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Context: Many biological pathways involved in regulating substrate metabolism display rhythmic oscillation patterns over a 24-hour period. In rodents, clock genes regulate circadian rhythms of metabolic genes and substrate metabolism. However, the inter-relationships among substrate metabolism, metabolic genes, and clock genes have not been fully explored in people.
Objective: We tested the hypothesis that diurnal expression pattern of pyruvate dehydrogenase kinase 4 (*PDK4*), a key metabolic enzyme involved in fuel switching between glucose and free fatty acids (FFA), is associated with plasma FFA concentration and clock genes.
Design and Methods: We analyzed peripheral blood mononuclear cells (PBMC), subcutaneous adipose tissue, and plasma samples obtained serially over 24 h from metabolically-healthy women (n=10, BMI=28.0 ± 1.1) and evaluated the inter-relationships among *PDK4*, plasma FFA, and clock genes. We also determined the potential mechanisms responsible for *PDK4* transcriptional regulation by using primary human PBMC and adipocytes.
Results: We found *PDK4* diurnal expression patterns were similar in PBMC and adipose tissue (ρ=0.84, P<0.001). The diurnal variation in PBMC *PDK4* expression correlated more strongly with plasma FFA (ρ=0.86, P<0.001) and insulin (ρ=0.63, P<0.001) concentrations than clock genes. Data obtained from primary human PBMC and adipocyte culture experiments demonstrated that FFA directly induced *PDK4* gene expression (P<0.001), at least in part, through activation of peroxisome proliferator-activated receptor alpha (PPAR-α).
Conclusions: Our results suggest that plasma FFA availability is an important regulator of diurnal expression patterns of *PDK4*, and identify a novel interaction between plasma FFA and cellular diurnal rhythms in regulating substrate metabolism.

This study demonstrates a novel interaction between plasma FFA availability and circadian rhythms in cellular gene expression of factors that regulate substrate selection in people.

INTRODUCTION

Daily energy homeostasis is maintained by alterations in the use of exogenous and endogenous fuels in response to nutrient availability and hormonal control during the feeding-fasting cycle (1,2). In the postprandial state, insulin stimulates glucose uptake and oxidization and enhances lipogenesis and triglyceride storage in adipose tissue. In the postabsorptive state, lipolysis of adipose tissue triglycerides releases free fatty acids (FFA) into the circulation, which are then preferentially used for mitochondrial β-oxidation and ATP production. Accordingly, the key biological pathways involved in regulating substrate metabolism display rhythmic oscillation patterns over a 24-hour period (1,3-5).

Pyruvate dehydrogenase kinase 4 (PDK4) is a key mitochondrial enzyme that is involved in regulating the shift in substrate oxidation between carbohydrate and fat as needed in response to the metabolic environment (6). PDK4 inactivates the pyruvate dehydrogenase complex and inhibits the entry of pyruvate into the TCA cycle, thereby preventing the oxidation of glucose and promoting the oxidation of FFA. Dysregulation of *PDK4* expression is associated with alterations in substrate metabolism that occur in people with obesity, insulin resistance, and type 2 diabetes (7,8). Data from studies conducted in rodents have shown that clock genes (2,9,10), master regulators of circadian rhythm, are directly involved in controlling circadian variations in *Pdk4* gene expression and whole-body and cellular substrate metabolism (11-13). Although data from several studies have found that clock genes oscillate in human tissues and cells (4,14-17), the diurnal regulation of *PDK4* and its relation with substrate metabolism and clock genes in people are not known.

The purpose of this study was to investigate the mechanisms responsible for the diurnal expression pattern of *PDK4* in metabolically-healthy people throughout the normal 24-h fed and fasted conditions. We hypothesized that diurnal variation in *PDK4* expression is associated with diurnal variations in plasma FFA concentrations and clock genes. Peripheral blood mononuclear cells (PBMC), adipose tissue, and plasma samples were obtained serially for 24h to determine the inter-relationships among diurnal variations in expression of *PDK4* and clock genes and plasma FFA and insulin concentrations. In addition, we determined the potential mechanisms responsible for *PDK4* transcriptional regulation by conducting studies in primary PBMC and human subcutaneous preadipocytes.

MATERIAL AND METHODS

Study subjects

Ten women who were overweight participated in this study (Table 1). All subjects had normal oral glucose tolerance and ≤ 1 metabolic syndrome criteria. Adipose tissue and blood samples analyzed for this study were obtained while subjects participated in a study that involved evaluating diurnal variations in insulin sensitivity (4). Written informed consent was obtained from all subjects before their participation in this study, which was approved by the Institutional Review Board of Washington University School of Medicine.

PBMC isolation and adipose tissue biopsies

Subjects were admitted to the Clinical Research Unit (CRU) in the evening the day before the study, consumed a standard dinner at 1800 h, and fasted until the next morning. At 2100 h, a Teflon catheter was inserted into a radial artery for blood sampling. Subjects were instructed to sleep at 2200 h and were awakened between 0530 h and 0600 h the next morning. During the day of the study, subjects ingested three identical liquid meals, which were consumed within 20 min at 0700 h (breakfast), 1230 h (lunch), and 1900 h (dinner). Each meal was comprised of 55% of total energy as carbohydrates, 15% as protein, and 30% as fat, and contained one-third of each subject's estimated total daily energy requirement, calculated as 1.2 x measured resting energy expenditure, determined by using a metabolic measurement cart (TrueOne 2400; ParvoMedics, Sady, UT). All meals were prepared in the metabolic kitchen of the CRU. To minimize the impact of physical activity, subjects were asked to rest in bed during the study. Blood samples were obtained through the radial artery catheter every 3 hours from 0600h to 0300h and at 0500h (0600 h, 0900 h, 1200 h, 1500 h, 1800 h, 2100 h, 2400 h, 0300 h, 0500 h) and PBMC were isolated through a density gradient centrifugation by using the Histopaque-1077 (#10771; Sigma, St. Louis, MO). Abdominal subcutaneous adipose tissue samples were

obtained every 6 hours (0600 h, 1200 h, 1800 h, 2400 h). Each abdominal subcutaneous adipose tissue sample was obtained from a different periumbilical quadrant to avoid the potential confounding effects of post-traumatic injury and inflammation on our outcome measures. After the biopsy site was cleaned and sterilized, the skin and underlying tissues were anesthetized by cutaneous and percutaneous injection of 1% lidocaine. Abdominal subcutaneous adipose tissue was aspirated through a 4-mm liposuction cannula (Tulip Medical Products, San Diego, CA) connected to a 30cc syringe from the periumbilical area. Tissue samples were immediately rinsed with ice-cold saline, and frozen in liquid nitrogen until subsequent analyses.

Real-time PCR

Total RNA was isolated from PBMC, adipose tissue, and cultured primary cells by using RNeasy Mini kit (Qiagen, Valencia, CA) or Trizol reagent (Invitrogen, Carlsbad, CA). Real-time PCR was performed on clock genes (*CLOCK*, brain and muscle Arnt-like protein-1 [*BMAL1*], Period 1 [*PER1*], Period 2 [*PER2*], and Rev-ErbA [*REV-ERBα*]), and *PDK4*. Gene expression was determined by using an ABI 7500 real-time PCR system (Invitrogen) with SYBR Green or TaqMan (Invitrogen) as we previously described (4,18). We purchased predesigned TaqMan probes from Invitrogen. Sequences of the primers are provided in Supplemental Table 1. The expression of each gene was normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All PBMC *PDK4* gene expression data are provided in Supplemental Table 2.

Plasma FFA, insulin, cortisol, and glucose concentrations

Plasma FFA was measured as previously described (19). Plasma insulin and cortisol concentrations were measured by using electrochemiluminescence technology (Elecsys 2010, Roche Diagnostics) (4,18). Plasma glucose concentration was determined by using an automated glucose analyzer (Yellow Spring Instruments Co, Yellow Springs, OH).

Primary culture of PBMC and human subcutaneous preadipocytes

PBMC were isolated from blood samples obtained from healthy non-obese people (BMI 22.7 ± 1.6 kg/m^2), after subjects fasted overnight. Isolated PBMC were immediately cultured in RPMI-1640 medium containing glucose (5 mmol/L), glutamine (2 mmol/L), and 0.5 % fatty acid-free, low endotoxin (≤ 0.1 ng/mg), BSA (#A8806; Sigma) (basal medium). After a 3-hour incubation with the basal medium, cells were treated with basal medium containing physiological concentrations (50 or 250 μmol/L) of fatty acids (palmitate [#P9767; Sigma]: oleate [#O7501; Sigma]: linoleate [#L8134, Sigma], 2:2:1 ratio), physiological (0.1 or 1 nmol/L) or supra-physiological (10 nmol/L) concentrations of insulin, a peroxisome proliferator-activated receptor alpha (PPAR-α) antagonist GW6471 (10 μmol/L) (#4618; Tocris Bioscience, Ellisville, MO), or combination of them for 3 hours. All culture media were sterilized by using membrane filtration before usage.

Primary human subcutaneous preadipocytes were purchased from Lonza (#PT-5020; Walkersville, MD). Human preadipocytes were exposed to differentiation medium containing FBS and other growth factors, such as insulin and dexamethasone (#PT-8002; Lonza), and differentiated to mature adipocytes according to the manufacture's instruction. Differentiated cells were incubated in DMEM medium (#D6046; Sigma) containing 1% FBS and 0.5% fatty acid-free, low endotoxin, BSA for 24 hours before they were treated with FFA (250 μmol/L), insulin (10 nmol/L), or both for 3 hours.

Statistical Analyses

Shapiro-Wilk was used to verify normality of the data. One-way repeated measures analysis of variance (ANOVA) was used to evaluate diurnal variations (time effects). A cosinor analysis was performed by using the CircWave v1.4 software to assess the diurnal rhythms of *PDK4* and clock genes expression. Spearman's correlation coefficient (ρ) was calculated to examine correlations between outcomes of interest. Comparisons among three or more groups were performed by using one-way ANOVA followed by the Tukey's *post-hoc* test. Data are presented as mean ± SEM. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Diurnal expression pattern of PDK4 in PBMC is strongly correlated with plasma FFA and insulin concentrations in metabolically-healthy people

The expression of clock genes (*PER1*, *PER2*, and *REV-ERBα*) and *PDK4* showed distinct diurnal variations in PBMC obtained from metabolically-healthy women (Figure 1) (P<0.05, one-way repeated measures ANOVA). PBMC *PER2* and *PDK4* had robust diurnal rhythms (P<0.001, cosinor analysis). We found that PBMC *PDK4* expression was moderately but significantly correlated with *PER2* (ρ=0.27, P=0.009) and *REV-ERBα* (ρ=0.28, P=0.008) (Supplemental Figure 1). Plasma concentrations of FFA and insulin showed reciprocal diurnal changes during the 24-h feeding-fasting cycle (Figures 2A and B). Diurnal variations in PBMC *PDK4* expression and plasma FFA concentration were nearly identical (Figure 2C) and strongly correlated with each other (ρ=0.86, P<0.001) (Figure 2D). Moreover, there was a robust correlation between PBMC *PDK4* expression and plasma FFA concentrations in each participant (Supplemental Tables 2 and 3). We found a significant, but weaker, correlation between PBMC *PDK4* expression and the inverse values of plasma glucose concentrations (ρ=0.47, P<0.001) (Supplemental Figure 2). PBMC *PDK4* expression also correlated strongly with the inverse values of plasma insulin concentrations (ρ=0.63, P<0.001) (Figures 2E and 2F and Supplemental Tables 3). Although we found a robust diurnal variation in plasma cortisol concentrations (P<0.001, one-way repeated measures ANOVA), PBMC *PDK4* expression did not correlate with plasma cortisol concentrations (ρ=0.08, P=0.43) (Supplemental Figure 3).

FFA induces PBMC PDK4 gene expression by activating PPARα

To determine the mechanism responsible for the observed relationships between *PDK4* and plasma FFA and insulin concentrations, we isolated PBMC from metabolically-healthy people and developed a primary human PBMC culture system that included the physiological range of FFA and insulin concentrations. We found that physiological concentrations of FFA (50-250 μmol/L) markedly (~66 fold) increased PBMC *PDK4* gene expression in a dose-dependent manner (Figure 3A). In contrast, insulin treatment did not affect PBMC *PDK4* gene expression at physiological (0.1 or 1 nmol/L) and even supra-physiological (10 nmol/L) concentrations (Figure 3B). FFA-induced PBMC *PDK4* gene expression was inhibited by a PPAR-α antagonist GW6471, but not insulin (Figure 3C), suggesting that FFA directly induces PBMC *PDK4* gene expression, at least in part, through activation of PPAR-α.

Adipose tissue PDK4 expression is associated with plasma FFA concentrations and PBMC PDK4 expression.

The expression of clock genes (*CLOCK*, *BMAL1*, *PER1*, *PER2*, *REV-ERBα*) showed diurnal variations (P<0.05, one-way repeated measures ANOVA) and rhythms (P<0.05, cosinor analysis) in subcutaneous adipose tissue (Figure 4). The anti-phase expression patterns of *BMAL1* and *PER1/PER2* were consistent with those observed in PBMC (Figure 1) and the expression patterns of clock genes were also similar to those previously found in human adipose

tissue (14,17). Adipose tissue *PDK4* expression also displayed a robust diurnal variation (P<0.001, one-way repeated measures ANOVA) and rhythm (P=0.003, cosinor analysis) (Figure 4). Adipose tissue *PDK4* expression correlated with *PER1* (ρ=0.58, P<0.001) and *PER2* (ρ=0.62, P<0.001) (Figure 5A and 5B). There was a stronger positive correlation between adipose tissue *PDK4* expression and plasma FFA concentrations (ρ=0.80, P<0.001) (Figure 5C) and between adipose tissue and PBMC *PDK4* expression (ρ=0.84, P<0.001) (Figure 5D and Supplemental Table 3). In addition, we found a significant, but weaker, correlation between adipose tissue *PDK4* expression and the inverse values of plasma insulin concentrations (ρ=0.47, P=0.002) and plasma cortisol concentrations (ρ=0.37, P=0.019) (Figures 5E and 5F). However, adipose tissue *PDK4* expression was not correlated with the inverse values of plasma glucose concentrations (ρ=0.06, P=0.71) (Supplemental Figure 4). Finally, FFA (250 μmol/L) acutely induced *PDK4* expression and insulin (10 nmol/L) partly inhibited FFA-induced, but not basal, *PDK4* expression in cultured human subcutaneous adipocytes (Figure 5G). Taken together, these results suggest that diurnal variation in adipose tissue *PDK4* expression is, at least in part, regulated by plasma FFA availability.

DISCUSSION

The results from this study demonstrate that *PDK4*, a key mitochondrial enzyme involved in fuel switching between glucose and FFA, oscillates in PBMC and adipose tissue in metabolically-healthy people. Moreover, the diurnal expression patterns of *PDK4* were significantly correlated with plasma FFA and insulin concentrations, and clock genes. Data obtained from primary cell culture experiments demonstrated that FFA induces *PDK4* transcription, at least in part, through PPAR-α signaling. Although several circulating hormones are known to affect *PDK4* expression, such as growth hormone (20,21), thyroid hormone (22), glucocorticoid (23,24), adiponectin (25), and epinephrine (26), our results suggest that plasma FFA availability is a key regulator of diurnal variation of *PDK4* during normal 24-h fed and fasted conditions. These findings demonstrate a novel interaction between plasma FFA concentrations and diurnal variations in cellular gene expression of factors that regulate FFA metabolism in people, and underscore the importance of plasma FFA availability in regulating whole-body and cellular substrate metabolism.

Our findings are consistent with data from previous studies conducted in healthy people that found *PDK4* expression is induced during experimental conditions that increase plasma FFA concentrations, such as a lipid emulsion infusion (27), starvation (28,29), and high-fat diet feeding (30,31). In addition, FFA-induced *PDK4* expression is likely mediated by PPAR-α in PBMC, which is consistent with the results from previous studies that found *PDK4* expression is induced by the selective PPAR-α ligand WY-14643 in human PBMC (32). However, in contrast to the profound inhibitory effect of insulin on *PDK4* expression in human skeletal muscle (30,33), we found insulin had a minimal effect on *PDK4* expression in cultured PBMC and adipocytes. The mechanism responsible for these apparent differences is not known, but could involve cell type-specific differences in *PDK4* transcriptional regulation. Indeed, it was reported that PPARα-independent activation of *PDK4* transcription involves the estrogen-related receptor-α (ERR-α)/peroxisome proliferator-activated receptor gamma coactivator-1 (PGC1A) pathway in myoblasts but not in hepatocytes (34). Therefore, postprandial insulin secretion could inhibit *PDK4* expression in a tissue-specific manner, contributing to the diurnal alterations in whole-body substrate metabolism during the normal fed-fasted cycle. However, we cannot exclude the possibility that our primary culture system does not accurately reflect *in vivo* conditions and that

the presence of insulin or FBS during the adipocyte differentiation process dilutes the effect of insulin on *PDK4* expression in mature adipocytes.

Emerging evidence from mouse models has suggested that clock genes regulate FFA metabolism by modulating transcription of key metabolic enzymes, including *Pdk4* (1,9-11). We found expression of clock genes, such as *PER1*, *PER2*, and *REV-ERBα*, correlated with *PDK4* expression in PBMC and adipose tissue, although these relationships were weaker than those between plasma FFA concentrations and *PDK4* expression. These findings indicate that peripheral clock genes could be involved in regulating diurnal variations in *PDK4* and FFA metabolism in people. In addition, we found adipose tissue *PDK4* expression correlated with plasma cortisol concentrations, suggesting the central circadian clock could be also involved in regulating the diurnal variation of *PDK4* expression in some peripheral organs. A limitation of our study is that we did not use a constant routine protocol, which would have allowed an assessment of the endogenous component of circadian rhythms without environmental influences (35), because we specifically chose to evaluate metabolic outcomes in a real world setting. We cannot exclude the possibility that timing of meals and sampling in relationship to waking-time varied among subjects, or that subjects experienced sleep deprivation during the study, which could have potentially affected clock genes expression. Therefore, it is possible that endogenous expression rhythms of clock genes are more tightly linked to the metabolic regulators, including *PDK4* expression and FFA metabolism during constant feeding and lighting conditions. Additional studies are needed to determine whether misalignment of fasting-feeding or sleep-awake cycles with endogenous circadian rhythms affects diurnal variations of *PDK4* expression and its relationship with FFA metabolism.

In this study, we analyzed the expression of clock genes and *PDK4* in circulating PBMC, because frequent sampling is required to evaluate the full dynamics of diurnal variation in cellular function, which is not possible with tissue biopsies because of the burden on study participants. Our results demonstrate that diurnal expression patterns of *PDK4* in PBMC are significantly correlated with those in adipose tissue. In addition, *PDK4* gene expression patterns observed in PBMC and adipose tissue found in the present study are consistent with the expression pattern observed in skeletal muscle samples that we reported previously (4). Taken together, these results suggest that PBMC can provide an accessible surrogate for other tissues to investigate the diurnal variations in cellular events involved in regulating substrate metabolism.

In summary, the results from the present study suggest that plasma FFA availability is an important physiological regulator of diurnal expression patterns of *PDK4* in both PBMC and adipose tissue. These findings demonstrate the importance of interaction among organ systems in regulating metabolic function and energy homeostasis in people. Additional studies that involve a systems biology approach are needed to determine whether the inter-relationships among diurnal rhythms of clock genes, metabolic genes, and circulating metabolites are altered in people who have metabolic dysfunction, such as those with insulin resistance and type 2 diabetes.

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S.Y. was involved in designing and conducting *in vitro* experiments, sample processing and data analysis. A.C.M. was involved in tissue sample processing and data analysis. P.A. conducted the metabolic studies and data analysis. K.L.S. and M.P.F. were involved in sample processing and data analysis. A.L.O. and B.W.P. contributed to plasma sample processing and data analysis. S.K. obtained funding for the studies, was involved in conducting the metabolic studies and data analysis. All authors reviewed and edited the manuscript. J.Y. is the guarantor of this

work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure 1. Diurnal expression patterns of clock genes and *PDK4* in PBMC. Gene expression of selected clock genes (*CLOCK*, *BMAL1*, *PER1*, *PER2*, and *REV-ERBα*) and *PDK4* normalized to *GAPDH* expression in PBMC obtained from metabolically-healthy women (n=10). Subjects consumed identical meals at 0700 h, 1230 h, and 1900 h. †,‡Values significantly different over time assessed by using one-way repeated measures ANOVA, $^{\dagger}P<0.05$ and $^{\ddagger}P<0.001$. Data are means ± SEM.

Figure 2. Diurnal variation of PBMC *PDK4* expression is associated with plasma FFA and insulin concentrations. Plasma FFA (**A**) and insulin (**B**) concentrations over 24 hours in metabolically-healthy women (n=10). Arrows indicate PBMC collection. Comparison between diurnal variations in PBMC *PDK4* gene expression (red line) and plasma FFA concentrations (blue line) (**C**) and inverse plasma insulin concentrations (**E**) (green line). Relationship between PBMC *PDK4* gene expression and plasma FFA concentrations (**D**) and inverse values of plasma insulin concentrations (**F**). †Values significantly different over time assessed by using one-way repeated measures ANOVA, P<0.001. Data are means ± SEM.

Figure 3. FFA induces *PDK4* gene expression through activation of PPARα in primary PBMC. *PDK4* gene expression in PBMC isolated from metabolically-healthy people after they fasted for ~12 h overnight. PBMC were cultured and treated with FFA (**A**) or insulin (**B**) for 3 hours (n=8 per group). (**C**) Effects of insulin (10 nmol/L) or a PPAR-α antagonist (10 μmol/L GW6471) on FFA-induced PBMC *PDK4* gene expression (n=7 per group). *Value significantly different from corresponding value assessed by using one-way ANOVA followed by the Tukey's *post-hoc* test, P<0.01. Data are means ± SEM.

Figure 4. Diurnal expression patterns of clock genes and *PDK4* in adipose tissue. Gene expression of selected clock genes (*CLOCK*, *BMAL1*, *PER1*, *PER2*, and *REV-ERBα*) and *PDK4* normalized to *GAPDH* expression in subcutaneous adipose tissue obtained from metabolically-healthy women (n=10). †,‡Values significantly different over time assessed by using one-way repeated measures ANOVA, $^{\dagger}P<0.05$ and $^{\ddagger}P<0.001$. Data are means ± SEM.

Figure 5. Adipose tissue *PDK4* gene expression is associated with plasma FFA concentrations and PBMC *PDK4* gene expression. Relationship between adipose tissue *PDK4* gene expression and adipose tissue *PER1* (**A**) and *PER2* (**B**) gene expression, plasma FFA (**C**), PBMC *PDK4* gene expression (**D**), plasma insulin (**E**), and cortisol concentrations (**F**). (**G**) *PDK4* gene expression in differentiated human subcutaneous adipocytes treated with FFA (250 μmol/L), insulin (10 nmol/L), or both for 3 hours (n=3 per group). *,**Values significantly different from corresponding value assessed by using one-way ANOVA followed by the Tukey's *post-hoc* test, $^{*}P<0.05$ and $^{**}P<0.001$. Data are means ± SEM.

Table 1. Study subject characteristics (n=10)

Age (years)	42.3 ± 5.9
Body mass index (kg/m^2)	28.0 ± 1.1
Fat-free mass (kg)	46.1 ± 5.3
Total body fat (%)	40.5 ± 4.6
Visceral adipose tissue volume (cm^3)	524 ± 252
Intrahepatic triglyceride content (%)	1.59 ± 1.51
Glucose (mg/dl)	90.3 ± 5.0
Insulin (mU/l)	7.5 ± 1.1
HOMA-IR	1.79 ± 0.82
Triglyceride (mg/dl)	70 ± 30
HDL-cholesterol (mg/dl)	64 ± 22
LDL-cholesterol (mg/dl)	107 ± 44

Values are means ± SD.

Abbreviations: HOMA-IR, homeostasis model assessment of insulin resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Figure 1

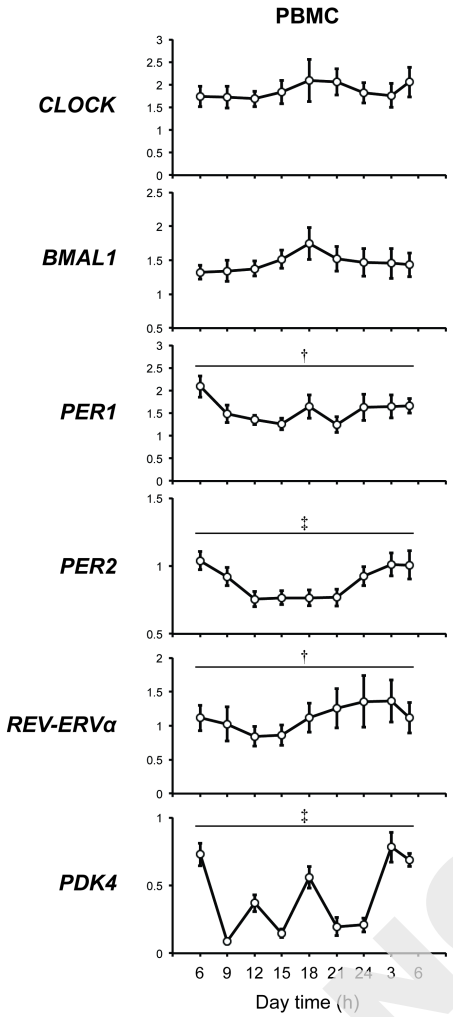

Figure 2

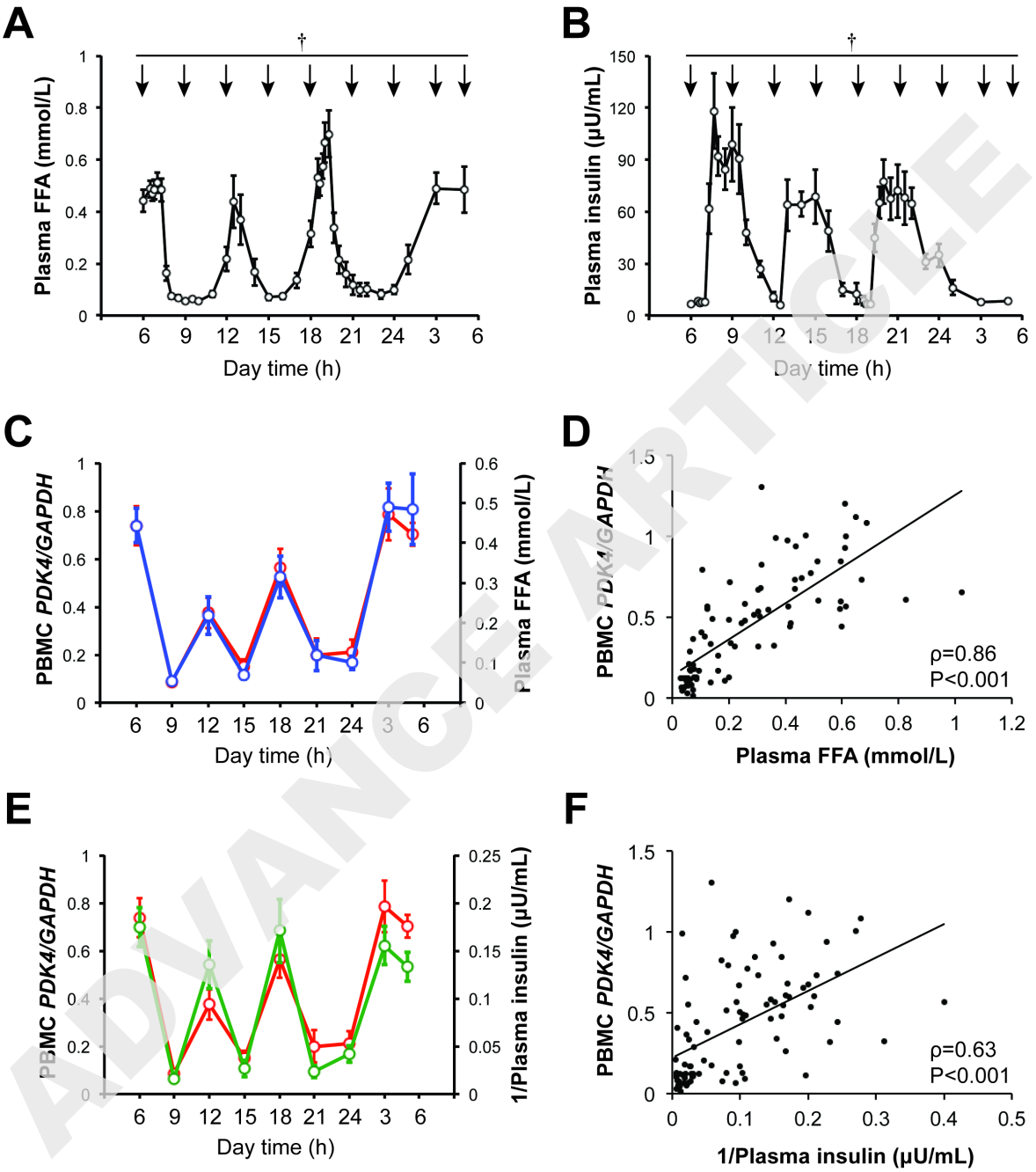

Figure 3

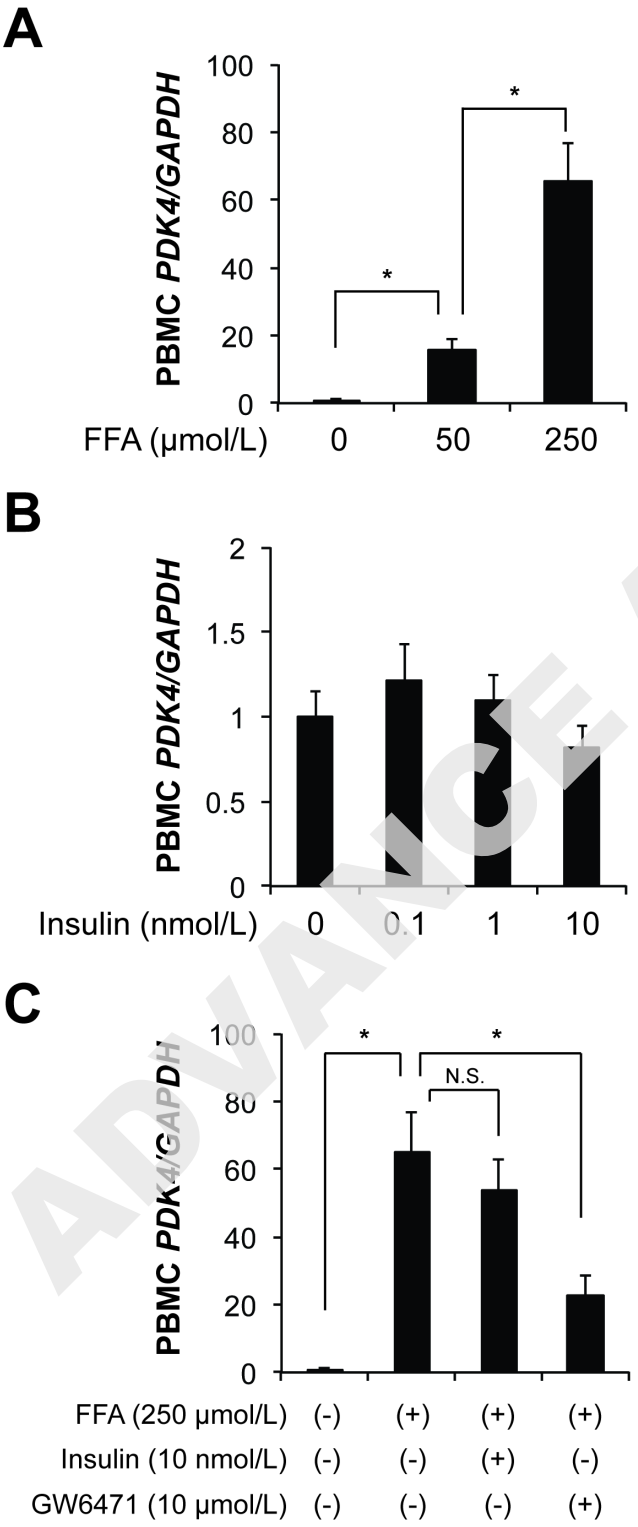

Figure 4

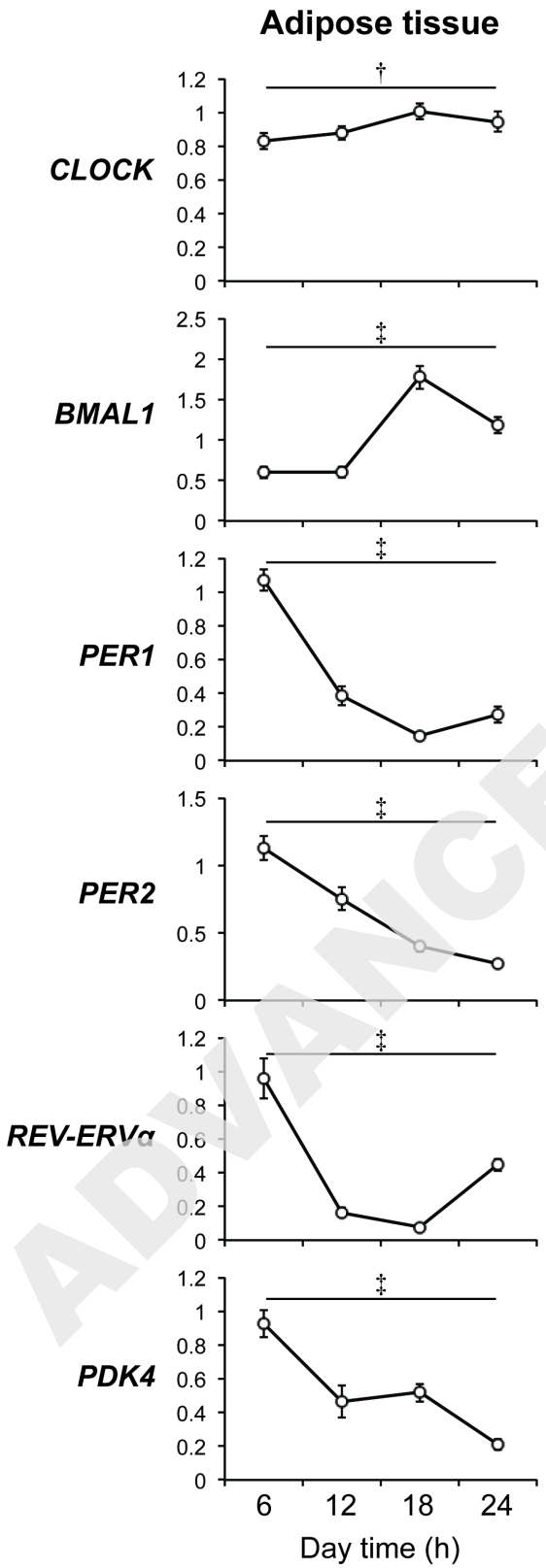

Figure 5