High-Fat Diet and Palmitate Alter the Rhythmic Secretion of Glucagon-Like Peptide-1 by the Rodent L-cell

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Secretion of the incretin hormone, glucagon-like peptide-1 (GLP-1), by the intestinal L-cell is rhythmically regulated by an independent molecular clock. However, the impact of factors known to affect the activity of similar cell-autonomous clocks, such as circulating glucocorticoids and high-fat feeding, on GLP-1 secretory patterns remains to be elucidated. Herein the role of the endogenous corticosterone rhythm on the pattern of GLP-1 and insulin nutrient-induced responses was examined in corticosterone pellet-implanted rats. Moreover, the impact of nutrient excess on the time-dependent secretion of both hormones was assessed in rats fed a high-fat, high-sucrose diet. Finally, the effects of the saturated fatty acid, palmitate, on the L-cell molecular clock and GLP-1 secretion were investigated in vitro using murine GLUTag L-cells. Diurnal variations in GLP-1 and insulin nutrient-induced responses were maintained in animals lacking an endogenous corticosterone rhythm, suggesting that glucocorticoids are not the predominant entrainment factor for L-cell rhythmic activity. In addition to hyperglycemia, hyperinsulinemia, insulin resistance, and disorganization of feeding behavior, high-fat high-sucrose-fed rats showed a total abrogation of the diurnal variation in GLP-1 and insulin nutrient-induced responses, with comparable levels of both hormones at the normal peak (5:00 PM) and trough (5:00 AM) of their daily pattern. Finally, palmitate incubation induced profound derangements in the rhythmic expression of circadian oscillators in GLUTag L-cells and severely impaired the secretory activity of these cells. Collectively our findings demonstrate that obesogenic diets disrupt the rhythmic activity of the L-cell, partially through a direct effect of specific nutritional components. **(*Endocrinology* 157: 586–599, 2016)**

The activity of many metabolic tissues demonstrates circadian rhythmicity. This time-dependent regulation permits tissues to anticipate metabolic reactions during the normal 24-hour period and therefore to optimize their use of energy. The daily patterns in the function of metabolic tissues are orchestrated by circadian oscillators, the so-called clock genes, which constitute the molecular machinery of cell-autonomous clocks (1). A relatively small number of core clock genes regulate their own transcription by self-sustained feedback loops. However, they also modulate the expression of a large number of genes involved in energy homeostasis, either directly or through subordinate transcription factors (2). Cell-autonomous clocks governing the daily activity of metabolic tissues, collectively known as peripheral metabolic clocks, have been identified in the intestine, pancreatic islets, liver, muscle, adipose tissue, adrenal glands, and kidney (3–10). These clocks are entrained by inputs coming from the suprachiasmatic nuclei (the master clock) as well as by behavioral and humoral peripheral factors such as the feeding-fasting cycle (6) and variations in the glucocorticoid levels (11). The role of the peripheral entrainment factors is particularly relevant for metabolic clocks, and they often dominate over the synchronizing cues emitted by the suprachiasmatic nuclei (1).

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Abbreviations: AUC, area under the curve; B, corticosterone; DPP IV, dipeptidyl peptidase IV; FBS, fetal bovine serum; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; HFHS, high-fat, high-sucrose; HOMA-IR, homeostatic model assessment for insulin resistance; OGTT, oral glucose tolerance test; ZT, zeitgeber time.

Maintenance of robust diurnal rhythms is essential for energy homeostasis (12). Consistent with this, circadian disruption is associated with alterations in the function of metabolic tissues and represents a major risk for several metabolic disorders including diabetes and obesity (13). Moreover, attenuation in the pattern of self-sustained circadian oscillators in liver and adipose tissue has been observed in obese animals (14, 15). Because circadian clocks in metabolic tissues are heavily influenced by the feeding cycle, it is not surprising that disruptions not only in the timing but also in the composition and caloric content of nutrients alter the function of these cell-autonomous oscillators, as found with high doses of sugar and palmitate (16–18), common components of obesogenic diets. However, neither the effects of a Western diet nor those of specific nutrients on the functioning of molecular clocks in endocrine cells have been extensively studied to date.

Glucagon-like peptide-1 (GLP-1) is an essential regulator of fuel homeostasis in the body, serving as a link between the intestine and the pancreatic β-cells. GLP-1 is released by the intestinal L-cell in response to ingested nutrients and subsequently stimulates insulin secretion in a glucose-dependent manner (19). Interestingly, rodent L-cell activity has been recently demonstrated to be regulated in a circadian manner (20). Thus, circadian expression of self-sustained oscillators has been found in murine GLUTag cells, a well-established model of the intestinal L-cell (21, 22). Moreover, both a circadian rhythm in the L-cell response to well-established secretagogues in vitro and a diurnal variation in the GLP-1 response to oral nutrients in rats have also been demonstrated (20). Specifically, the highest GLP-1 secretory response to identical glucose loads is observed immediately before the normal feeding period and the lowest at the end of the active period, closely matching the daily profile in corticosterone levels in rats (23). However, a potential role of glucocorticoids as an entrainment factor in the diurnal variation of GLP-1 responses remains to be investigated. Remarkably, GLP-1 also appears to be instrumental in the diurnal variation of the insulin secretory response in rats because both patterns are highly correlated under physiological conditions (20). Furthermore, when the pattern in GLP-1 secretion is either shifted or disrupted by experimental manipulation, the pattern in insulin secretion changes accordingly (20). GLP-1 is thus a candidate mediator of the alterations in energy homeostasis produced by circadian disruption.

In the present study, the diurnal variations in GLP-1 and insulin responses were first examined in rats implanted with corticosterone pellets to suppress the endogenous rhythm in corticosterone secretion. This procedure maintains constant corticosterone levels within the physiological range (24) and was used to determine whether glucocorticoids play a role as an entrainment factor for the rhythmic activity of the L-cell. The impact of nutrient excess in the time-dependent secretion of GLP-1 and insulin was then assessed in rats maintained on a high-fat, high-sucrose (HFHS) diet. All animals were studied at the time points previously demonstrated to represent the peak (zeitgeber time [ZT] 10) and trough (ZT22) responses of GLP-1 and insulin during an oral glucose tolerance test (OGTT) in normal male rats (20). Finally, to investigate the effects of a specific component of this obesogenic diet on the function of the cell-autonomous clock in the L-cell, murine GLUTag L-cells were incubated with palmitate, which has been previously demonstrated to disrupt the molecular clocks of other cells in vitro (16–18), followed by the determination of canonical clock gene expression and GLP-1 secretory activity.

Materials and Methods

In vivo studies

Animals

Adult male Wistar rats (250–275 g) were purchased from Charles River Laboratories and were acclimated to the animal facility for a week before use. The animals were housed two to three rats per cage under a 12-hour light, 12-hour dark cycle with lights on at 7:00 AM (ZT0) and controlled room temperature (20–22°C). They were maintained with free access to tap water and standard chow (Tekland Global 18% protein rodent diet; Harlan), except as otherwise indicated. All experimental procedures were approved by the Animal Care Committee of the University of Toronto.

Corticosterone pellet study

Corticosterone (B; 50 mg) or placebo (cholesterol) pellets (Innovative Research of America) were sc implanted under isoflurane anesthesia (n = 7–8 rats/group). B pellets were designed to release 2.3 mg corticosterone per day for 3 consecutive weeks. The animals were housed individually after pellet implantation, and body weight and food intake were monitored daily. On experimental day 10, food intake was recorded every 4 hours to determine the daily pattern of feeding. On experimental days 13 and 19, rats were fasted for 4 hours and administered an OGTT (3 g/kg glucose) by gavage at either 5:00 PM (ZT10) or 5:00 AM (ZT22), previously established as the peak and trough, respectively, of the normal diurnal variations in the GLP-1 and insulin responses to oral glucose (20). This fasting period was selected to allow circulating levels of GLP-1, insulin, and glucose to return to basal prior to each OGTT (20). Each animal was studied at both time points, allowing 6 days of recovery between tests. For sample collection, the rats were placed in a tube restrainer and blood (300 μL) was extracted from a small cut in a lateral tail vein at t = 0 and then 10, 20, 30, and 60 minutes after glucose administration. Blood samples were collected into 30 μL of an anticoagulant and protease inhibitor solution (0.1 mM diprotin

A [Sigma-Aldrich], 5000 IUK/mL aprotinin [Sigma-Aldrich] and 0.03 M EDTA). Plasma was stored at −20°C until use.

High-fat high-sucrose study

Rats (n = 7–8 per group) were individually housed and maintained on either regular chow (58% kcal coming from carbohydrates, 24% from protein, and 18% from fat) with free access to tap water or on a HFHS diet (35% kcal coming from carbohydrates, 20% from protein, and 45% from fat (including 40% from lard, which contains 30% palmitate; D12451; Research Diets, Inc), with free access to both regular tap water and 30% sucrose-water. Body weight, food intake, and 30% sucrose-water intake were monitored daily. On days 22 and 23, caloric consumption (food and 30% sucrose-water intake) was recorded every 4 hours to determine the daily distribution of feeding. At the end of weeks 4 and 5, OGTTs were conducted as above. On day 38, the rats were euthanized and tissues were collected and stored at −80 C until use.

Glucose and hormone determinations

Plasma glucose levels were measured by an enzymatic assay using a glucose analyzer (Analox Instruments). The insulin and total GLP-1 levels were determined by a sandwich immunoassay (MesoScale Discovery) and corticosterone by an ELISA (DRG International, Inc.).

In vitro studies

Cell cultures

Murine GLUTag cells were used as a model of the rodent enteroendocrine L-cell based on the existence of a cell-autonomous clock in these cells, which drives a circadian variation in their GLP-1 secretory activity (20). GLUTag cells were propagated in DMEM (Gibco) containing 25 mmol/L glucose and supplemented with 10% fetal bovine serum (FBS; Gibco).

Effects of palmitate pretreatment on clock gene expression

Cells plated in 10-cm plates were incubated with 0.5 mM palmitate (Sigma-Aldrich) or vehicle (DMEM with 10% FBS) for 12 hours and then synchronized by overnight serum deprivation (in DMEM with 0.5% FBS) followed by a 1-hour shock with 10 μM forskolin (Sigma-Aldrich) in DMEM with 10% FBS, as previously described (20). Cells were then washed and incubated again with 0.5 mM palmitate in regular DMEM with 10% FBS or vehicle. Total RNA was extracted at time 0, 4, 8, 12, 16, 22, 25, 28, 31, and 35 hours.

Effects of palmitate on GLP-1 secretion

GLUTag cells were plated on 24-well plates for 48 hours and then pretreated with 0.5 mM palmitate or vehicle, serum starved, and synchronized as above with 1 μM forskolin, followed by reincubation with 0.5 mM palmitate or vehicle for 4 hours. Cells were then washed and incubated with media alone (control), 1 mM bethanechol, or 1 μM glucose-dependent insulinotropic peptide (GIP) for 2 hours in the absence of the pretreatment. Peptides were extracted from the media and cells by reversed-phase adsorption, and the GLP-1 content was determined by RIA (Millipore), as previously reported (20). Secretion was determined as the percentage of total GLP-1 found in the media, whereas total content was calculated as media plus cellular levels of GLP-1.

Cell viability assay

The cells were plated in poly-D-lysine-coated 24-well plates for 24 hours, and the effect of palmitate on GLUTag cell viability was tested by incubating the cells with a range of different palmitate concentrations (0.1 mM, 0.5 mM, and 2.5 mM) or vehicle (DMEM, 10% FBS) for 48 hours. Cell viability was then determined by a neutral red assay, as reported (25).

RNA analysis

Total mRNA was extracted from rat tissues and GLUTag cells using an RNeasy Plus minikit with QIAshredder (QIAGEN) and then quantified by a spectrophotometric assay, obtaining purity ratios above 2.0 for all samples. Reverse transcription was performed using Superscript II reverse transcriptase and random primers (Invitrogen). Semiquantitative real-time PCR was conducted using Taqman assays (primers listed in Table 1) with Taqman universal master mix (Applied Biosystems). Data were analyzed using Opticon software (Bio-Rad Laboratories), and relative expression was determined following the 2(-δδC[t]) method (26). The *H3f3a* amplicon was used as internal control.

Statistical analyses

Data are expressed as mean ± SE. Areas under curves (AUC) were calculated by the trapezoid rule. Hepatic insulin resistance

Table 1. Primers Used for Quantitative RT-PCR Analysis

Gene Symbol (Species)	NCBI mRNA Accession Number	Amplicon Length	Assay ID
Arntl (mouse)	NM_001243048.1 NM_007489.4	87	Mm00500226_m1
Arntl (rat)	NM_02436.2	137	Rn00577590_m1
Per2 (mouse)	NM_011066.3	73	Mm00478113_m1
Per2 (rat)	NM_031678.1	100	Rn01427704_m1
Gcg (mouse)	NM_008100.3	62	Mm00801712_m1
Gcg (rat)	NM_012707.2	65	Rn00562293_m1
Nr1d1 (rat)	NM_001113422.1 NM_145775.2	64	Rn01460662_m1
H3f3a (mouse)	NM_008210.4	82	Mm01612808_g1

Abbreviation: NCBI, National Center for Biotechnology Information.

was estimated by the homeostatic model assessment (HOMA-IR) index, and whole-body insulin resistance was calculated by Matsuda's composite index, as previously validated (27, 28). A Student's *t* test or a one-way or two-way ANOVA followed by Fisher's post hoc analyses were conducted as appropriate to compare the different groups. Significance was set at $P < .05$. Nonlinear curve fitting calculation was performed using OriginPro 8.5 software.

Results

Suppression of the circadian pattern in corticosterone does not prevent the diurnal variation in GLP-1 and insulin secretory responses to an OGTT

Placebo- and B pellet-implanted rats showed comparable body weights prior to surgery. Although the body weight of B pellet-implanted animals dropped immediately after the surgery, it steadily increased thereafter, returning to that of the placebo-implanted animals by experimental day 14 (Figure 1A). Both groups of animals consumed a comparable number of calories daily throughout the study (Figure 1B). Moreover, the timing of caloric intake was similar because both groups showed a similar distribution of feeding during the light and the dark phases of the day (Figure 1C).

Control animals showed the expected pattern in corticosterone levels, with significantly ($P < .001$) higher concentrations in the late afternoon (ZT10, 5:00 PM) as compared with the early morning (ZT22, 5:00 AM). In contrast, the normal circadian variation in corticosterone levels was totally prevented in B pellet-implanted animals, which showed comparable concentrations of corticosterone at the peak (ZT10) and the trough (ZT22) of the normal circadian pattern (Figure 1D). Thus, although the plasma

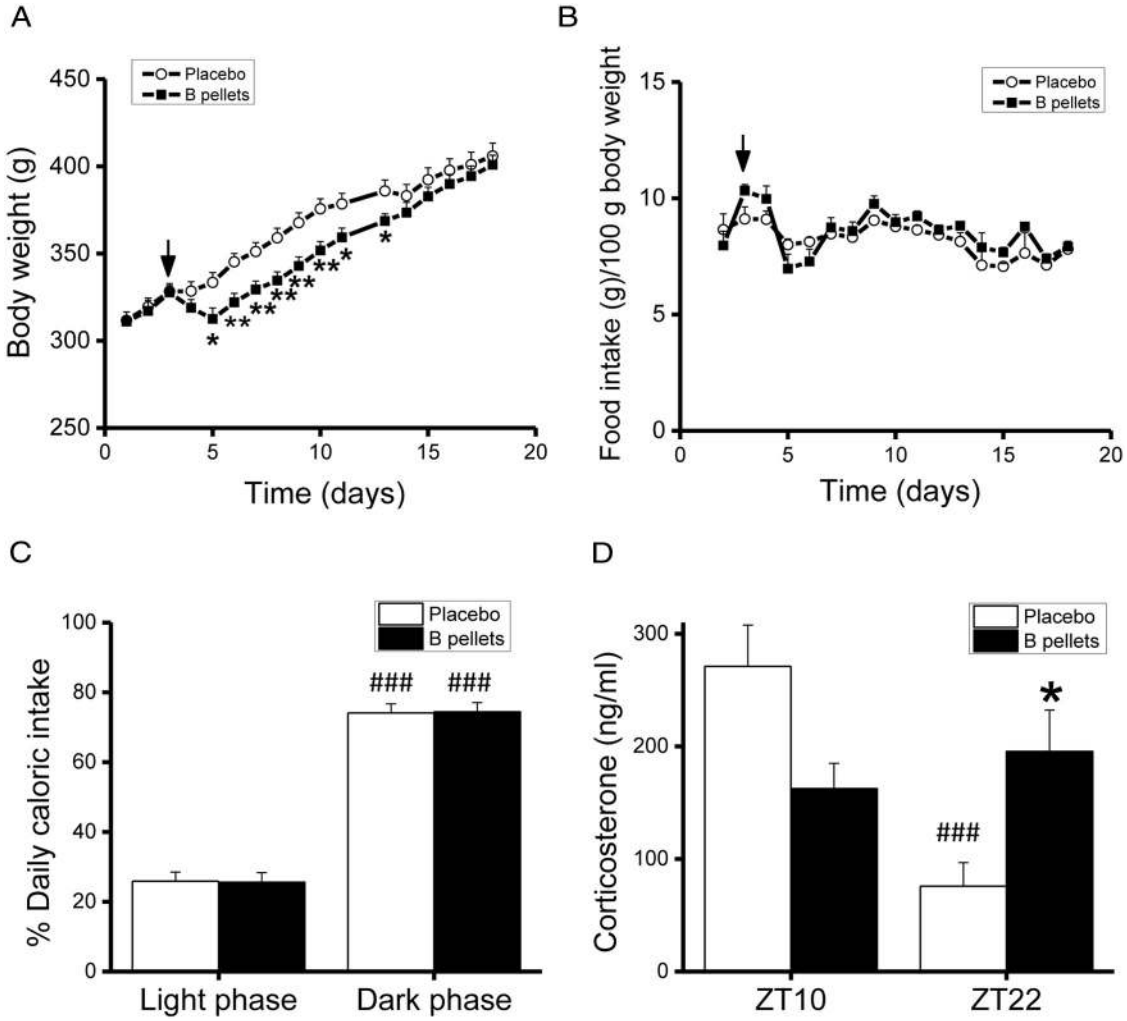

Figure 1. Body weight (A), food intake per body weight (B), and temporal distribution of feeding during the light-dark cycle (C) of rats sc implanted with either placebo or corticosterone pellets. A and B, Arrows indicate the day of pellet implantation. D, Circulating corticosterone levels in the same animals at the normal peak (ZT10) and trough (ZT22) of the circadian rhythm of corticosterone secretion (n = 7–8 rats per group). *, $P < .05$, **, $P < .01$ vs placebo group; ###, $P < .001$ vs the other time point within the same group.

levels of corticosterone in B pellet-implanted rats remained constant around the daily average of placebo-implanted animals, they were significantly ($P < .05$) higher at the normal trough, ZT22, with respect to the control group.

To establish whether the diurnal variation in GLP-1 and insulin nutrient-induced responses is affected by the endogenous rhythm in corticosterone levels, the responses of both hormones to identical oral glucose loads were examined at the previously established peak (ZT10) and trough (ZT22) of their daily patterns (20), in placebo- and B pellet-implanted rats. Both groups of animals showed comparable basal levels of GLP-1 (Figure 2A), insulin (Figure 2B), and glucose (Figure 2C) as well as similar HOMA-IR index values (Figure 2D) at the two time points studied. Placebo-implanted rats showed the expected variation in GLP-1 and insulin secretory responses to oral glucose, such that both responses were higher at ZT10 as compared with ZT22 (by 1.4-fold, $P < .05$, and by 1.8-

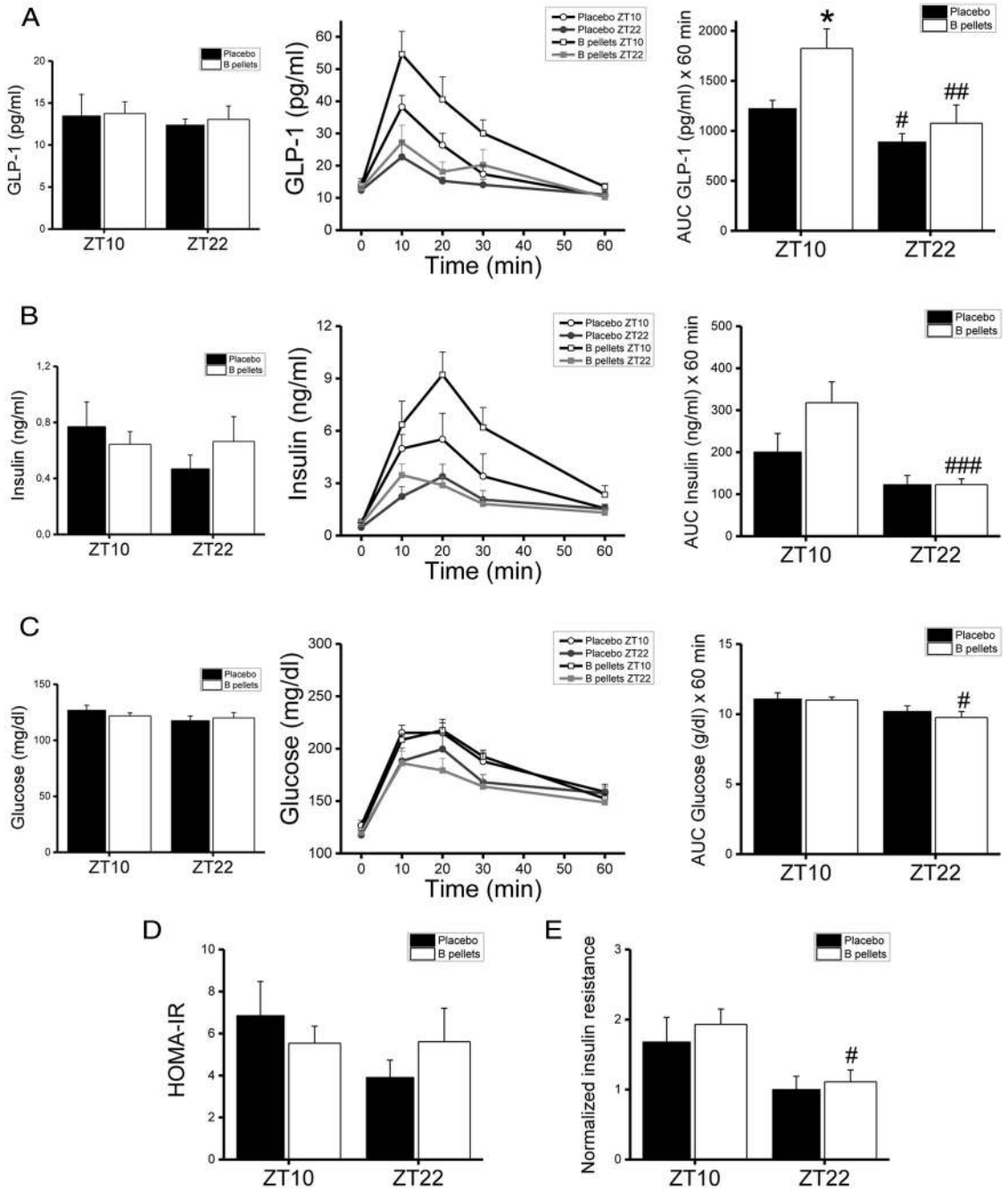

Figure 2. Basal plasma levels and time-course profiles as well as corresponding AUCs of GLP-1 (A), insulin (B), and glucose (C) excursions in response to identical oral glucose loads conducted at two different time points (ZT10 and ZT22) in 4-hour-fasted rats implanted with either placebo or B pellets. Hepatic (D) and whole-body (E) insulin resistance of the same animals (n = 7–8 rats per group). *, $P < .05$ vs placebo group; #, $P < .05$, ##, $P < .01$, ###, $P < .001$ vs the other time point within the same group.

fold, respectively). Moreover, the diurnal variations in both GLP-1 and insulin responses were maintained in animals with complete abrogation of the endogenous corticosterone rhythm, such that the GLP-1 response to identical glucose loads was 1.7-fold higher at ZT10 as compared with ZT22 ($P < .01$; Figure 2A), and the insulin response was 2.6-fold higher at the same time point ($P < .001$; Figure 2B). Despite higher GLP-1 and insulin responses at ZT10 as compared with ZT22 in the rats with constant corticosterone levels, an antiphasic pattern for glucose disposal was found in these animals, which showed significantly improved glucose tolerance at ZT22 ($P < .05$; Figure 2C) in association with reduced insulin resistance at the same time point ($P < .05$; Figure 2E). Furthermore, the enhanced GLP-1 response observed in placebo-implanted animals at ZT10 was further potentiated (by 1.5-fold, $P < .05$) in animals with a disrupted corticosterone rhythm, suggesting that the normal circadian pattern of corticosterone, although not a zeitgeber for the daily pattern of activity of the L-cell, may play a role in regulating the amplitude of GLP-1 secretion; a similar trend was observed for insulin levels in the B pellet-implanted rats at ZT10, although this did not reach statistical significance.

HFHS diet increases body weight and alters the temporal distribution of feeding

Both groups of animals (HFHS and regular chow diet) showed comparable body weights at the beginning of the study (338.9 ± 4.5 g and 330.5 ± 5.5 g, respectively); however, rats on the HFHS diet gained significantly more weight than control rats from the first week of feeding ($P < .05$). The increment in body weight of animals on the HFHS diet continued throughout, and by the end of the study, the difference in body weight was maximal (575.5 ± 12.7 g vs 513.1 ± 14.3 g, $P < .01$, Figure 3A). Consistent with the increased body weight, rats on a HFHS diet consumed significantly more calories than the animals fed regular chow (Figure 3B). The difference in caloric consumption was maximal at the end of the first week (35% increment, $P < .001$) and dropped steadily afterward, although it continued to be significantly higher at the end of the experimental period (14% increment, $P < .01$). Animals on a HFHS diet also showed an alteration of their feeding pattern, consuming 8% more of their total calories during the inactive (light) phase ($P < .05$; Figure 3, C and D).

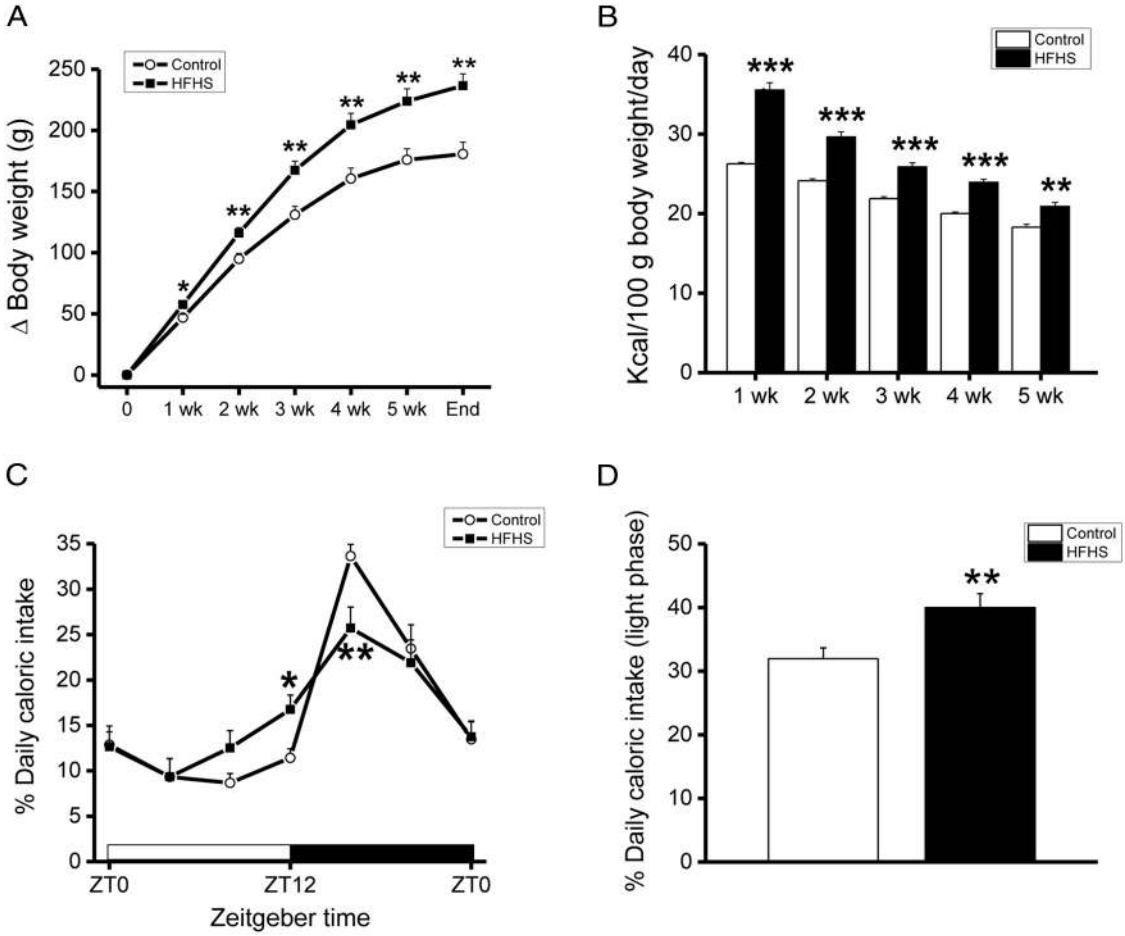

Figure 3. Weekly increment in body weight (A) and average daily caloric consumption per body weight (B) of rats fed regular chow (control) or a HFHS diet. C and D, Diurnal feeding pattern of these animals (n = 6–7 rats per group). ***, $P < .001$, **, $P < .01$, *, $P < .05$ vs animals fed regular chow.

HFHS alters the diurnal variation in GLP-1 and insulin secretory responses to an OGTT

To determine the effects of nutrient excess on the GLP-1 and insulin responses to identical oral glucose loads, rats on a chow or HFHS diet were studied at the peak (ZT10) and trough (ZT22) of the normal secretory patterns. Rats fed regular chow showed no significant differences in basal GLP-1 (Figure 4A), insulin (Figure 4B), and glucose levels (Figure 4C) between the two time points. Similarly, no differences were detected in the basal GLP-1 and insulin levels of animals fed a HFHS diet. However, these animals showed significantly elevated glucose concentrations at ZT22 ($P < .05$) as compared with the chow-fed rats (Figure 4C), whereas HOMA-IR, an indicator of hepatic insulin resistance, was significantly increased ($P < .05$) at ZT10 (Figure 4D).

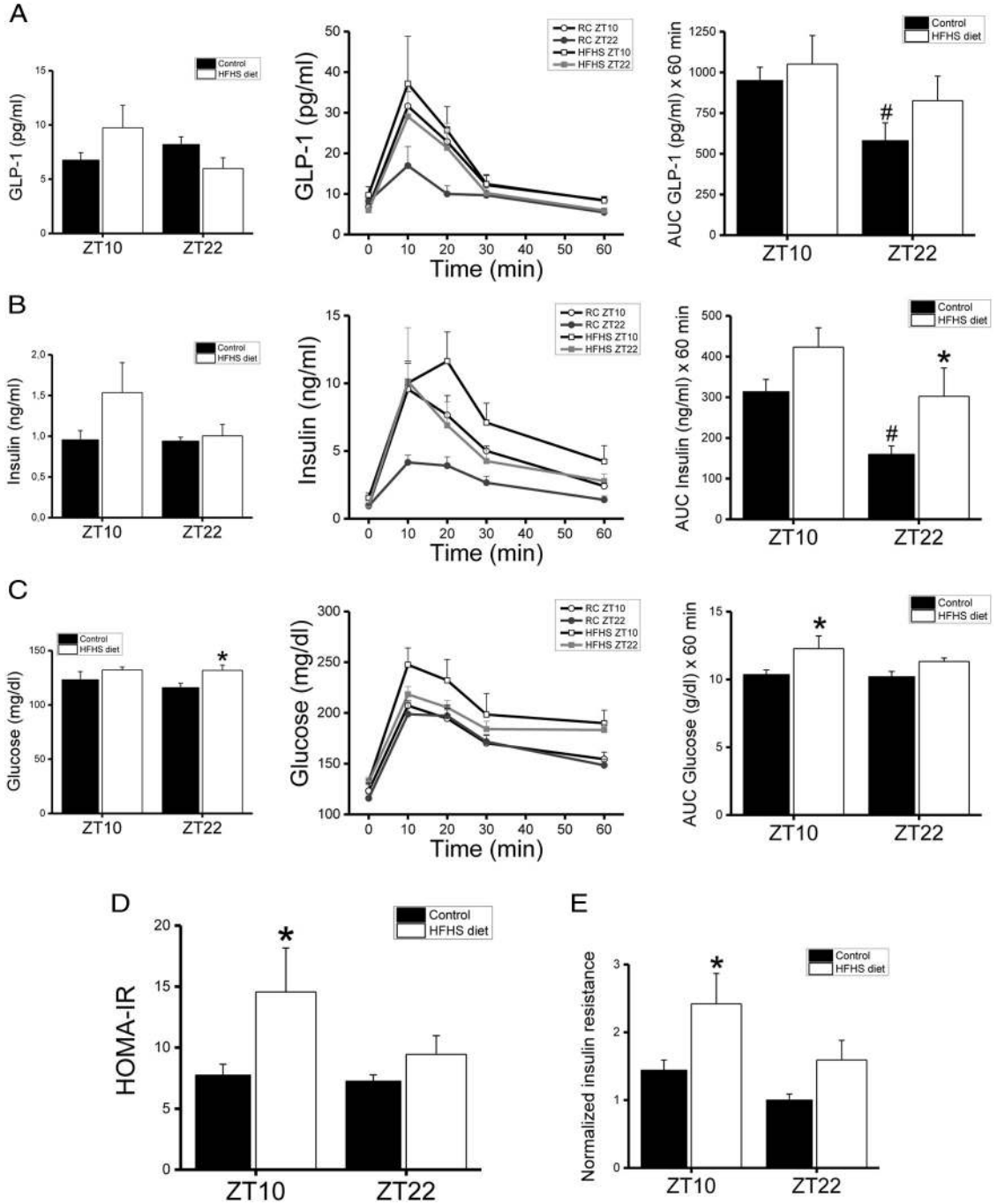

Figure 4. Basal plasma levels and time-course profiles as well as corresponding AUCs of GLP-1 (A), insulin (B), and glucose (C) excursions in response to identical oral glucose loads conducted at two different time points (ZT10 and ZT22) in 4-hour-fasted rats fed regular chow (control) or a HFHS diet for 5 weeks. Hepatic (D) and whole-body insulin resistance (E) of the same animals (n = 6–7 rats per group) are shown. *, $P < .05$ vs the control group; #, $P < .05$ vs the other time point within the same group.

Rats fed regular chow showed the expected diurnal variation in GLP-1 and insulin nutrient-induced responses, with significantly ($P < .05$) enhanced GLP-1 (by 1.6-fold, Figure 4A) and insulin (by 1.9-fold, Figure 4B) secretion at ZT10 as compared with ZT22 in response to identical glucose loads. In contrast, the variation in GLP-1 and insulin secretory responses was totally abrogated in HFHS diet-fed rats, which showed comparable hormone levels at both time points. Moreover, at the trough of the normal daily pattern (ZT22), the insulin response was significantly augmented (by 1.7-fold, $P < .05$) in rats fed a HFHS diet as compared with the control group. Along with the disruption in GLP-1 and insulin secretory patterns observed in the HFHS-fed animals, glucose tolerance was also significantly impaired, such that the glucose levels after the OGTT at ZT10 were significantly higher (by 1.2-fold, $P < .05$) in these animals with respect to chow-fed rats (Figure 4C). In association with the impairment in glucose tolerance, whole-body insulin resistance was also significantly elevated at ZT10 (by 1.7-fold, $P < .05$) in rats fed the HFHS diet (Figure 4E).

High-fat diets have previously been linked to alterations in the molecular clock of metabolic tissues, including liver and fat (14, 15). The effects of the HFHS diet on the expression of clock genes were therefore examined in the ileal mucosa, the section of the intestine in which GLP-1-secreting cells are most abundant (29). In chow-fed rats, the mRNA levels of three representative clock genes (*Bmal-1*, *Per-2*, and *Rev-erb[alpha]*) were found to oscillate, with levels of *Bmal-1* showing an antiphase pattern with respect to *Per2* and *Rev-erbα* (Figure 5, A–C), as previously reported (20). Unexpectedly, this pattern was not disrupted in the HFHS rats. Finally, no obvious patterns or effects of diet were detected at the same time points in the mRNA levels of *proglucagon*, the gene encoding GLP-1 (Figure 5D).

Pretreatment with palmitate disrupts the L-cell molecular clock

To examine the effects of a specific component of the HFHS diet on the molecular clock of the L-cell, murine GLUTag cells were incubated with palmitate, the most

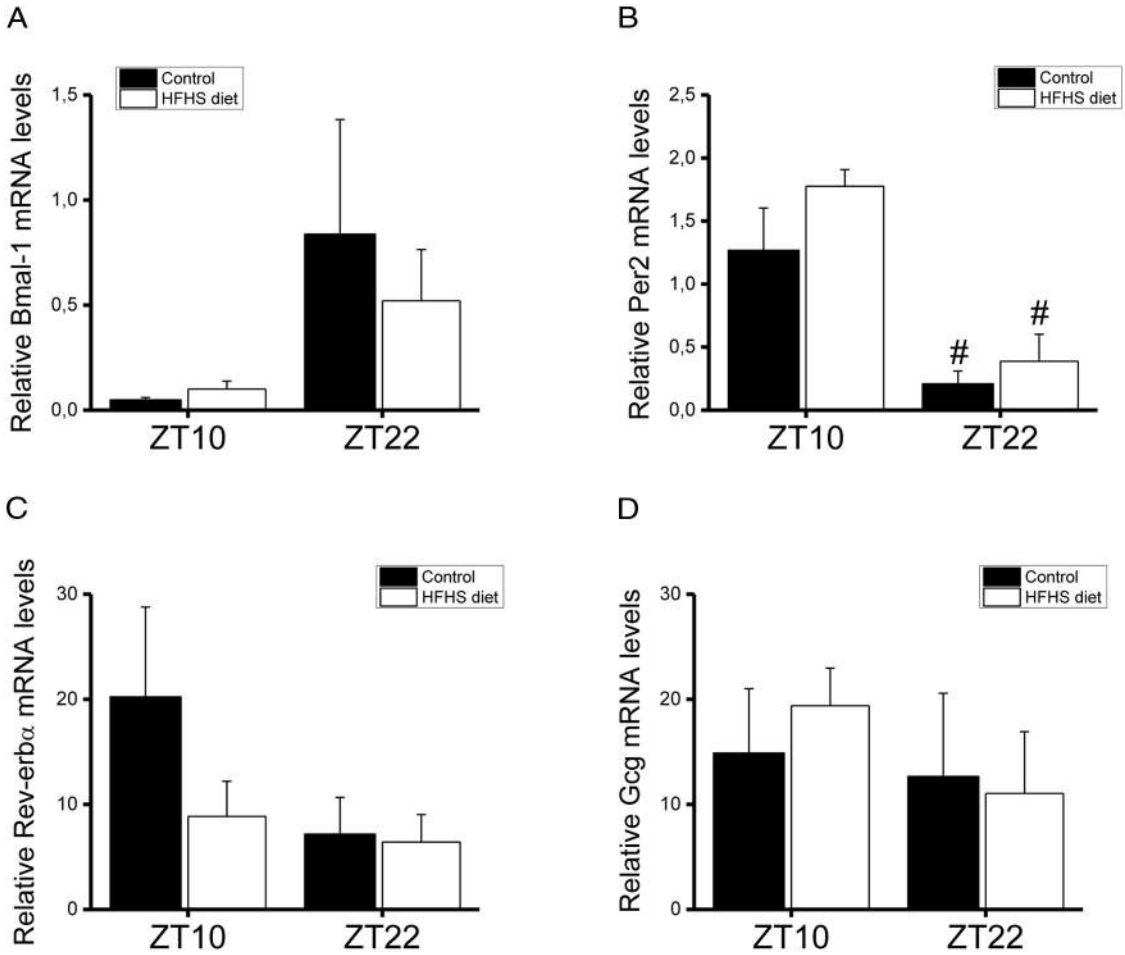

Figure 5. Relative mRNA levels of *Bmal-1* (A), *Per2* (B), *Rev-erbα* (C), and *proglucagon* (D) in the intestinal mucosa of rats fed regular chow (control) or a HFHS diet for 5 weeks (n = 3–5 rats per group). #, $P < .05$ vs the other time point within the same group).

abundant saturated fatty acid in the circulation of obese animals (30), and which has been previously demonstrated to alter the function of other cell-autonomous clocks (16–18). A dose of 0.5 mM palmitate was used to mimic the circulating levels of free fatty acids in obese animals (31). After pretreatment with vehicle, synchronized GLUTag cells showed the expected antiphasic fluctuations in the mRNA levels of two canonical clock genes, *Bmal-1* and *Per-2* (Figure 6, A and B), as previously reported (20). When the patterns of expression of both genes were fit to a 24-hour period curve, correlation coefficients of 76% and 92% were obtained, respectively, indicating a bona fide circadian periodicity. In contrast, the same fitting approach failed in palmitate-incubated cells (Figure 6, A and B), which showed reduced correlation coefficients (37% and 57%, respectively), suggesting an alteration in the circadian expression of these genes. A role for palmitate as a disruptor of the cell-autonomous clock in GLUTag cells was further supported when the pattern of expression of both clock genes was adjusted to the best-fitting approach. Thus, in vehicle-treated cells, the pattern of expression of *Bmal-1* fit (R^2 = 79%) to a waveform function with a period of 29.5 ± 4.2 hours and an amplitude of 1.8 ± 0.3. However, the period was slightly longer (34.0 ± 8.8 h) and the amplitude was significantly reduced (0.6 ± 0.2; $P < .05$) in the cells that were incubated with palmitate (Figure 6A). Similarly, the pattern of *Per-2* expression, which, in vehicle-treated cells, was fit (R^2 = 91%) to a waveform function with a period of 23.8 ± 0.8 hours and an amplitude of 1.1 ± 0.1, was markedly impaired by palmitate incubation, showing a significant ($P < .05$) reduction in both parameters (20.7 ± 1 h and 0.69 ± 0.1 h, respectively, Figure 6B). Overall, our data indicate that the activity of the cell-autonomous clock in the GLUTag cells is significantly attenuated by pretreatment with palmitate.

Because palmitate can cause lipotoxicity (32, 33), GLUTag cells were exposed to a range of palmitate concentrations over a 48-hour period. The highest dose of palmitate tested (2.5 mM) significantly ($P < .01$) reduced cell viability by 24%, but lower concentrations, including 0.5 mM palmitate, had no significant effect on the viability of the GLUTag cells (Figure 7A). Furthermore, palmitate pretreatment actually induced a consistent elevation in the mRNA levels of *proglucagon* in the GLUTag cells ($P < .05$, Figure 7B).

Pretreatment with palmitate disrupts GLP-1 secretory responses

To determine whether palmitate alters the L-cell response to known secretagogues, GLUTag cells were pretreated with vehicle or 0.5 mM palmitate and the secretory response to bethanechol and GIP were examined at time 4 hours after synchronization, which represents the peak of

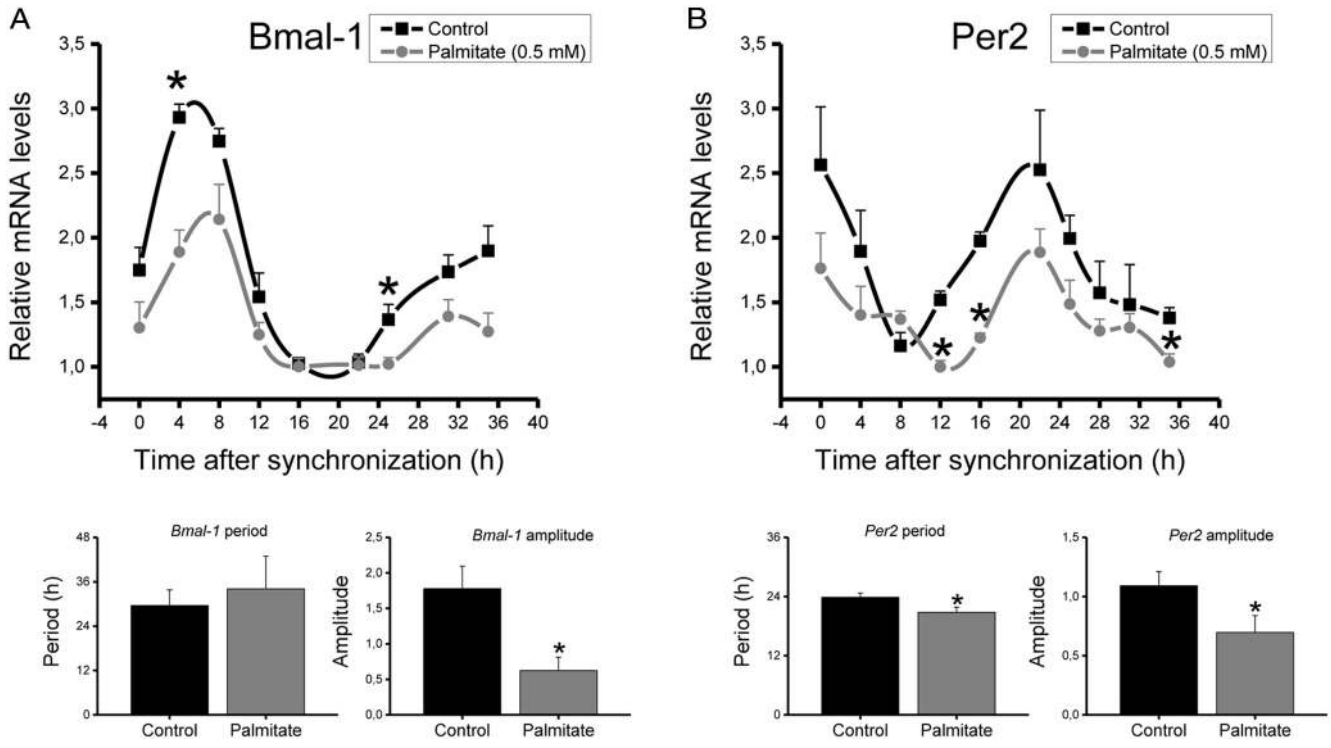

Figure 6. Rhythmic fluctuations in the mRNA levels of *Bmal-1* (A) and *Per2* (B) in synchronized GLUTag cells incubated under control conditions (black lines) or pretreated with 0.5 mM palmitate (gray lines). The time point with the lowest average value was set as 1 for each gene. The insets at the bottom of each graph represent the period and the amplitude of the waveform function calculated by nonlinear curve fitting for each pattern of expression (n = 4). *, $P < .05$ vs the control group.

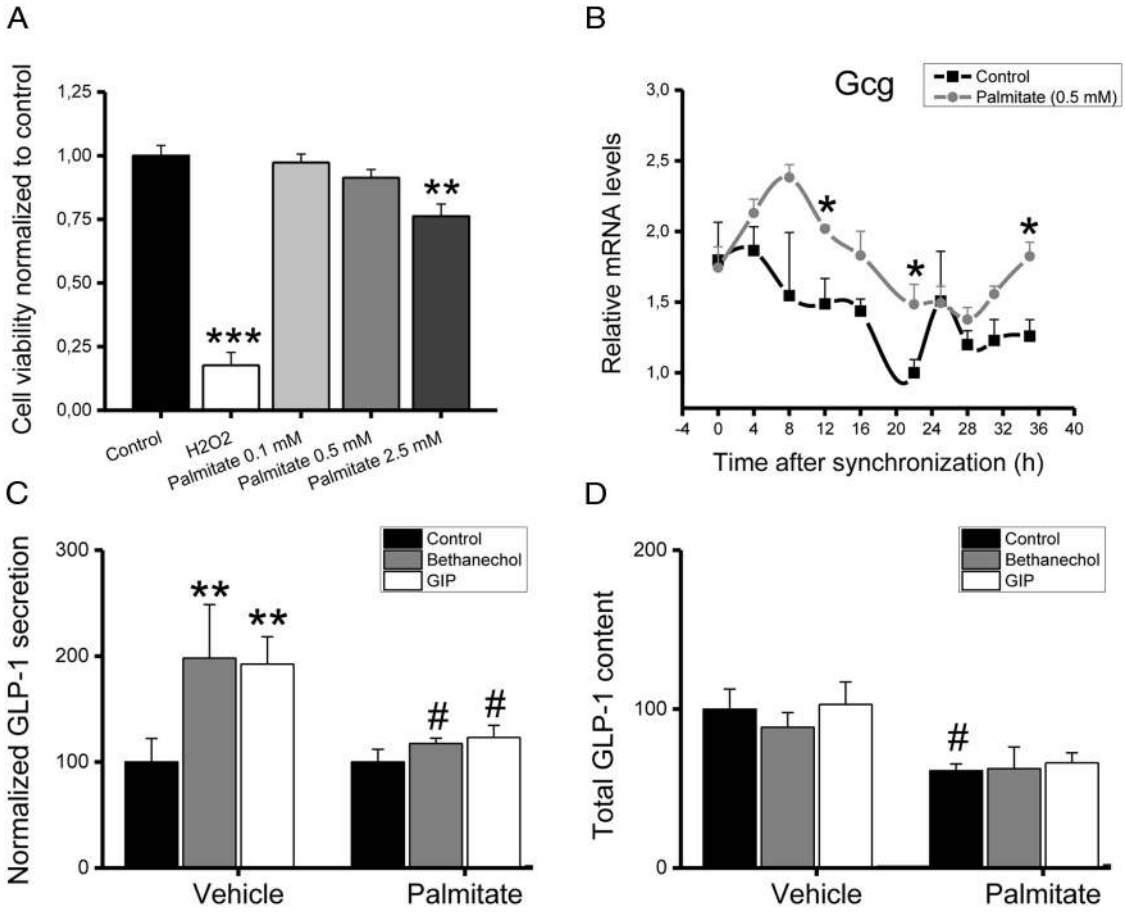

Figure 7. Cell viability determined by a neutral red viability assay in GLUTag cells exposed to a range of palmitate concentrations for 48 hours (A; n = 8). ***, $P < .001$, **, $P < .01$ vs the control wells. B, Relative mRNA levels of *proglucagon* in GLUTag cells pretreated with 0.5 mM palmitate or vehicle (control) (n = 4). *, $P < .05$ vs the control group). GLP-1 secretory response to bethanechol and GIP (C) and total GLP-1 content (D) of GLUTag cells pretreated with 0.5 mM palmitate or vehicle (control; n = 4) are shown. **, $P < .01$ vs the control group; #, $P < .05$ vs the same treatment after the vehicle preincubation.

the rhythm in GLP-1 responses (20). Both bethanechol and GIP were found to stimulate GLP-1 release by vehicle-pretreated cells ($P < .01$, Figure 7C). Basal levels of GLP-1 in the media were not significantly affected by palmitate pretreatment (187 ± 19 pg/well vs 232 ± 37 pg/well; P = NS). However, whereas both bethanechol and GIP stimulated GLP-1 release by vehicle-pretreated cells ($P < .01$), the response to these secretagogues was significantly impaired ($P < .05$) in the cells pretreated with palmitate (for both absolute GLP-1 levels, not shown, and when normalized to control values; Figure 7C). Unexpectedly, despite the enhanced expression of proglucagon that was observed in the palmitate-pretreated cells, significant ($P < .05$) reductions in total GLP-1 content were also observed in these cells (Figure 7D), suggesting a posttranscriptional effect of palmitate on GLP-1 biosynthesis.

Discussion

It has recently been demonstrated that the activity of the rodent intestinal L-cell is regulated in a circadian manner and that the normal pattern in the GLP-1 responses to ingested nutrients is highly correlated to the pattern in the insulin responses (20). Furthermore, circadian disruption associated with alterations in the timing of food intake, such as induced by daytime feeding, perturbs the patterns in both GLP-1 and insulin secretion, leading to the suggestion that the feeding-fasting cycle is a major zeitgeber for this incretin axis (20). However, glucocorticoids are known to be one of the principal entrainment factors for peripheral clocks (11, 34) and the variations in glucocorticoid levels govern the metabolic rhythms in many metabolic tissues (35, 36). Additionally, the saturated fatty acid, palmitate, has been shown to alter circadian clock activity in several metabolic cell types (16–18). Hence, the aim of the present study was to determine the impact of these factors on the circadian regulation of the GLP-1-insulin axis in rats.

Interestingly, the normal peak in corticosterone levels closely corresponds with the timing of the highest GLP-1 and insulin secretory responses in rats (20, 23), suggesting that circulating corticosterone levels may be one of the

factors contributing to the pattern in GLP-1 secretion. However, herein we provide evidence that the diurnal variation in GLP-1 and insulin nutrient-induced responses is maintained after suppressing the circadian pattern in corticosterone levels, thereby precluding a preponderant role of glucocorticoids as an entrainment factor for the rhythmic activity of the L-cell. Of importance, corticosterone treatment did not alter either the timing of or total caloric intake by the rats. However, a permissive role for corticosterone in GLP-1 release cannot be discounted because corticosterone levels were not reduced by implantation of the pellets but remained constant within the physiological range, and indeed, significantly enhanced GLP-1 responses were found at the peak (ZT10) of the normal diurnal pattern in L-cell sensitivity in corticosterone pellet-implanted animals. Nonetheless, the potentiation of GLP-1 secretion promoted by long-term corticosterone treatment may not be due to a direct effect on the L-cell because, in contrast to our findings in vivo, previous in vitro studies have found reduced GLP-1 secretion in response to prolonged incubation of GLUTag cells with dexamethasone along with no effect in GLP-1 responsiveness after incubation with antagonists of the glucocorticoid receptor (37). Further studies are therefore required to determine the potential indirect mechanism mediating the effect of prolonged corticosterone treatment on L-cell responsiveness.

Previous reports have identified obesity and nutrient excess as particularly strong disruptors of circadian behavioral rhythms and cell-autonomous clocks in metabolic tissues (14, 15). In the present work, our rat model of dietary fat and sucrose excess demonstrated several of the metabolic alterations characteristic of severe obesity (ie, hyperglycemia, hyperinsulinemia, and insulin resistance). More importantly, rats fed a HFHS diet showed total abrogation of the diurnal patterns in GLP-1 and insulin nutrient-induced secretory responses, with comparable GLP-1 secretion detected at both the peak and nadir of the normal daily pattern, suggesting that the rhythmic regulation of L-cell activity is clearly affected under these conditions. Because we have previously shown that daytime feeding inverts the peak GLP-1 and insulin responses to an OGTT (ie, to highest levels at ZT22 instead of ZT10) but does not abolish the diurnal patterns, this finding expands the number of circadian-related perturbations that affect the GLP-1-insulin axis to include dietary composition. This conclusion is further supported by our observations that chronic exposure to palmitate attenuates the self-sustained oscillation of clock genes in GLUTag cells and profoundly impairs the GLP-1 secretory responses to well-established L-cell secretagogues. Alternatively, the disruption in the diurnal variation of GLP-1 responses might also be a consequence of the disorganization in the feeding regimen found in HFHS diet-fed animals; nonetheless, our data demonstrate that obesogenic diets are detrimental to rhythmic regulation of L-cell activity. Whether the disruption of GLP-1 secretory patterns may eventually lead to deficient GLP-1 secretion if the metabolic insult is maintained, as is commonly found in patients with type 2 diabetes (38) and in obese nondiabetic subjects (39, 40), remains to be clarified.

Of note, together with suppression of the diurnal variation in GLP-1 responses, the daily pattern in insulin responses was also abolished in our HFHS diet-fed animals, in agreement with previous studies showing parallel disruptions in the secretory rhythms of both hormones in response to circadian disruption (20). Because the existence of an inherent molecular clock in the β-cell, essential for the regulation of insulin production and secretion, is well established (5, 41), the physiological relevance of the GLP-1 diurnal pattern with respect to the rhythmic activity of the β-cell remains as an interesting point of discussion. We (20) have previously shown that, during an ip glucose tolerance test, in the absence of stimulated GLP-1 secretion, the rhythm in glucose-stimulated insulin release is abolished; however, the normal pattern in insulin levels is restored when GLP-1 is coadministered with the ip glucose load. Hence, GLP-1 appears to be a major physiological entrainment factor for the functional clock in the β-cell. Nevertheless, one limitation of the present study is our lack of a determination of GIP levels, the second major incretin hormone, which, in rodents, also stimulates GLP-1 release (42). Therefore, a potential role for GIP in the daily pattern of both GLP-1 and insulin responses remains to be clarified.

In contrast to what might be expected, the daily patterns in GLP-1 and insulin nutrient-induced responses described herein were not associated with a parallel pattern in glucose tolerance, indicating that other factors, such as insulin sensitivity, may be modulating glucose disposal. Accordingly, the calculation of two well-validated surrogate measures of insulin resistance, homeostatic model assessment and Matsuda's composite index, showed a consistent trend of higher insulin resistance in the late afternoon (ZT10). In support of this, previous studies using the gold standard methodology for the calculation of insulin sensitivity, the euglycemic-hyperinsulinemic clamp, have demonstrated that glucose disposal is significantly potentiated during the dark period in rodents (43). Thus, it seems that the patterns in GLP-1 and insulin secretory responses follow the daily variation in insulin resistance to optimize nutrient disposition. In agreement with this hypothesis, the up-regulation of insulin-secretory responses is a well-known adaptive response in the settings of acute

insulin resistance (44). Our observation that alterations in the normal pattern of GLP-1 responses after different types of circadian disruption, such as consumption of an obesogenic diet (present study), continuous light exposure, or feeding at inappropriate times (20), are associated with significantly impaired glucose tolerance, supports the importance of maintaining robust rhythms in L-cell activity for a healthy disposition of nutrients. Importantly, the present study demonstrates that the alterations in GLP-1 secretory rhythm induced by circadian disruption are not produced by changes in the stress hormones. Finally, it remains unclear as to whether mimicking the normal rhythm in GLP-1 levels may improve the efficacy of long-acting GLP-1 receptor agonists and GLP-1/GIP degradation (dipeptidyl peptidase IV [DPP IV]) inhibitors in patients with type 2 diabetes. However, a recent study has demonstrated that sitagliptin, a commonly used DPP IV inhibitor, functions mainly through the prevention of endogenous GLP-1 degradation after its release from the L-cell but prior to its diffusion into the circulation (45). It therefore appears likely that DPP IV inhibitors already enhance the diurnal rhythms in GLP-1 release that have been demonstrated in healthy subjects (46).

As observed in most metabolic tissues, the intestinal mucosa expresses self-sustained circadian oscillators (3, 4, 20). Consistent with these reports, we found that *Bmal-1* expression at ZT10 and ZT22 was antiphasic to that of *Per2* and *Rev-erbα*. However, no significant differences between control and HFHS diet-fed rats were detected in any of the three clock genes examined. In contrast, pretreatment of GLUTag L-cells with palmitate induced profound derangements in the rhythmic expression of circadian oscillators, both attenuating the amplitude of the fluctuations and shortening their period. Because L-cells are only a minor fraction of the epithelial cells in the intestinal mucosa, a specific effect of the HFHS diet on the molecular clock of the L-cell in vivo cannot be ruled out. Furthermore, in agreement with previous findings showing no direct relationship between the pattern of GLP-1 secretory responses and proglucagon expression (20), no significant differences in the transcript levels of *proglucagon* were detected between ZT10 and ZT22 in either control or HFHS diet-fed animals.

Plasma levels of nonesterified fatty acids commonly range between 0.1 and 1 mM in healthy individuals, with higher levels observed during the fasting state, although levels are often elevated in obesity (47); a similar profile is observed in rats on a fat-enriched diet (31). Moreover, because the high-fat diet used in the present study was lard based and saturated fatty acids grossly represent 50% of lard composition, it seemed reasonable to assume that the L-cells, which directly face the intestinal lumen, may be exposed to high concentrations of palmitate. Consistent with our findings in HFHS diet-fed rats, we found that pretreatment of the GLUTag cells with palmitate induced profound derangements in the rhythmic expression of circadian oscillators, both attenuating the magnitude of the fluctuations in the mRNA levels and shortening their period. Although it was previously suggested that long-term palmitate incubation may promote apoptosis in GLUTag cells, this toxic effect of palmitate was demonstrated in the setting of very low (0%–2%) serum concentrations (32, 33). In our hands, in the context of normal serum levels for these cells (10%), only the highest dose of palmitate tested (2.5 mM) reduced cell viability after a 48-hour incubation period. It therefore seems unlikely that the observed effects of palmitate on the expression of clock genes were provoked by an increased rate of cell death. Finally, disruption of metabolic clocks has been previously associated with defective hormone secretion in different endocrine cells (5, 9, 41), and we have demonstrated that the silencing of circadian-regulated genes in the L-cell is also linked to the down-regulation of GLP-1 secretory responses (20). We therefore hypothesized that the malfunction in the internal clock of the L-cell induced by palmitate exposure may lead to defective GLP-1 secretion.

Consistent with this postulate, we found that short-term palmitate pretreatment was detrimental for the secretory activity of the L-cell, reducing the sensitivity of these cells to a subsequent stimulus, in agreement with a previous report (48). Alternatively, although not necessarily incompatible with this hypothesis, the deficient GLP-1 response of these cells may be secondary to the diminished cellular GLP-1 content induced by palmitate pretreatment.

In summary, our data show that an intact rhythm in corticosterone levels is not required for the diurnal variations in the GLP-1 and insulin nutrient-induced responses in rats, thereby supporting a role for the feeding-fasting cycle as the main entrainment factor for the daily pattern in GLP-1 secretory responses. Furthermore, in the setting of a hypercaloric and obesogenic diet, the normal pattern is disrupted, such that comparable GLP-1 levels are observed at both the normal peak and nadir. This alteration in the rhythmic activity of the L-cell may be a consequence of the malfunction of its internal molecular clock provoked by the exposure to high doses of palmitate. Because the normal diurnal pattern in insulin responses is also disrupted by an obesogenic diet in rats, our findings suggest that GLP-1 is a potential mediator of the metabolic alterations triggered by circadian disruption.

Acknowledgments

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