Diurnal Variation in Vascular and Metabolic Function in Diet-Induced Obesity

Divergence of Insulin Resistance and Loss of Clock Rhythm

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Circadian rhythms are integral to the normal functioning of numerous physiological processes. Evidence from human and mouse studies suggests that loss of rhythm occurs in obesity and cardiovascular disease and may be a neglected contributor to pathophysiology. Obesity has been shown to impair the circadian clock mechanism in liver and adipose tissue but its effect on cardiovascular tissues is unknown. We investigated the effect of diet-induced obesity in C57BL6J mice upon rhythmic transcription of clock genes and diurnal variation in vascular and metabolic systems. In obesity, clock gene function and physiological rhythms were preserved in the vasculature but clock gene transcription in metabolic tissues and rhythms of glucose tolerance and insulin sensitivity were blunted. The most pronounced attenuation of clock rhythm occurred in adipose tissue, where there was also impairment of clock-controlled master metabolic genes and both AMPK mRNA and protein. Across tissues, clock gene disruption was associated with local inflammation but diverged from impairment of insulin signaling. We conclude that vascular tissues are less sensitive to pathological disruption of diurnal rhythms during obesity than metabolic tissues and suggest that cellular disruption of clock gene rhythmicity may occur by mechanisms shared with inflammation but distinct from those leading to insulin resistance. ***Diabetes* 62:1981–1989, 2013**

Much research in the field of obesity is directed toward the role of obesity in the development of cardiovascular disease and insulin resistance. A critical early step in atherosclerotic cardiovascular disease is endothelial dysfunction, the hallmark of which is impaired nitric oxide (NO) production by endothelial NO synthase (eNOS) in vascular endothelial cells. We and others have shown that obesity is associated with endothelial dysfunction in human (1,2) and animal studies (3). A further subject of investigation is the sequence of tissue-specific events in obesity that lead to insulin resistance in the canonical insulin-sensitive tissues: liver, adipose tissue, and skeletal muscle. Insulin also acts on the endothelium to promote NO release through a phosphoinositol-3-kinase (PI3K)-dependent pathway (4), and a causal link is established between vascular insulin resistance and endothelial dysfunction (5–7). Insulin resistance thus stands at the crossroads of cardiovascular and metabolic disease.

Circadian rhythms are pervasive in physiological processes. In the cell, timekeeping is maintained by an autoregulatory transcriptional-translational oscillator. The nucleus of this mechanism consists of a positive limb, composed of heterodimers of *BMAL1* (brain and muscle aryl hydrocarbon receptor nuclear translocator [ARNT]-like) with either *CLOCK* (circadian locomotor output cycles kaput) or *NPAS2* (neuronal PAS domain-containing protein), which promote transcription of *PER* (period) and *CRY* (cryptochrome) genes, which then close the negative feedback loop by inhibiting *BMAL1* and *CLOCK* (8). Rhythm is passed downstream through control of transcription of client clock-controlled genes, allowing the clock to influence a wide nexus of cellular physiology. In the endothelium, there is diurnal variation in NO production (9) and endothelial-dependent vascular tone (10,11). Blood pressure (BP) and heart rate dip during the inactive or sleep phase. Responses to glucose and insulin challenge display a clear diurnal pattern (12) and many gate-keeping enzymes in metabolic pathways are under clock control (13). There is some evidence that components of intracellular insulin signaling pathways, such as PI3K and its downstream kinase Akt, are similarly regulated by the clock (14), but diurnal variation in these pathways is incompletely characterized.

Evidence is mounting that normal physiological rhythm may be lost in disease (15). In transgenic mouse models, mutation of core clock genes leads to endothelial dysfunction (14,16) and obesity (17) with abnormal systemic glucose and insulin homeostasis (18). Human genetic studies report associations between polymorphisms of *CLOCK* and obesity (19), and *BMAL1* and diabetes and hypertension (20). Diet-induced obesity in wild-type mice leads to secondary blunting of rhythmic clock gene transcription in liver and adipose tissue (21) and in energy-sensing hypothalamic regions (22). These studies complement old observations of a coexistence between rhythm loss and disease in humans. Obese humans show blunting of the normal diurnal variation in response to glucose challenge (23). Nondipping, or loss of normal diurnal variation in BP, is associated with diabetes (24) and is linked to hypertensive complications (25). There is a rhythm of onset of myocardial infarction, stroke, and other adverse cardiovascular events, which cluster in the

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early hours of the morning when endothelial reactivity falls and BP and hemostatic activity rise (26), which too is lost in diabetes (27).

Although knowledge of the association between rhythm loss and cardiovascular and metabolic disease is longstanding, it remains nonetheless poorly explored. As yet, no studies have examined the effect of obesity upon normal physiological variation in vascular function or upon rhythmic transcription of core clock genes in cardiovascular tissues. It is not known whether tissues vary in their sensitivity to clock disruption in disease. It is unknown whether the loss of clock rhythm occurs in conjunction with other pathological events in obesity, notably insulin resistance and inflammation. In a C57BL6J mouse model of diet-induced obesity, we examined the effect of obesity upon *1*) rhythmic transcription of core clock genes; *2*) diurnal variation in physiological measures of vascular and metabolic function; *3*) rhythmic transcription of metabolic master regulatory genes; *4*) rhythms of AMP-activated protein kinase (AMPK) mRNA and protein; *5*) inflammation; and *6*) insulin signaling and its diurnal variation in tissues.

RESEARCH DESIGN AND METHODS

Animals. Three-week-old male C57BL6J mice were purchased from Harlan Laboratories UK and randomized either to high-fat diet (Bio-serv, Frenchtown, NJ; nutritional content by weight: protein, 20%; fat, 35.5%; carbohydrate, 36.3%; fat content by calories: 60%) or standard laboratory chow (Special Diet Services, Essex, U.K.) as previously described (3,28). Animals were housed in a standard animal facility under controlled temperature and humidity with a regular lighting schedule of 12 h light:12 h dark (lights on 7:00 A.M. and lights off 7:00 P.M.). Experiments were performed after 10 weeks of diet in accordance with U.K. Home Office regulations for animal care. Aortic vasomotion studies, in vivo metabolic tests, and Akt immunoblotting were performed at 8:00 A.M. and 8:00 P.M., which in nocturnal mice correspond to the beginning of the inactive and active phases, respectively. BP measurement, quantitative PCR, AMPK immunoblotting, and plasma insulin measurement were performed at 8:00 A.M., 2:00 P.M., 8:00 P.M., and 2:00 A.M. to yield 24-h expression profiles.

Quantitative PCR. Tissues were snap-frozen in liquid nitrogen and prepared by mechanical homogenization (TissueLyser; Qiagen, Dusseldorf, Germany). RNA was extracted using Trizol reagent (Invitrogen, Paisley, U.K.) and reverse transcribed using a kit (Applied Biosystems, Warrington, U.K.). Quantitative PCR was performed in triplicate in a thermal cycler (ABI Prism 7900HT; Applied Biosystems) using custom-synthesized oligonucleotides (Invitrogen, Paisley, U.K.) with the SybrGreen fluorescent dye system (Applied Biosystems), and results were normalized against the reference gene *Gapdh*. Primer sequences are given in Supplementary Fig. 1.

Ex vivo aortic vasomotion studies. Vasomotor activity was measured as previously described (3,5–7,28,29). The thoracic aorta was debrided of adherent connective tissue and cut into rings of 5-mm length. Rings were mounted in an organ bath apparatus (PanLabs, Barcelona, Spain) immersed in Krebs-Henselheit solution (composition in mmol/L: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $NaHCO_3$ 25, $MgSO_4$ 1.19, $CaCl_2$ 2.5, glucose 11.0) maintained at 37°C and bubbled with 95% O_2/5% CO_2. After incubation at a resting tension of 3 g for 45 min, vasoconstriction was assessed by cumulative stimulation with the α-adrenoceptor agonist phenylephrine (PE) (1 nmol/L to 1 μmol/L). After washout and reincubation at resting tension, rings were preconstricted with 300 nmol/L PE, and endothelial-dependent vasodilation was assessed by cumulative stimulation with the vasodilator acetylcholine (ACh; 1 nmol/L to 1 μmol/L). Insulin-mediated vasodilation was examined by incubation for 2 h with insulin (100 mU/mL; Actrapid) followed by cumulative stimulation with PE. Basal NO release was examined by incubation with the nonspecific NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA; 0.1 mol/L) followed by cumulative stimulation with PE.

Noninvasive BP and heart rate measurement. Systolic and diastolic BP and heart rate were measured by tail-cuff plethysmography (Kent Scientific, Torrington, U.K.) as previously described (3,5–7). Conscious mice were placed in a cylindrical restraining device on a warmed holding platform, and mean values for systolic and diastolic BP and heart rate were calculated from 30 recorded cycles. Three training sessions were performed before measurements were obtained.

Metabolic profiling. In vivo metabolic testing was performed as previously described (3,5–7,28,29). For glucose tolerance testing, mice were fasted for 12 h, followed by intraperitoneal injection of 1 mg/kg glucose. For insulin tolerance testing, mice were fasted for 4 h, followed by intraperitoneal injection of 0.75 units/kg insulin (Actrapid; NovoNordisk, Bagsvaerd, Denmark). Whole-blood glucose was determined sequentially by tail vein sampling using a portable meter (Accu-chek Aviva; Roche Diagnostics, Burgess Hill, U.K.). For plasma insulin quantification, blood was collected by terminal cardiac puncture and plasma was obtained by centrifugation. Insulin was measured using an ultrasensitive mouse ELISA kit (CrystalChem, Downers Grove, IL) as previously described (6,7,28,29).

Protein immunoblotting. Protein was extracted in lysis buffer by mechanical homogenization and quantified according to colorimetric assay (BCA protein assay kit, Calbiochem, San Diego, CA). Protein (30 μg) was loaded into 4–12% Bis-Tris gels (NuPage; Invitrogen Life Sciences, Carlsbad, CA), separated by electrophoresis, and blotted onto polyvinylidene fluoride membrane as previously described (28,29). Blots were probed with primary antibody as follows: eNOS 1:1,000 and Ser1177-phosphorylated eNOS 1:666 (both BD Biosciences, San Jose, CA); AMPK α-subunit 1:5,000 (a gift of Grahame Hardie, University of Dundee, Dundee City, U.K.); Akt 1:1,000 and Thre308-phosphorylated Akt 1:1,000 (both Cell Signaling Technology, Danvers, MA); and β-actin 1:3,000 (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were incubated with 1:1,000 secondary antibody conjugated to horseradish peroxidase (Dako, Glostrup, Denmark) and visualized with SuperSignal West Pico chemiluminescent substrate (ThermoFisher Scientific, Rockford, IL). Protein band densitometry data were normalized against β-actin.

Statistical analysis. Data are expressed as mean ± SEM. Analysis was performed by unpaired Student *t* test or ANOVA with Bonferroni post hoc correction as indicated. Significance was accepted at $P < 0.05$.

RESULTS

After 10 weeks of high-fat diet, animals were significantly obese in comparison with chow-fed controls (41.8 ± 0.3 vs. 29.8 ± 0.2 g, $P < 0.0001$) (Supplementary Fig. 2*A*). Abdominal obesity was pronounced (epididymal fat pad mass 1.30 ± 0.02 vs. 0.34 ± 0.01 g, $P < 0.0001$) (Supplementary Fig. 2*B*), and obese animals were markedly hyperinsulinemic over the 24-h day in comparison with controls (Supplementary Fig. 2*C*).

Rhythmic transcription of core clock genes is not impaired in cardiovascular tissues of obese mice but is attenuated in some metabolic tissues. There was no significant impairment of cycling of *Bmal1* and *Per2* in aorta (Fig. 1*A*). Adipose tissue showed marked and significant attenuation of cycling of *Bmal1* (two-way ANOVA interaction $F = 7.24$, $P < 0.01$; obesity $F = 4.86$, $P < 0.05$; time $F = 441.16$, $P < 0.001$) and *Per2* (two-way ANOVA interaction $F = 6.64$, $P < 0.01$; obesity $F = 56.25$, $P < 0.001$; time $F = 44.58$, $P < 0.001$) (Fig. 1*C*), but there was no significant disruption of rhythm in liver or muscle (Fig. 1*B* and *D*). There was no diurnal variation in the reference gene *Gapdh* and its expression did not differ between obese and lean animals (data not shown).

Diurnal variation in cardiovascular physiological measures is preserved in obesity. Lean aortas showed diurnal variation in constriction to stimulation with PE (maximal constriction at 8:00 A.M. 1.10 ± 0.08 vs. 0.94 ± 0.05 g at 8:00 P.M.) (Fig. 2*A*), and this variation was not attenuated by obesity (maximal constriction at 8:00 A.M. 0.54 ± 0.03 vs. 0.44 ± 0.04 g 8:00 P.M.; ANOVA $P < 0.001$) (Fig. 2*A*). Obese aortas showed marked impairment of constriction to PE, which was reversed by stimulation with the nonspecific NOS inhibitor L-NMMA (constriction at 8:00 A.M.: obese 1.03 ± 0.07 with and 0.49 ± 0.07 g without L-NMMA; lean 1.37 ± 0.08 with and 0.99 ± 0.11 g without L-NMMA; $P < 0.001$; data not shown). Expression of inducible NO synthase (iNOS) mRNA was increased in obese aortas over 24 h (data not shown). The daily pattern of BP did not differ significantly between obese and lean animals (Fig. 2*B*). Heart rate was significantly elevated in obese animals

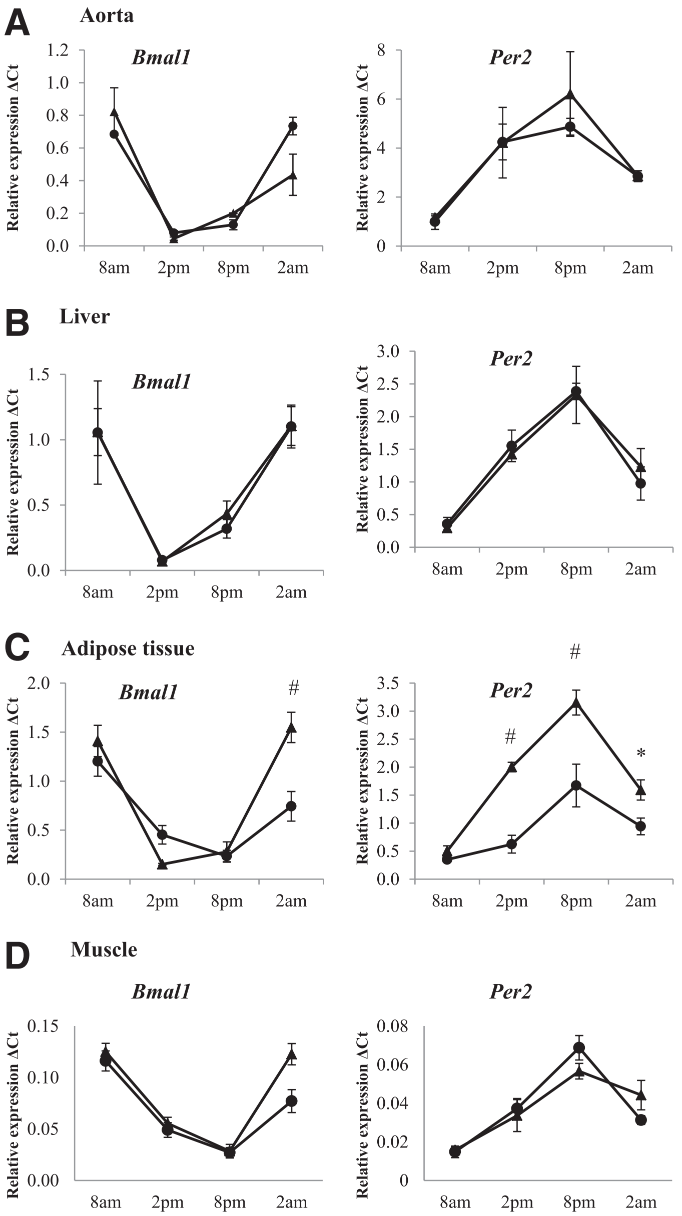

FIG. 1. The effect of obesity on rhythmic transcription of the core clock genes *Bmal1* and *Per2* in vascular and metabolic tissues. Aorta (*A*); liver (*B*); adipose tissue (*C*); skeletal muscle (*D*). Results from obese animals are denoted by circles and lean by triangles. Two-way ANOVA with Bonferroni post hoc correction: *P < 0.05 and #P < 0.001; n = 4 in each group.

(two-way ANOVA, obesity F =18.27, $P < 0.001$) (Fig. 2*C*) with no impairment of diurnal variation. Diurnal variation in endothelial-dependent vasodilation to ACh was less evident (Fig. 2*D*), but there was statistically significant diurnal variation in the early phase of the curve, which was preserved in obese aortas; upon stimulation with 3 nmol/L ACh, there was a convincing vasodilator response at 8:00 P.M. (13.7 ± 3.3% in lean vs. 13.4 ± 2.4% in obese) but minimal vasodilation at 8:00 A.M. (0% in lean vs. 1.5 ± 0.9% in obese, $P < 0.001$) (Fig. 2*E*). There was no diurnal variation in endothelial-independent vasodilation to the NO donor sodium nitroprusside (data not shown). Both lean and obese aortas showed rhythmic phosphorylation of eNOS with an increase in the ratio of Ser1177 phospho-eNOS to total eNOS at 8:00 P.M.; however, this rhythm was not statistically significant in either group (Fig. 2*F*). Phospho-eNOS abundance was mildly reduced in obesity at both time points. Obese aortas were insulin resistant and showed no significant attenuation of constriction in response to insulin treatment at either time point (Fig. 2*G*). There was no evident diurnal variation in the magnitude of response to insulin in lean aortas.

Diurnal variation in systemic glucose and insulin homeostasis is impaired in obesity. A distinct diurnal pattern of response to glucose challenge was seen both in lean and obese mice (ANOVA $P < 0.001$) (Fig. 3*A*); at 8:00 A.M., fasting blood glucose was lower but 30-min glucose peak was higher than at 8:00 P.M. Diurnal variation was further expressed as the percentage difference between the maximal 30-min glucose peaks attained at 8:00 A.M. vs. 8:00 P.M. (Fig. 3*B*). Obese animals showed blunting of this diurnal variation in peak glucose (Student t test $P < 0.05$). Insulin sensitivity also showed diurnal variation with increased sensitivity to insulin challenge at 8:00 P.M., both in lean and obese animals (ANOVA $P < 0.001$) (Fig. 3*C*). The percentage difference between the 60-min glucose nadirs attained at 8:00 A.M. vs. 8:00 P.M. revealed significant blunting of diurnal variation in insulin sensitivity in obesity (Student t test $P < 0.01$) (Fig. 3*D*).

Rhythmic cellular metabolism is dysregulated in obesity. Dysregulation of rhythmic clock gene transcription in metabolic tissues due to obesity was associated with effects upon downstream clock-controlled genes with major roles in control of glucose and lipid metabolism: *Rev-erbα*, *Dbp* (D-site albumin promoter binding protein), *Pparα* (peroxisome proliferator-activated receptor α), and *Pepck* (phosphoenolpyruvate carboxykinase). In adipose tissue, there was marked and statistically significant attenuation of rhythmic transcription of all genes in obese animals (two-way ANOVA: *Rev-erbα* interaction F = 8.15, $P < 0.001$; obesity F = 14.41, $P < 0.001$; time F = 11.61, $P < 0.001$; *Dbp* obesity F = 76.58, $P < 0.001$; time F = 8.52, $P < 0.01$; *Pparα* interaction F = 4.22, $P < 0.05$; obesity F = 13.69, $P < 0.01$; time F = 5.21, $P < 0.01$; *Pepck* obesity F = 33.76, $P < 0.001$; time F = 3.37, $P < 0.05$) (Fig. 4*B*). Rhythms of AMPK were significantly blunted both in mRNA (two-way ANOVA obesity F = 7.74, $P < 0.05$) and protein (two-way ANOVA interaction P = NS; obesity F = 5.34, $P < 0.05$; time F = 4.92, $P < 0.01$) (Fig. 5*B*), and peak expression of protein lagged 6 h behind that of mRNA. In liver, rhythmic gene transcription was largely unaffected by obesity, with the exception of *Pepck*, which was significantly blunted in obese animals (two-way ANOVA interaction P = NS; obesity F = 8.63, $P < 0.01$; time F = 3.20, $P < 0.05$) (Fig. 4*A*). There were significant differences between obese and lean in protein levels (obesity F = 12.89, $P < 0.001$) but not mRNA of AMPK (Fig. 5*A*).

Expression of adipokines is dysregulated in obesity. We investigated the effect of obesity upon diurnal profiles of transcription of the adipokines leptin and adiponectin, which are known to be under clock control. Transcription of leptin was significantly upregulated in obese animals over the full 24-h day (two-way ANOVA obesity F = 18.76, $P < 0.001$) (Fig. 6*A*) and that of adiponectin was downregulated in comparison with lean (two-way ANOVA obesity F = 5.35, $P < 0.05$) (Fig. 6*B*). Diurnal variation was not impaired.

Local inflammation in obesity is most pronounced in adipose tissue. *F4-80*, a marker of macrophage infiltration, was markedly upregulated in obese adipose tissue but not in other tissues ($P < 0.001$) (Fig. 7*A*). The complement protein *C3* was expressed most strongly in liver, consistent with its predominant hepatic synthesis,

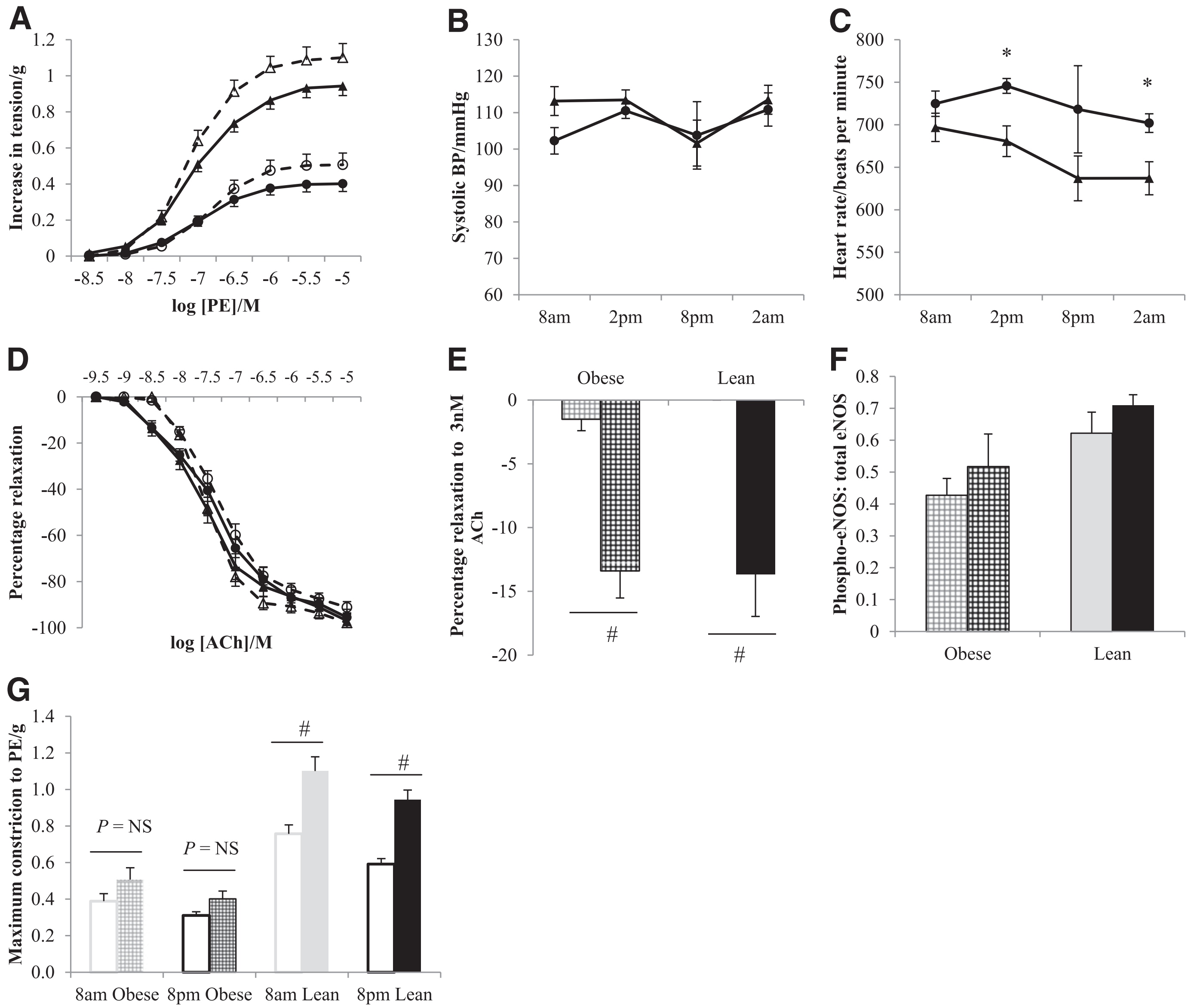

FIG. 2. The effect of obesity on diurnal variation in cardiovascular indices. ***A*****: Diurnal variation in constriction to PE.** ***B*****: Diurnal variation in systolic BP.** ***C*****: Diurnal variation in heart rate.** ***D*****: Diurnal variation in endothelial-dependent vasodilation to ACh.** ***E*****: Diurnal variation in endothelial vasodilation; vasodilator response to 3 nmol/L ACh.** ***F*****: Diurnal variation in eNOS activation (ratio of phospho-eNOS to total eNOS protein).** ***G*****: Diurnal variation in vasomotor response to insulin.** ***A–D*****: Results from obese animals are denoted by circles and lean by triangles, and, where appropriate, values at 8:00 A.M. by open symbols and those at 8:00 P.M. by closed symbols.** ***E–G*****: Obese animals are denoted by checkered bars and lean by solid bars, and 8:00 A.M. values are denoted by gray and 8:00 P.M. by black; in** ***G*****, open bars denote insulin and filled bars vehicle treatment. Two-way ANOVA: *$P < 0.05$ and #$P < 0.001$.** ***A*****,** ***D*****,** ***E*****, and** ***G*****:** ***n*** **= 8–13 per group.** ***B*****,** ***C*****, and** ***F*****:** ***n*** **= 4–6 per group.**

but was upregulated in obesity only in adipose tissue ($P < 0.01$) (Fig. 7*B*). Neither gene showed diurnal variation in transcription in any tissue (data not shown). In aorta, the vascular inflammatory marker *VCAM-1* (vascular cell adhesion molecule 1; $P < 0.05$) (Fig. 7*C*) was upregulated in obesity but *ICAM-1* (intercellular adhesion molecule) (Fig. 7*D*) and *E-selectin* (data not shown) did not differ between obese and lean. Expression of *TNF-α* was markedly and significantly elevated in obese adipose tissue (two-way ANOVA interaction $F = 1.66$, P = NS; obesity $F = 15.48$, $P < 0.001$; time $F = 0.93$, P = NS) (Fig. 7*E*).

Obesity is associated with impairment of insulin signaling in liver and aorta but not in adipose tissue or skeletal muscle. Diurnal variation in insulin signaling did not show statistically significant differences but aorta and adipose tissue showed a trend toward increased Thre308 phosphorylation of Akt at 8:00 P.M. (Fig. 8*A* and *C*). Abundance of the control protein β-actin did not show diurnal variation (data not shown). Impairment of insulin signaling was found both in vascular and metabolic systems, with significantly reduced phospho-Akt abundance in the aorta and liver of obese animals ($P < 0.05$) (Fig. 8*A* and *B*) but no statistically significant changes in adipose tissue or muscle (Fig. 8*C* and *D*). The ratio of phospho-Akt to total Akt was not significantly altered in obesity, since in liver and aorta, abundance of total Akt as well as of phospho-Akt was reduced in obesity (data not shown).

DISCUSSION

This study demonstrates novel findings with regard to the role of circadian clock dysfunction in the pathophysiology

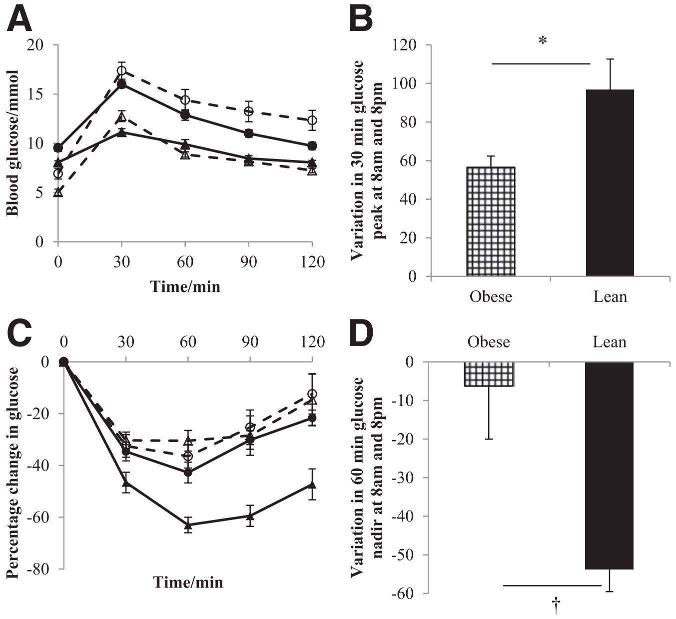

FIG. 3. The effect of obesity on diurnal variation in response to glucose and insulin challenge at 8:00 A.M. and 8:00 P.M. *A*: Glucose tolerance test; diurnal response to intraperitoneal glucose challenge in obese and lean animals. *B*: Glucose tolerance test; diurnal variation in glucose peak at 30 min is blunted in obesity. *C*: Insulin tolerance test; diurnal response to intraperitoneal insulin challenge in obese and lean animals. *D*: Insulin tolerance test; diurnal variation in glucose nadir at 60 min is blunted in obesity. *A* and *C*: Results from obese animals are denoted by circles and lean by triangles and values at 8:00 A.M. by open symbols and those at 8:00 P.M. by closed symbols. *B* and *D*: Obese animals are denoted by checkered bars and lean by solid bars. Student *t* test: *$P < 0.05$ and †$P < 0.01$; $n = 16$ in each group.

of vascular and metabolic disease in obesity. *1*) Loss of diurnal rhythm associated with obesity is found in measured physiological indices in the metabolic system but not in the cardiovascular system, which corresponds to the preservation of core clock gene cycling in vascular tissues but disruption in some metabolic tissues. *2*) Adipose tissue is most vulnerable to clock gene disruption secondary to obesity, which is associated with marked disruption of downstream clock-regulated genes in cellular metabolic homeostasis including *AMPK* and of AMPK protein. *3*) There is divergence between rhythm loss and impairment of tissue insulin signaling, with adipose tissue most sensitive to rhythm loss and liver to insulin resistance, suggesting that insulin resistance and clock gene dysfunction arise by different mechanisms. *4*) Tissue inflammation coincides with rhythm loss, suggesting possible common influences on inflammation and the cellular process of clock gene dysfunction.

Preservation of diurnal variation in cardiovascular measures in obesity. The diurnal variation observed in this study is consistent with previous reports of increased endothelial responsiveness during the active period. Thus at 8:00 P.M., we found reduced vasoconstriction, increased NO-dependent vasodilation, and a trend toward greater phosphorylation of eNOS. The aortic vasomotor phenotype of our obese mice differs from other studies that have reported impaired endothelial-dependent vasodilation, marked eNOS protein dysfunction, hyperconstriction, and hypertension (30,31). However, the findings of our study are consistent with our previous report of an obese vascular phenotype characterized by upregulation of iNOS in obese aortas, leading to increased NO production by iNOS rather than eNOS, hypoconstriction, and an exaggerated response to the NOS inhibitor L-NMMA (3). Tumor necrosis factor-α (TNF-α) and leptin were found to be elevated and make a possible link between obese adipose tissue and the vasculature. Both are circulating mediators secreted by adipose tissue that are known to cause endothelial dysfunction, and furthermore, TNF-α has been shown to induce iNOS expression (32,33).

Two alternatives may explain why diurnal rhythms were preserved in the cardiovascular system despite clear

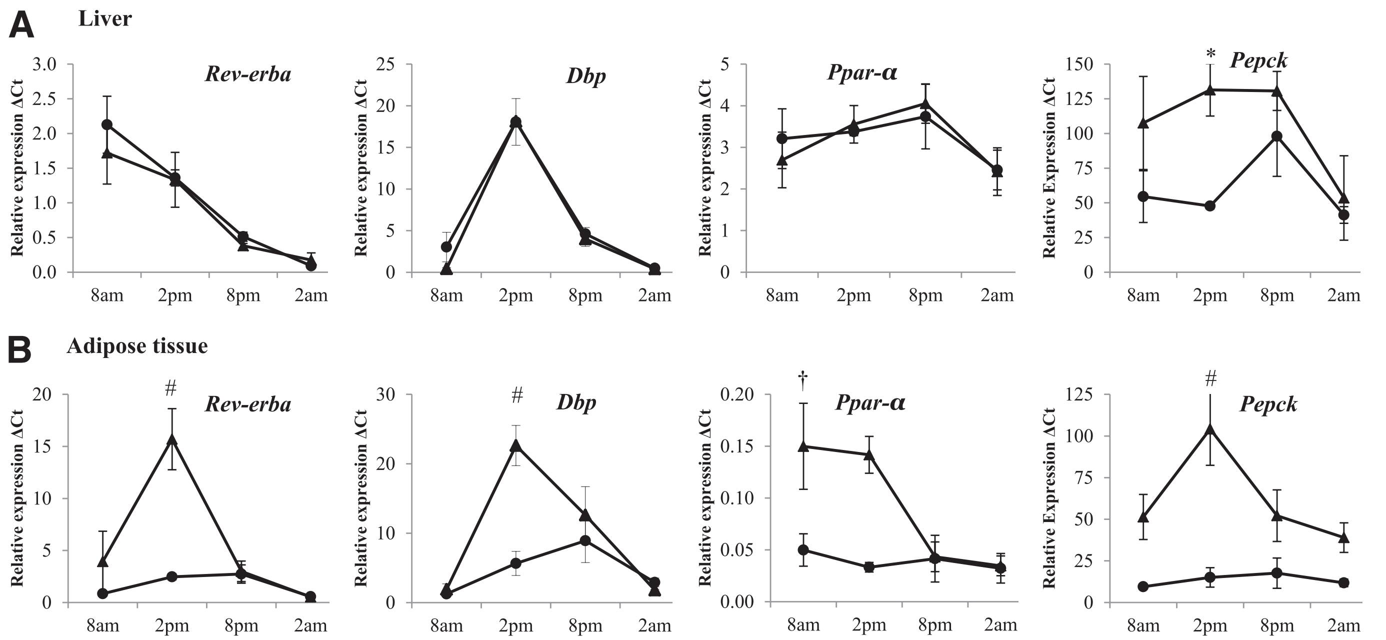

FIG. 4. The effect of obesity on rhythmic transcription of clock-controlled genes regulating glucose and lipid homeostasis. *A*: Liver; *Rev-erbα*, *Dbp*, *Ppar-α*, and *Pepck*. *B*: Adipose tissue; *Rev-erbα*, *Dbp*, *Pparα*, and *Pepck*. Results from obese animals are represented by circles and lean by triangles. Two-way ANOVA with Bonferroni post hoc correction: *$P < 0.05$, †$P < 0.01$, and #$P < 0.001$; $n = 4$ in each group.

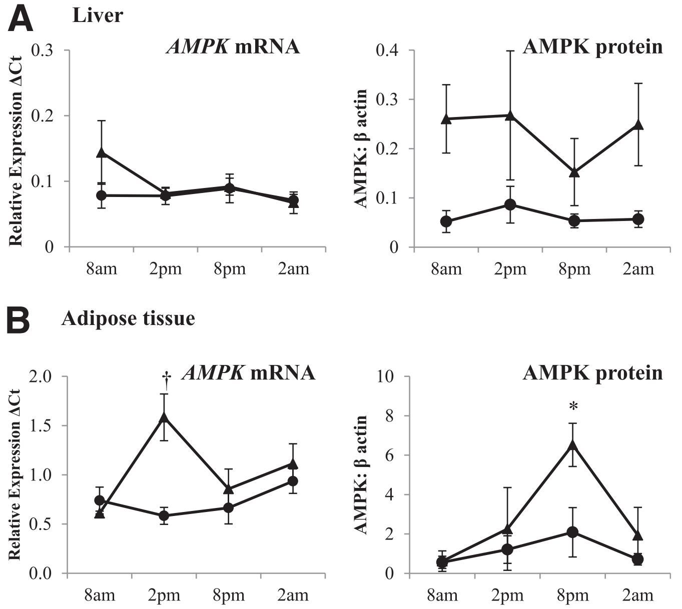

FIG. 5. The effect of obesity on mRNA and protein rhythms of AMPK. ***A*: Liver. *B*: Adipose tissue. Results from obese animals are represented by circles and lean by triangles. Two-way ANOVA with Bonferroni post hoc correction: *P < 0.05 and †P < 0.01; *n* = 4–6 in each group.**

disruption of metabolic indices: vascular tissues are either resilient to disruption of the clock or they require longer exposure to develop the pathological effects of obesity upon diurnal variation. Hsieh et al. (34) found in mice that exposure to long-term high-fat diet for 11 months resulted in disruption of clock gene transcription in the liver and kidney, which was not evident with a shorter duration of obesity. Nondipping BP is associated with diabetes in humans but was not seen in these obese mice despite hyperglycemia and hyperinsulinemia consistent with type 2 diabetes, perhaps because a longer period of obesity is required. It is consistent with the narrative of cardiovascular disease caused by metabolic disease that loss of rhythm should occur first in metabolic tissues and subsequently appear in the vasculature.

Loss of diurnal variation in metabolic indices. Diurnal variation in insulin sensitivity was best seen in responses to metabolic challenge, consistent with previous reports of heightened insulin sensitivity during the active period, but diurnal variation was less clear in aortic vasomotion and Akt signaling. The primary mechanism by which obesity altered Akt signaling appears to be through suppression of the expression of Akt protein and not through impairment of its phosphorylation by upstream kinases in the insulin signaling pathway. Although there was a convincing loss of rhythm in glucose tolerance and insulin sensitivity in obesity, identification of the insulin-sensitive tissue primarily responsible for this defect is complex. Tissue insulin resistance, as measured by impairment of Akt signaling, was greatest in liver, but loss of rhythm was greatest in adipose tissue. Skeletal muscle demonstrated neither insulin resistance nor disruption of clock gene rhythms. The two major tissue events contributing to the loss of systemic insulin sensitivity are held to be failure to suppress hepatic glucose output and impairment of skeletal muscle glucose uptake in response to insulin stimulation, and adipose accounts for ~10% of insulin-induced glucose disposal and is thought to have a minor role in systemic glucose homeostasis (35). Interestingly, rhythmic transcription of *Pepck* was disrupted in liver despite tight preservation of clock gene rhythms in this tissue.

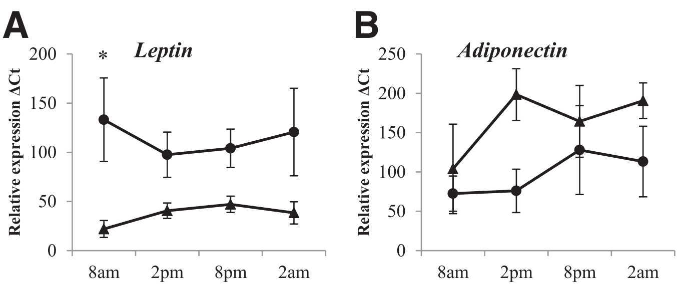

FIG. 6. The effect of obesity on rhythmic transcription of adipokines in adipose tissue. A: *Leptin*. B: *Adiponectin*. Results from obese animals are represented by circles and lean by triangles. Two-way ANOVA with Bonferroni post hoc correction: *P < 0.05; *n* = 4 in each group.

Metabolic master genes and AMPK make a bidirectional link between the clock and metabolism. The link between the clock and cellular metabolism is an evolving area of research. Evidence of the intimate link between clock function and cellular energy balance comes from the discovery of NAMPT, NAD^+ (36), and cAMP (37) as clock inputs and of heme as the ligand activating the accessory clock protein REV-ERBα (38). Numerous master metabolic genes with wide-ranging effects in determining systemic glucose and lipid homeostasis are known to display rhythmic transcription (39), and in this study, we confirm the findings of Kohsaka et al. (21) of the deleterious effect of obesity upon their diurnal profiles in metabolically active tissues. AMPK is of special interest because it embodies the two-way traffic between the clock and cellular redox status. As an energy-sensing kinase, it is activated by a rise in the intracellular AMP: ATP ratio indicative of falling energy stores; by direct binding to clock proteins, it enzymatically alters their stability (40), and transcriptome studies suggest that AMPK itself may be clock controlled (41). Its role in promoting systemic insulin sensitivity and impairment of its expression and activity in diet-induced obesity are recognized (42,43). Although the effect of obesity upon *AMPK* from the perspective of diurnal variation has received some attention (44,45), this study presents the first evidence of AMPK rhythms and loss of these rhythms in obesity. The 6-h lag observed between the peaks of mRNA and protein in this study is consistent with a delay required for translation.

Mechanism of cellular clock disruption in obesity. The finding of divergence of insulin resistance and loss of diurnal rhythm is novel and prompts discussion of how disruption of the clock fits into the context of what is already known about the cellular pathogenesis of obesity. Of the tissues studied, adipose was the most susceptible to cellular clock disruption, which suggests that the local adipose milieu in obesity may be especially pathogenic to the clock. Our finding of divergence between tissue-specific insulin resistance and inflammation is consistent with an elegant study that reported that in diet-induced obesity, insulin resistance developed early in liver but late in adipose tissue, and inflammation was concentrated in adipose tissue (46). Broad convergence of inflammation and clock dysfunction raises the question of whether the two processes may share a common pathway and merits further investigation.

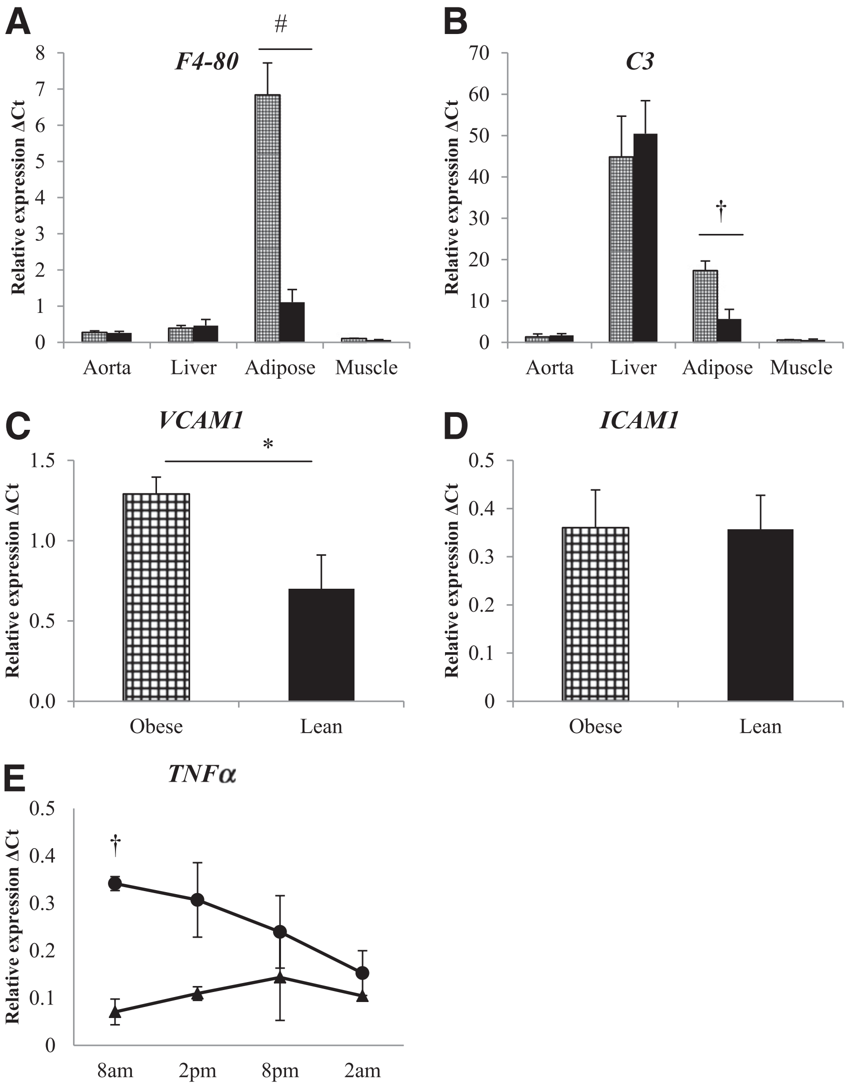

FIG. 7. Expression of inflammatory genes in obesity. *A*: Macrophage marker *F4-80*. *B*: Complement *C3*. *C*: *VCAM-1* in aorta. *D*: *ICAM-1* in aorta. *E*: *TNF-α* in adipose tissue. *A–D*: Results from obese animals are denoted by checkered bars and lean by solid bars. *E*: Results from obese animals are represented by circles and lean by triangles. Student *t* test (*A–D*); two-way ANOVA with Bonferroni post hoc correction (*E*): *$P < 0.05$, †$P < 0.01$, and #$P < 0.001$; $n = 4$ in each group.

Implications of this study. This animal study raises points directly applicable to human disease. Few studies have examined the effect of obesity on human clock gene function because of the requirement for repeated and invasive tissue sampling (47,48). Otway et al. (47) did not find abnormalities of rhythmic transcription of clock genes in adipose tissue of obese humans, although this may be due to sampling of subcutaneous adipose tissue, which is less strongly linked to insulin resistance and dyslipidemia than visceral adipose tissue (49). We found only mild impairment of core clock genes and metabolic genes in subcutaneous adipose tissue (data not shown). Indeed, differences in clock gene expression have been reported between visceral and subcutaneous depots in human adipose explants (50). The prevalence of loss of diurnal rhythm in obesity and type 2 diabetes is not known and it remains to be seen whether it is an integral feature of acquired metabolic disease in human populations. Although loss of rhythm in the cardiovascular system was not found in these mice, it is possible that in humans with chronic vasculopathy, loss of endothelial rhythms may be found in conjunction with atherosclerosis. AMPK is further of interest because several pharmacological agents used in the treatment of diabetes, such as metformin and thiazolidinediones, exert their effects through its activation. Our findings prompt further investigation in human disease.

Conclusion. This study establishes important differences in the susceptibility of vascular and metabolic tissues to pathological loss of diurnal variation in diet-induced obesity. The novel finding that tissue-specific clock disruption occurs in conjunction with inflammation but not with insulin resistance demands further investigation of the underlying cellular mechanisms. We argue that loss of diurnal rhythm is an integral component of the pathophysiology of obesity and deserves closer attention.

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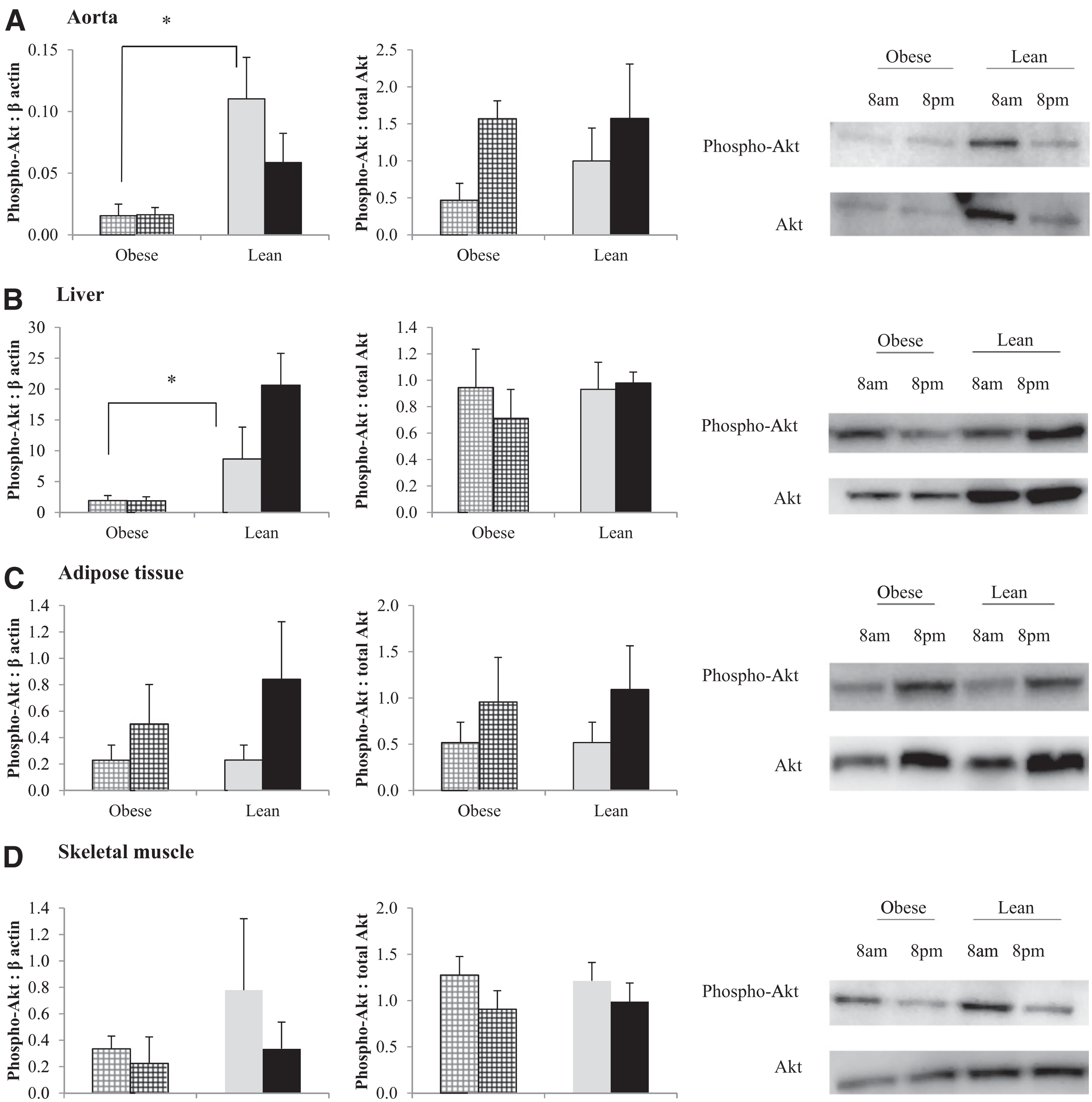

FIG. 8. The effect of obesity on Akt signaling and its diurnal variation at 8:00 A.M. and 8:00 P.M. *A*: Aorta. *B*: Liver. *C*: Adipose tissue. *D*: Skeletal muscle. Data are presented as expression of phospho-Akt normalized to β-actin or as the ratio of phospho-Akt to total Akt. Results from obese animals are denoted by checkered bars and lean by solid bars, and 8:00 A.M. values are denoted by gray and 8:00 P.M. by black. Student *t* test: $*P < 0.05$; $n = 6$ in each group.

No potential conflicts of interest relevant to this article were reported.

M.J.P. designed and performed experiments, analyzed data, and wrote and revised the manuscript. R.S.M. performed experiments, contributed to discussion, and reviewed the manuscript. S.B.W. and M.T.K. contributed to discussion, reviewed the manuscript, and secured funding. P.J.G. and E.M.S. secured funding, supervised the project, and reviewed the manuscript. E.M.S. 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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