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Obesity alters the peripheral circadian clock in the aorta and microcirculation

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Abstract

Perturbation of daily rhythm increases cardiovascular risk. The aim of this study was to determine whether obesity alters circadian gene expression and microvascular function in lean mice and obese (*db/db*) mice. Mice were subjected to normal light-dark cycle or constant darkness (DD) to alter circadian rhythm. Metabolic parameters and microvascular vasoreactivity were evaluated. Array studies were conducted in the AM and PM cycles to assess the rhythmicity of the entire genomics. Rhythmic expression of specific clock genes (*Bmal1*, *Clock*, *Npas2*, *Per1*, *Per2*, and *Cry1*), clock output genes (*dbp*), and vascular relaxation-related genes (*eNOS*, *GTPCH1*) were assessed. Obesity was associated with metabolic dysfunction and impaired endothelial dilation in the microvasculature. Circadian rhythm of gene expression was suppressed 80% in both macro- and microcirculations of obese mice. Circadian disruption with DD increased fasting serum glucose and HbA1C in obese but not lean mice. Endothelium-dependent dilation was attenuated in obese mice and in lean mice subjected to DD. Rhythmic expression of *per1* and *dbp* was depressed in obesity. Expression of *eNOS* expression was suppressed and *GTPCH1* lost rhythmic expression both in obesity and by constant darkness. These results suggest that obesity reduces circadian gene expression in concert with impaired endothelial function. The causal relationship remains to be determined.

Keywords

Circadian disruption; Clock genes; Obesity; Vascular function; eNOS

Introduction

As a consequence of living on a planet that rotates, living organisms are intrinsically programmed to coordinate biological processes to the periodic light cycle. This circadian system acts as a daily endogenous timekeeper that allows organisms to anticipate environmental and internal biological cues and respond accordingly. While light is the primary time keeper for daily rhythms, [9,10,18,34], most peripheral tissues, including cardiovascular organs, contain a circadian clock which acts either in concert or independent of the central clock [19,31]. The circadian clock machinery is controlled by interlocking transcriptional/translational feedback loops based and is driven by the transcription *Clock* and Brain and Muscle Aryl Hydrocarbon Receptor Nuclear Translocator-Like Protein 1 (*Bmal1*) and repressed by the *Period* genes (*Per* 1–3), the *Cryptochrome* genes (*Cry1* and *Cry2*) [24]. While circadian gene oscillations can be controlled by photic signals at the whole body level, in the periphery, non-photic stimuli such as behaviors, physiological cues, signaling molecules, and diet are also involved in circadian regulation [9,18]. These genes can directly drive clock output genes or downstream target genes to influence physiology beyond the sleep-wake cycle [19,23].

Hemodynamic parameters such as blood pressure and heart rate exhibit diurnal variation as does the incidence of cardiovascular events. [14,22,24,25]. Moreover, mutation of clock genes have detrimental effects on vascular function in mice [19]. In terms of metabolism, humans switching from a daytime schedule to a nighttime schedule show higher insulin and glucose levels in otherwise healthy adults, consistent with the known harmful impact of shift

work increased on cardiovascular diseases [11,13,21,32,33]. Obese individuals frequently exhibit erratic eating habits that include late night food excursions which can additionally be associated with sleep apnea and cardiovascular disease [12,15]. Obesity is well documented to impair vascular function in obesity, especially endothelial function.[28]. The extent to which circadian asynchrony contributes this vascular disease is unknown.

The goal of this study was to determine if 1 – obesity impacts circadian gene oscillation across the full spectrum in vascular tissue, 2 – obesity specifically impairs clock gene oscillation in the microcirculation and 3 - the impact of altering circadian rhythm on gene expression and microvascular function in obese mice.

Materials and methods

Animals

All animal studies were performed according to protocol approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved and monitored by the Georgia Regents University, Institutional Animal Care and Use Committee. Male leptin-receptor mutant (*db/db*) mice bred on a C57BL/6 background used as obese controls and dual heterozygous littermates used as lean controls were purchased at 10-11 weeks of age from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in an animal room controlling temperature at 75 ± 5 °F and humidity 50 ± 5% in 12-12-hour light-dark cycle or constant darkness for 4 weeks to alter circadian gene expressions [16]. Standard diet and filtered water were provided *ad libitum*. The animals were divided into four groups: lean mice in light-dark cycle (LM-LD), lean mice in constant darkness (LM-DD), obese mice in light-dark cycle (Ob-LD), and obese mice in constant darkness (Ob-DD).

Array studies

Large scale gene expression analysis was conducted by the Integrated Genomics Shared Resource in the GRU Cancer Center using Affymetrix Technology. Aortae from 5 mice were harvested from control (*db*/+) and obese (*db/db*) at 7 AM and 7 PM. Total mRNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and frozen until analysis. Genes were ranked with exclusion criteria of two-fold difference between AM and PM points. Data were plotted as scatterplots and tabulated for qualitative assessment.

Morphology and Metabolic Parameters

After 4 weeks of light regimens, animals were fasted 12 hours for metabolic testing [5]. Body weight was determined in living animals before anesthetizing with 5% vapor isoflurane and euthanized by decapitation. Blood from aorta was used to determine fasting plasma glucose level with an enzyme-electrode sensor based on enzyme oxidation using an AlphaTRAK glucose meter (Abbott, Chicago, IL, USA) and glycated hemoglobin (HbA1C) with immunoassay method by an A1cNOW+ monitor (Bayer, Tarrytown, NY, USA). The rest of blood was centrifuged at 13,000 × g for 2 minutes at 4 °C. The supernatant was collected and stored at −20 °C until insulin and leptin levels were determined by enzyme-linked immunosorbent assay (ELISA), (Alpco Diagnostics, USA) as well as triglyceride,

cholesterol, and non-esterified fatty acid (NEFA) levels were determined by in vitro enzymatic method (Wako Chemicals, USA).

Vascular Reactivity of Mesenteric Artery

Second- or third-order mesenteric arteries were cleaned and cut into 2.0 mm in length and mounted in a small-vessel arteriography (Living System Instrumentation, VT) between two glass micropipettes (25 μm-diameter tip) and secured with 10–0 silk ophthalmic suture. The vessels were placed in oxygenated (21% O_2, 5% CO_2, and 74% N_2) Krebs-Ringer bicarbonate solution (KRBS) composed of (in mmol/L) 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11.1 D-glucose at 37°C and maintaining intraluminal pressure at 60 mmHg. Vessels were monitored under a Nikon inverted light microscope (Melville, NY) connected to a video monitor. Internal diameter and wall thickness were continually measured using video calipers and expressed in micrometers. After developing myogenic tone, the vascular viability was tested by administration of saturated KCL solution. The vascular responses were measured in sequential doses of: phenylephrine (PE, 1 nM −100 μM), acetylcholine (Ach, 1 nM −100 μM), and sodium nitroprusside (SNP, 1 nM −100 μM). In the dilation experiments, the vessels were precontracted at a similar level by PE (1 μM). To verify the role of nitric oxide synthase (eNOS), the vessels were pretreated with ω-Nitro-L-arginine methyl ester (L-NAME) 100 μM for 30 min before Ach administration. Passive tone was measured in calcium-free KRBS with ethylene glycol-bis (β-aminoethyl ether)-N, N, N’,N’-tetraacetic acid (EGTA) 0.1 mM. Dose responses are expressed as a percentage of dilation compared to their maximum passive diameter while a percentage of the constriction was compared to the maximum response to saturated KCl solution [1].

Real-Time Polymerase Chain Reaction

Mesenteric arteries were harvested, immediately removed of non-vascular tissues, and snap-frozen in liquid nitrogen. Total mRNA was extracted using Trizol (Invitrogen, Carlsbad, CA), and PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA) and cDNAs were synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). cDNAs were used as the template to amplify a gene of interest using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and the Thermal iCycler (Bio-Rad, Hercules, CA) for real-time RT-PCR experiments. Primer sequences (Invitrogen, Carlsbad, CA) for the selected genes were described in Table 1. Relative gene expression levels were quantified using the 2-ΔΔCt approximation method. The data were represented as the ratio of a gene of interest to control group (LM-LD) at 6 AM.

Statistics

Data were expressed as mean ± standard error of mean (SEM). The differences between groups of LM-LD versus Ob-LD, LM-LD versus LM-DD, and Ob-LD versus Ob-DD were assessed by unpaired t-test. Comparison of the vasodilation effect in the presence and absences of L-NAME was analyzed by paired t-test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Effects of obesity on the circadian pattern of aortic gene expression

A scatterplot reflecting the expression of all genes is shown in Figure 1, with genes oscillating more than 2-fold highlighted in red. In normal lean mice, between 7 AM and 7 PM, 130 genes oscillated by at least two fold. Genes that oscillated at least 2.5-fold are tabulated in Table 1. In contrast, in obese mice, only 28 genes oscillated. As shown in Table 1, many of these genes are associated with the molecular components of the circadian clock – *bmal*, *per*, *dbp*. When the oscillatory expression of the genes is lost, a host of genes entrained to them cease to be expressed.

Effects of disrupted circadian rhythm on morphology and metabolic parameters

Metabolic parameters in all mice groups are summarized in Table 2. Under normal conditions, obese leptin resistant mice have an approximately 2-fold increase in body weight (BW) compared to lean mice. To normalized BW, obese animals had significant higher body mass index (BMI). Obese animals had significantly elevated in blood glucose and HbA1C compared to lean animals about 1.4-fold and 1.6-fold, respectively. Plasma profiles of insulin, cholesterol, leptin were also significantly elevated in obese mice. After four weeks of constant darkness, fasting blood glucose of obese mice in constant darkness raised approximately 1.4-fold compared to those in light dark cycle. They also exhibited significantly increased in HbA1c levels approximately 1.1-fold compared to those in light-dark cycle (Table 2).

Effects of disrupted circadian rhythm on microvascular function

Cumulative concentration-response curve of PE-induced contractile reactivity was similar in all animals (Fig. 2A). Obesity impaired vasodilator to Ach in a dose dependent manner compared to lean mice. Under DD, pressurized mesenteric arteries from lean mice were significantly decreased relaxation to Ach, while no additive effect in obese animals (Fig. 2B). Pre-incubation of nitric oxide synthase inhibitor, L-NAME, the vascular response to Ach was significantly attenuated in all groups. The largest magnitude was demonstrated in lean mice under regular light-dark cycle (Fig. 2C). There was no significantly difference in SNP-mediated dilation between lean and obese mice in normal light-dark cycle. Notably, pressurized mesenteric relaxation to NO donor was attenuated only in lean mice subjected to DD (Fig. 2D).

Effects of disrupted circadian rhythm on a daily rhythmic expression of circadian clock genes and circadian output gene

The complex expression patterns of circadian genes in shown in Figure 3. The daily rhythm of *Bmal1*, and *Per2* was largely preserved, while *Cry1* was not a major oscillator in all groups (Fig. 3A, 3E, 3F). Obesity did not alter the *Clock* rhythmic expression but tended to depress in constant darkness both in lean and obese mice (Fig. 3B). *Npas2* rhythm was preserved in obesity compared to lean mice. After 4 week in constant darkness, the rhythmic expression of *Npas2* was significantly attenuated in lean mice but had no effect in obese mice (Fig. 3C). The daily rhythm of *Per1* expression was lost in obesity. In constant

darkness, the expression of *Per1* was modestly suppressed in both lean and obese mice (Fig. 3D). Most importantly, the daily rhythm of clock output gene, *dbp*, was significantly suppressed in obese mice. After circadian disruption in constant darkness, the rhythmic expression of *dbp* in lean mice was blunted closely to the level of obese mice. In obese mice under constant darkness, *dbp* expression had a minor reduction from their obese level but not significantly approached (Fig. 3G).

Effects of disrupted circadian rhythm on a daily rhythmic expression of vascular relaxation-related genes

The daily rhythm of *eNOS* in mesenteric artery was significantly suppressed in obese mice compared to lean mice under normal light dark condition. After disruption of circadian rhythm in constant darkness, rhythmic expression of eNOS was significantly suppressed in lean mice but not change in obese mice (Fig. 4A). *GTPCH1* tended to loss their rhythmic expressions both in obesity and in constant darkness (Fig. 4B).

Discussion

In the present study, we performed an extensive characterization of circadian gene expression and vascular function in obese mice. The key findings are 1) obesity markedly suppresses the vascular circadian clock, 2) circadian depression extends into the microcirculation, 3) disruption of circadian rhythm impairs gene expression and endothelial function in the microcirculation and 4) the effect of circadian disruption and obesity are not additive. These observations suggest that a novel and underappreciated mechanism of vascular disease may be dysfunction in daily patterns of gene expression.

Emerging evidence points to the molecular clock as a basis for disease. Conditions in which normal daily rhythms are disrupted – shift work, frequent international travel, frequent interruptions of sleep – are associated with asynchrony or desynchrony of circadian signaling. [7,8,17,20]. Moreover, these conditions are likewise associated an in increase in vascular disease [11,13,21,32,33]. While environmental changes caused by alterations in daily rhythms may contribute, recent data argue that variations in expression of clock genes also play a role in cardiometabolic dysfunction [26,27,35]. In the case of obesity, alteration of peripheral clock gene components have been reported in obese animals [9,30,36]. In the current study, obese animals displayed flattened circadian expression *Per1* and have blunted clock output gene *dbp* expression. As a clock output gene, suppression of *dbp* may indicate a generalized diminution of clock performance in obesity. While the loss of circadian rhythm of *Per1* was the most striking of the genes analyzed, further studies are necessary to clarify a particular role of *Per1* as a specific mechanism of vascular disease.

Additional support for links between circadian dysfunction and cardiovascular disease can be found in genetic models of altered circadian rhythm. [19,24]. Mice deficient in circadian control genes *bmal* and *clock* have impaired endothelial function as assessed by reduced vasodilation to acetylcholine and augmented neointimal responses to vascular injury [4]. Impairment extended into the microcirculation, notably with evidence of stiffness and altered MMP activation in the knockout mice [2]. In the current study, responses to acetylcholine are markedly reduced in the microcirculation of obese mice, a finding echoed

elsewhere[29]. This deficit correlates with a generalized deficit of clock gene expression in obesity and a depression of clock readout genes like *dbp*. While correlative at the current level of resolution, these data suggest that endothelial function is associated with loss of peripheral clock gene function in obesity.

In an effort to determine if microvascular endothelial function as seen in obesity, environmental circadian disruption was accomplished by four weeks of constant darkness as described previously[3]. This circadian disruption reduced endothelium-dependent dilation in lean mice but no additional effect in obesity. In the presence of L-NAME, all mesenteric artery responses to acetylcholine were similar, suggesting that a deficit in NO production and/or signaling in both obesity and constant darkness. Loss of NO is a well-documented deficit in both obesity and circadian dysfunction and the parallel occurrence in both models raises the prospect of a common insult.

Further support of this notion is found in the analysis of markers of endothelial function. In mesenteric arteries, *eNOS* is fairly consistent throughout the diurnal cycle, maintaining a fairly constant supply of eNOS enzyme. Arteries from obese animals showed a generalized depression of this expression and constant darkness showed at least some partial depression throughout the day. *GTPCH1* is the rate-limited enzyme for the production of BH4 in which an essential cofactor for nitric oxide synthase to generate NO in endothelial cells [6]. Suppression of *GTPCH1* expression causes a reduced BH4 bioavailability [6,37]. While minor variations in *GTPCH1* are observed, these did not achieve significance and likely contribute little to the observed defects.

The picture is complicated by the analysis of circadian genes. The clock output gene *dbp* was blunted in lean mice acclimated to constant darkness, to levels that mimicked the obese mice. Thus both obesity and constant darkness produce similar degrees of circadian disruption as assessed by a classic output gene. On the other hand, individual clock components are much more complicated. While obesity markedly depressed *Per1* cycling, constant darkness had no major effect in either group. While other genes showed varying degrees of alteration, no pattern emerged that tracked that changed seen in *dbp* or in vascular function. Constant darkness pushed *clock* gene expression toward an antiphase pattern and generally depressed *Npas2* expression but had no real effect in obese samples. Thus, while the general pattern mirrors itself between obesity and circadian disruption at a broad strokes level, the molecular details paint a picture that is much less clear. Because the precise relationship between individual clock genes and cellular function remains to full determined, more study is needed to determine the precise molecular mechanisms of clock dysfunction in obesity.

Conclusion

Obesity is the most significant risk factor for vascular disease in industrialized cultures. The mechanisms by which obesity impedes vascular function are incompletely understood. The data provided by these studies provide a novel clue into these mechanisms – the loss of circadian control of gene expression. Obesity significantly dampens circadian gene expression in both the aorta and the microcirculation in concert with reduced in vasomotor

dysfunction. Circadian disruption with constant darkness caused similar impairments that were non-additive with the effects of obesity. While it is tempting to conclude that the lack of additive effect indicates that obesity and circadian dysfunction have overlapping mechanisms, more studies are need to fully unravel the role of circadian dysfunction in obesity-related microvascular disease.

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Non-abbreviations and Acronyms

Ach	Acetylcholine
BH4	Tetrahydrobiopterin
BMAL1	Brain and Muscle Aryl Hydrocarbon Receptor Nuclear Translocator-Like Protein 1
CLOCK	Circadian Locomotor Output Cycle Kaput
CRY1	Cryptochrome 1
DBP	D Site Albumin Promoter –Binding Protein
EGTA	Ethylene Glycol-bis (β-aminoethyl ether)-N, N, N’,N’-tetraacetic Acid
eNOS	Endothelial Nitric Oxide Synthase
FFA	Free Fatty Acids
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GTPCH1	Guanosine-5′-Triphosphate Cyclohydrolase 1
HbA1c	Hemoglobin A1c
LM-DD	Lean Mice in Constant Darkness
LM-LD	Lean Mice in Light-dark Cycle
L-NAME	ω-Nitro-L-Arginine Methyl Ester
NPAS2	Neuronal PAS Domain Protein 2
Ob-DD	Obese Mice in Constant Darkness
Ob-LD	Obese Mice in Light-dark Cycle
PE	Phenylephrine
PEROID	Period Homolog
SNP	Sodium Nitroprusside

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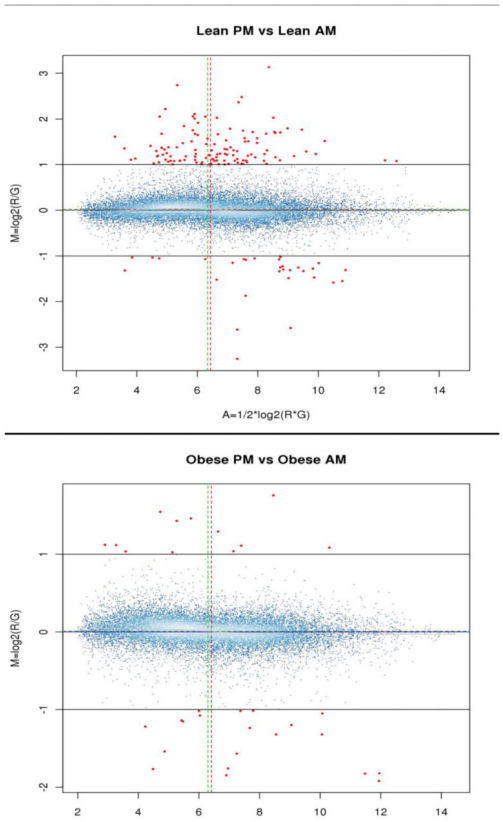

Figure 1. Obesity reduces oscillation of gene expression in the aorta

Differences in expression by more than 2-fold (red) and less than 2-fold (Blue) in lean (Panel A) and obese (Panel B) mice between 7 AM and 7 PM. Data represent pooled samples from 5 mice in each group. A total of 20963 genes are included.

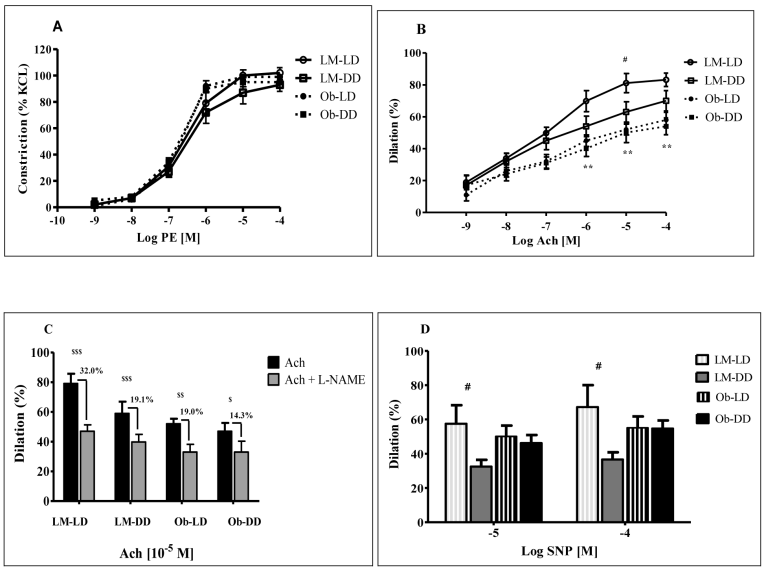

Figure 2. Circadian disruption and obesity impair endothelium-dependent and independent dilation in lean mice

Cumulative concentration–response curves of pressurized-mesenteric arteries to the vasoconstrictor phenylephrine, PE (A), the endothelium-dependent dilator acetylcholine, Ach (B) and the endothelium-independent dilator sodium nitroprusside, SNP (D) were performed in LM-LD, LM-DD, Ob-LD, and Ob-DD (n=8). Responses to acetylcholine were determined in the absence or presence of L-NAME 100 μM (C), (n=6-8). Data were shown as mean± SEM. $^{\#\#}P<0.01$, LM-LD vs Ob-LD; $^{*}P<0.05$, LM-LD vs LM-DD; $\$P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$; presence vs absence L-NAME; L-NAME, Nω-nitro-L-arginine methyl ester; LM-LD, lean mice in light-dark cycle; LM-DD, lean mice in constant darkness; Ob-LD, obese mice in light-dark cycle; Ob-DD, obese mice in constant darkness.

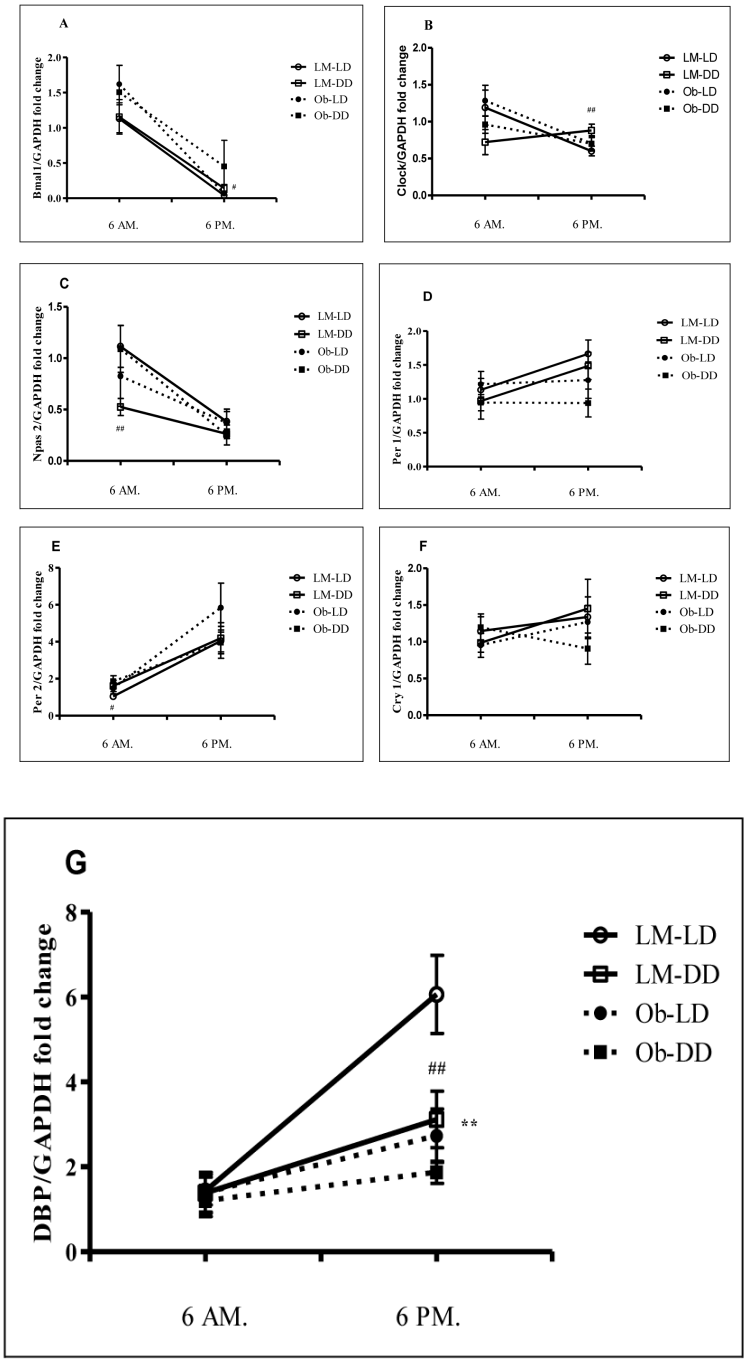

Figure 3. Circadian disruption altered daily rhythm of select vascular circadian gene expression

Vascular circadian expression in LM-LD, LM-DD, Ob-LD, and Ob-DD was assessed by real-time PCR. mRNA circadian profiles of BMAL1 (A), CLOCK (B), NPAS2 (C), PER1 (D), PER2 (E), CRY1 (F), and clock output gene; DBP (G) were studied at 06.00 AM., and 06.00 PM. in mesenteric arteries. Relative gene expression as the fold changes was quantified using the 2-ΔΔCt approximation method. Gene expression was normalized twice to GAPDH and additionally normalized to a referent control LM-LD at 06.00 AM. Data were shown as mean± SEM. The member of animals is 10 per group at each time point.

**$P<0.01$, LM-LD vs Ob-LD at corresponding time; $^{\#}P<0.05$, $^{\#\#}P<0.01$, LM-LD vs LM-DD at corresponding time; LM-LD, lean mice in light-dark cycle; LM-DD, lean mice in constant darkness; Ob-LD, obese mice in light-dark cycle; Ob-DD, obese mice in constant darkness; PCR, polymerase chain reaction.

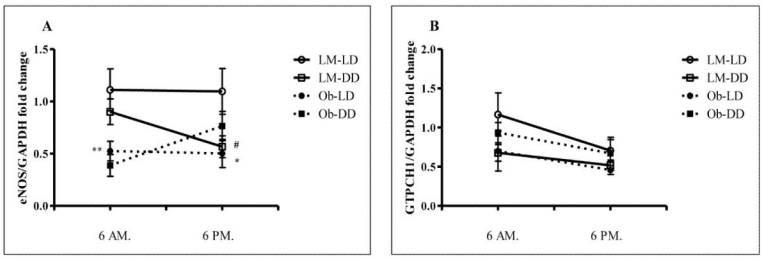

Figure 4. Circadian disruption altered daily rhythm of the vascular relaxant enzyme eNOS and GTPCH1

Vascular relaxant enzyme expression in LM-LD, LM-DD, Ob-LD, and Ob-DD was assessed by real-time PCR. mRNA oscillations of eNOS (A), and GTPCH1 (B) were studied at 06.00 AM., and 06.00 PM. in mesenteric arteries. Relative gene expression as the fold changes was quantified using the 2-ΔΔCt approximation method. Gene expression was normalized twice to GAPDH and additionally normalized to a referent control LM-LD at 06.00 AM. Data were shown as mean± SEM. The member of animals is 8 per group at each time point. *P<0.05 and **P<0.01, LM-LD vs Ob-LD at corresponding time; $^{\#}$P<0.05, $^{\#\#}$P<0.01, LM-LD vs LM-DD at corresponding time; LM-LD, lean mice in light-dark cycle; LM-DD, lean mice in constant darkness; Ob-LD, obese mice in light-dark cycle; Ob-DD, obese mice in constant darkness; PCR, polymerase chain reaction.

Table 1

Representative Gene Expression from Affymetrix Gene arrays using a 2.5 cut-off. Gene identification performed using NetAffx Query at https://www.affymetrix.com.

Lean Mice AM vs. PM			
Transcript Cluster ID	**gene_assignment**	**Gene Symbol**	**Fold-Change(L-AM vs. L-PM)**
10345675	NM_008719 // Npas2 // neuronal PAS domain protein 2 // 1 B|1 20.0 cM // 18143 //	Npas2*	8.63483
10551197	Cyp2b6	Cyp2b10*	6.32741
10556463	Arntl(Bmal1)	Arntl(Bmal1)*	4.79271
10409278	NM_017373 // Nfil3 // nuclear factor, interleukin 3, regulated // 13 B1|13 32.0	Nfil3*	3.04133
10582231	Fbxo31(F-box only protein 31)	Fbxo31*	2.81508
10360631	NM_023341 // Cabc1 // chaperone, ABC1 activity of bc1 complex like (S. pombe) //	Cabc1*	2.81282
10402512	NR_028576 // Scarna13 // small Cajal body-specific RNA 1 // 12 E // 100306943	Scarna13*	2.77684
10598085	ATP6(ATP synthase F0 subunit 8, Mitochondrion)	ATP6*	2.76323
10443463	NM_007669 // Cdkn1a // cyclin-dependent kinase inhibitor 1A (P21) // 17 A3.3|17	Cdkn1a*	2.75607
10419854	NM_016972 // Slc7a8 // solute carrier family 7 (cationic amino acid transporter,	Slc7a8*	2.61035
10474700	NM_011580 // Thbs1 // thrombospondin 1 // 2 F1-F3|2 65.0 cM // 21825 /// ENSMUST	Thbs1*	2.60492
10522127	Klb(Isoform 1 of Beta-klotho gene)	Klb*	2.56751
10518781	Per3	Per3*	−2.49409
10402409	NM_009247 // Serpina1e // serine (or cysteine) peptidase inhibitor, clade A, mem	Serpina1e*	−2.56643
10548207	Pzp (pregnancy zone protein,Alpha-2-macroglobulin gene)	Pzp*	−2.57829
10589535	Ngp (neutrophilic granule protein gene)	Ngp*	−2.58713
10500565	NM_008293 // Hsd3b1 // hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroi	Hsd3b1*	−2.6116
10545177	unknown ENSMUST00000103331 U88067	*	−2.62185
10545247	LOC100047162 Igk-V19-14 (similar to immunoglobulin kappa-chain immunoglobulin kappa chain variable 19 (V19)-14)	*	−2.68254
10402406	NM_009245 // Serpina1c // serine (or cysteine) peptidase inhibitor, clade A, mem	Serpina1c*	−2.73098
10390691	NM_145434 // Nr1d1 // nuclear receptor subfamily 1, group D, member 1 // 11 D //	Nr1d1*	−2.73985
10438415	ENSMUST00000103752 // Igl-V2 // immunoglobulin lambda chain, variable 2 // 16 A3	Igl-V2*	−2.91297
10605571	Gyk(Isoform 2 of Glycerol kinase gene, glycerol kinase)	Gyk*	−2.94991
10549079	Gys2(glycogen synthase 2 gene)	Gys2*	−2.96305
10531149	Gc (Vitamin D-binding protein gene)	Gc*	−2.97501
10545215	Igkv12-46 Igk-V28 Igk (G8(Anti-MRBC hybridoma) light chain gene)	*	−2.99254

Lean Mice AM vs. PM			
Transcript Cluster ID	**gene_assignment**	**Gene Symbol**	**Fold-Change(L-AM vs. L-PM)**
10452854	NM_053188 // Srd5a2 // steroid 5 alpha-reductase 2 // 17 E2 // 94224 /// ENSMUST	Srd5a2*	−3.10559
10429538	NM_001033229 // Cyp11b1 // cytochrome P450, family 11, subfamily b, polypeptide	Cyp11b1*	−3.16881
10398032	NM_173024 // Serpina3b // serine (or cysteine) peptidase inhibitor, clade A, mem	Serpina3b*	−3.24955
10513521	Mup20(major urinary protein 20)	Mup20*	−3.44551
10450272	NM_009995 // Cyp21a1 // cytochrome P450, family 21, subfamily a, polypeptide 1 /	Cyp21a1*	−3.44805
10433172	NM_008134 // Glycam1 // glycosylation dependent cell adhesion molecule 1 // 15 F	Glycam1*	−3.61614
10545175	Igk-J1 Igk	Igk*	−3.67708
10500272	BC132471 // Gm129 // predicted gene 129 // 3 F2.1 // 229599 /// NM_001033302 //	Gm129*	−3.75504
10538965	Fabp1(Fatty acid-binding protein, liver gene)	Fabp1*	−3.79768
10571054	Star (Steroidogenic acute regulatory protein, mitochondrial gene)	Star*	−3.84016
10516064	Mfsd2a (adaptive thermogenesis)	Mfsd2a*	−4.58704
10463551	NM_007703 // Elovl3 // elongation of very long chain fatty acids (FEN1/Elo2, SUR	Elovl3*	−4.68888
10535704	Cyp3a4(Cytochrome P450 3A4 gene)	Cyp3a4*	−4.71989
10403006	ENSMUST00000103479 // Gm7112 // predicted gene 7112 // 12 F2 // 633457(C72-3A1 protein (Fragment) gene)immunoglobulin heavy variable 3-5	Gm7112*	−4.95395
10373452	BC132471 // Gm129 // predicted gene 129 // 3 F2.1 // 229599 /// NM_001033302 //	C1orf51/Gm129*	−6.34642
10553092	Dbp Sphk2 (D site-binding protein gene)	Dbp*	−6.52047
Obese mice AM vs PM			
Transcript Cluster ID	gene_assignment	Gene Symbol	
10345675	NM_008719 // Npas2 // neuronal PAS domain protein 2 // 1 B|1 20.0 cM // 18143 //	Npas2*	3.26192
10556463	Arntl(Bmal1)	*	3.0853
10538247	Npy (neuropeptide Y)*	*Npy (neuropeptide Y)	2.68052
10563955	Gm3079(ENSMUST00000101811 XM_001475615)	*	2.67764
10595202	cdna:Genscan chromosome(unknown)	*	−2.35786
10553092	Dbp(a member of the PAR bZIP (Proline and Acidic amino acid-Rich basic leucine ZIPper) transcription factor family)		−2.94079
10373452	BC132471 // Gm129 // predicted gene 129 // 3 F2.1 // 229599 /// NM_001033302 //	Gm129*	−3.15738

Table 2

Morphology and Metabolic Parameters at the Endpoint

Parameters	N	LM-LD	LM-DD	Ob-LD	Ob-DD
Age (wks)	38	15.0 ± 0.2	15.0 ± 0.2	15.3 ± 0.3	15.0 ± 0.2
BW (g	38	27.8 ± 0.4	28.5 ± 0.4	49.4 ± 0.6**	49.9 ± 0.6
BMI (kg/m2)	38	0.03 ± 0.0004	0.03 ± 0.0004	0.05 ± 0.001**	0.05 ± 0.001
Fasting glucose (mg/dl)	18	190.6 ± 9.5	202.4 ± 8.6	258.7 ± 20.1**	365.3 ± 25.5##
HbA1C (%)	18	4.5 ± 0.04	4.4 ± 0.05	7.3 ± 0.19 **	8.0 ± 0.22#
Insulin (ng/ml)	18	0.134 ± 0.03	0.074 ± 0.01#	3.924 ± 0.92**	4.779 ± 0.91
Cholesterol (mg/ml)	18	87.3 ± 7.4	65.523 ± 3.0	183.624 ± 9.8**	163.314 ± 11.1
Triglyceride (mg/ml)	18	62.8 ± 12.5	56.7 ± 15.2	107.5 ± 28.8	78.5 ± 18.1
NEFA (mEq/ml)	18	0.76 ± 0.12	0.69 ± 0.06	0.99 ± 0.09	0.92 ± 0.05
Leptin (pg/ml)	10	604.3 ± 91.2	1057.9 ± 387.8	7723.3 ± 711.1**	6969.8 ± 958.7

Data are shown as the mean ± SEM.

*P<0.05;

**P<0.01 compared between LM-LD versus Ob-LD.

#P<0.05;

##P <0.01 compared between LM-LD versus LM-DD or Ob-LD versus Ob- DD. N is a number of animals per group.