ADX-induced changes in intestinal apo A-IV gene and protein expression.

In conclusion, the present study clearly demonstrated that physiological fluctuation in the intestinal apo A-IV mRNA and
protein levels in rats fed ad libitum occurs diurnally. This diurnal rhythm is not regulated by light-dark phase but rather by the feeding regimen such as food restriction. The present study provided evidence that Cort is involved in entraining the diurnal rhythm of apo A-IV gene and protein expression, because the circadian rhythm of intestinal apo A-IV can be abolished by the depletion of glucocorticoids or constant Cort replacement. The precise mechanisms by which plasma Cort and intestinal apo A-IV interact and how they act together to regulate feeding behavior under physiological conditions remain to be determined.

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