MUTUAL INTERACTIONS BETWEEN CIRCADIAN CLOCKS, INFLAMMATORY SIGNALING PATHWAYS AND FATTY ACID METABOLISM

A Dissertation

by

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ABSTRACT

To examine mutual interactions between key mediators of inflammation and circadian clocks and their role in diet-induced metabolic disorders, we first determined whether circadian rhythm desynchronization exacerbates high fat diet (HFD)-induced proinflammatory and metabolic responses. In mice exposed to shifted light-dark (LD) cycles, the disrupted entrainment of circadian behavior and modulation of peripheral clock function was accompanied by further amplification of HFD-induced adipose tissue macrophage infiltration, proinflammatory M1 activation and cytokine expression along with corresponding increases in the severity of insulin resistance and glucose intolerance. Feedback modulation of circadian clock by diet-induced inflammatory signaling was next examined by determining whether HFD and different fatty acids alter fundamental properties of circadian behavior and peripheral clock gene rhythms in conjunction with their proinflammatory effects. The anti-inflammatory, polyunsaturated fatty acid, docosahexaenoic acid (DHA), had negligible effects on circadian rhythms. However, prolonged treatment with HFD or palmitate, the prevalent proinflammatory saturated fatty acid (SFA) in HFD, modulated suprachiasmatic nucleus (SCN)-regulated behavioral rhythmicity and peripheral clock gene rhythms, inducing small increases in free-running period of the activity rhythm and substantial lengthening (3-9hr) of the period of clock gene rhythms in macrophages, fibroblasts and differentiated adipocytes. Following acute treatment, palmitate had little or no effect on the phase of the activity rhythm but induced time-dependent phase shifts of clock gene rhythms in cultured fibroblasts and

differentiated adipocytes. The time-dependent phase shifting effects of acute palmitate were contemporaneous with rhythmic changes in palmitate-induced inflammatory signaling (NF-κB and IL-6). DHA and other inhibitors of inflammatory signaling repressed palmitate-induced proinflammatory responses and phase shifts of the fibroblast clock. Acute treatment with the proinflammatory cytokine IL-6 mimicked the peak phase shifting effects of palmitate alone and blockade of TNFα signaling using a neutralizing antibody repressed palmitate-induced phase shifts, suggesting that SFA-mediated inflammatory signaling via these proinflammatory cytokines may feed back to modulate circadian timekeeping in peripheral clocks. These findings suggest that key mediators of inflammation and inflammatory signaling may play a role in the mechanism by which HFD and SFAs modulate clock timekeeping properties, and in turn this dysregulation of circadian clocks exacerbates diet-induced systemic insulin resistance and glucose intolerance.

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NOMENCLATURE

Acsl1	Acyl CoA Synthetase
ANOVA	One-way Analysis of Variance
Atgl	Adipose Triglyceride Lipase
BMAL1	Brain, Muscle ARNT-Like protein 1
BMDM	Bone Marrow-Derived Macrophages
CARD	Cardamonin
CLOCK	Circadian Locomotor Output Cycles Kaput
CNS	Central Nervous System
CRY	Cryptochrome
CT	Circadian Time
DD	Constant Darkness
DHA	Docosahexaenoic Acid
DMEM	Dulbecco's Modified Eagle's Medium
ER	Endoplasmic Reticulum
FAA	Food Anticipatory Activity
Facl	Long Chain Fatty Acyl CoA Synthase
Fasn	Fatty Acid Synthase
FBS	Fetal Bovine Serum
FEO	Food-Entrainable Oscillator
FRP	Free-Running Period

HFD	High Fat Diet
Hsl	Hormone Sensitive Lipase
IL	Interleukin
IMDM	Iscove's Modified Dulbecco’s Medium
JNK	c-Jun N-terminal Kinase
LD	Light-Dark
LFD	Low Fat Diet
LL	Constant Light
LM	Levenberg-Marquardt (Damped Sine)
LPS	Lipopolysaccharide
Luc	Luciferase
M-CSF	Monocyte-Colony Stimulating Factor
NAD	Nicotinamide Adenine Dinucleotide
NAMPT	Nicotinamide Phosphoribosyltransferase
NES	Nighttime Eating Syndrome
PAL	Palmitate
PBS	Phosphate-Buffered Saline
PEPCK	Phosphoenolpyruvate Carboxykinase
PER	Period
PGC-1α	Peroxisome proliferator-activated receptor Gamma Coactivator 1-alpha
PMT	Photomultiplier Tube

RHT	Retinohypothalamic Tract
RORE	Retinoic acid-related Orphan receptor Response Elements
Scd1	Stearoyl-Coenzyme A Desaturase 1
SCN	Suprachiasmatic Nucleus
SE	Standard Error
SFA	Saturated Fatty Acid
SVC	Stromal Vascular Cells
TLR	Toll-Like Receptor
TNFα	Tumor necrosis factor α
VEH	Vehicle
ΔΦ	Phase Shift
τ	Tau

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE Page

FIGURE Page

CHAPTER I

INTRODUCTION

Circadian Rhythms

The earth rotates on its own axis once every 24 hours resulting in a daily cycle of day and night. Throughout evolution, in response to predictable changes in the environment on the rotating earth, rhythmicity has become a common property of many biological processes in living organisms including cyanobacteria, fungi, plants and animals. In particular, biological processes in these organisms fluctuate rhythmically so as to closely follow the daily solar cycle, and thus allow living organisms to anticipate and prepare in advance for predictable changes in the external environment.

These 24-hour rhythms are observed in some prokaryotic and almost all eukaryotic organisms. For example, the most primitive organism, cyanobacteria, has a daily rhythm in photosynthesis and nitrogen fixation, which peak during daytime and nighttime, respectively. The filamentous fungus *Neurospora crassa* is characterized by rhythmic production of conidiospores (Correa and Bell-Pedersen, 2002). In addition, *Drosophila* melanogaster displays robust rhythmicity in locomotor activity (Klarsfeld et al., 2003).

Similar to simple model systems, mammals exhibit daily rhythms in a wide array of biochemical, physiological and behavioral processes. The sleep-wake cycle is a prominent example of daily rhythmicity in mammals. Diurnal animals show a consolidated 8-12 hour interval of sleep at night and wakefulness during the daytime. Nocturnal animals show an

opposite pattern: sleep during the daytime and activity at night. The sleep-wake cycle is associated with other rhythmic biological processes such as body temperature and melatonin secretion (Wyatt et al., 1999). Body temperature peaks during the active period and remains low during sleep. Melatonin secretion from the pineal gland increases prior to sleep, reaches peak levels around midnight and declines before wakefulness in humans. Furthermore, other physiological and behavioral processes such as food intake, locomotor activity, and immune responses are marked by 24-hour rhythmicity in mammals (Scheiermann et al., 2013; Vollmers et al., 2009).

Fundamental Properties of Circadian Rhythms

From unicellular and multicellular organisms, rhythmic processes that oscillate with periods of approximately 24 hours are referred to as circadian (derived from Latin '*circa*' and '*diēm*' meaning 'about' and a 'day") rhythms. Circadian rhythms are distinguished by three fundamental properties:

1) Rhythmic processes persist in the absence of environmental time cues (e.g., solar cycle) with a period of about 24 hours. Under constant conditions, circadian rhythms "free-run," with a period, τ (tau), close to, but not exactly, 24 hours, indicating their endogenous regulation by an internal biological clock. For example, the circadian rhythm of mouse wheel-running activity persists in a constant darkness (DD) with a free-running period (FRP) of 23.5 hours (i.e., activity onsets occur 0.5 hour earlier each day). ;

2) Circadian rhythms are synchronized or entrained by environmental cycles with periods ranging from about 21-26 hours. In mammals, the daily solar or light-dark (LD)

cycle and other environmental stimuli such as food availability, temperature, humidity, and social cues are 'zeitgebers' (German term for 'time givers') that mediate the entrainment of circadian rhythms. During steady-state entrainment, circadian rhythms assume not only the exact periodicity of the external cycle, but also adopt a particular and characteristic phase relationship to environmental time cues (e.g., daily onset of rodent activity rhythm occurs shortly after lights-off during LD entrainment); and

3) Circadian rhythms are temperature compensated such that their endogenous periodicity remains fairly stable over a range of physiological temperatures. The rates for most biochemical and physiological processes are temperature-dependent and typically vary 2-3 fold (i.e., Q_{10} value of 2-3) with every 10º change in temperature. By comparison, temperature-induced modulation of circadian rhythms is much lower; the free-running period of circadian rhythms changes by only 2-3 hours with every 10º variation in temperature (i.e., $Q_{10} \approx 1.1$).

Functional Organization of Circadian System

Circadian rhythms are regulated by internal biological clocks. Even though the core clock mechanism differs among species, the basic functional organization of the circadian time keeping system is well-conserved in all living organisms and consists of three main components: clock or oscillator, sensory input/entrainment pathways and circadian output pathways. The oscillator is a pacemaker that generates circadian rhythms. Sensory input/entrainment pathways convey external time cues (e.g., light-dark information, food availability) that mediate the synchronization of the oscillator to environmental cycles.

Lastly, output pathways from the circadian clock are responsible for the regulation of overt rhythms in various molecular, cellular and behavioral processes, which reflect the "hands" of the clock.

Molecular Mechanism for Circadian Timekeeping in Mammals

The circadian timekeeping mechanism consists of interlocked transcription-translation feedback loops. In mammals, Brain, muscle ARNT-like protein 1 (*Bmal1*), circadian locomotor output cycles kaput (*Clock*), as well as the Period (*Per1* and *Per2*) and Cryptochrome (*Cry*) genes form the "gears" of the molecular clockworks that mediate circadian timekeeping throughout the body. These molecular components interact through positive- and negative-feedback loops in which gene transcription is rhythmically regulated by their protein products (Takahashi et al., 2008). In the positive arm, the helix-loop-helix transcription factors BMAL1 and CLOCK form heterodimers that up-regulate *Per* and *Cry* transcription by binding on E-box elements in the promoter region. Accumulated PER and CRY in cytoplasm then translocate to the nucleus and down-regulate their own transcription by inhibiting BMAL1-CLOCK transcriptional activity. In ancillary loops of the molecular clockworks, BMAL1-CLOCK heterodimers initiate transcription of the orphan nuclear receptors, *Rev-erbα* and *Rorα*. In turn, REV-ERBα and RORα bind to retinoic acid-related orphan receptor response elements (ROREs) present in *Bmal1* promoter, leading respectively to the repression and activation of *Bmal1* transcription.

Hierarchical Structure of Mammalian Circadian Clocks

The molecular feedback loops comprising the mammalian circadian clockworks are common to virtually all cells throughout the body and these cell-autonomous clocks are hierarchically organized. The ensemble of cellular clocks in the suprachiasmatic nucleus (SCN) of the hypothalamus functions as a master pacemaker in this hierarchical network and mediates the temporal synchronization of clocks located in other brain regions and peripheral tissues. In turn, these "peripheral" clocks provide for the local coordination of tissue- or cell-specific processes in time.

***SCN pacemaker*:** The SCN is located dorsal to the optic chiasm near the third ventricle in the anterior hypothalamus and consists of bilaterally paired structures containing approximately 20,000 neurons. The pacemaker function of the SCN was initially established through ablation studies demonstrating that complete lesions of this structure abolish overt circadian rhythms in many different processes including locomotor activity, feeding and body temperature (Moore and Eichler, 1972; Stephan and Zucker, 1972). However, conclusive evidence for the unique role of the SCN as a circadian pacemaker function was derived from observations indicating that fetal transplants of the SCN restore circadian behavioral rhythmicity in SCN-lesioned hosts (Lehman et al., 1987; Silver et al., 1990). Another important functional property of the SCN is that its cells are capable of generating self-sustained oscillations in various molecular and physiological processes including cellular metabolism, neuropeptide secretion, electrical activity, and gene

expression. Furthermore, the SCN communicates temporal information via diffusible factors to peripheral circadian clocks throughout the body.

***Peripheral clocks*:** Virtually all peripheral tissues and cells throughout the body contain self-sustained and cell-autonomous clocks similar to those in the SCN. For example, explanted tissues from *mPer2^{luc}* knock-in mice show persistent *Per2::Luc* rhythms in liver, muscle and adipose tissue *in vitro* (Yoo et al., 2004b). In addition, cell-autonomous clocks in the SCN and peripheral tissues share the same molecular mechanism responsible for their circadian timekeeping function. However, overt circadian rhythms regulated by the SCN or peripheral clocks are entrained by different signals.

Regulation of Circadian Clocks

The daily LD cycle is the predominant environmental signal that entrains SCN-regulated circadian rhythms such as the activity and body temperature rhythms. The SCN receives direct retinal innervation via the retinohypothalamic track (RHT), a monosynaptic projection from a subpopulation of retinal ganglion cells that contain the photopigment, melanopsin, and terminate bilaterally in the ventrolateral subregions of each nucleus. The RHT is necessary for circadian photoentrainment because transection of this visual pathway disrupts circadian entrainment to LD cycles without affecting image-forming visual functions.

Although the principal zeitgeber in mammals is the daily solar cycle, other non-photic environmental stimuli that oscillate daily, such as food availability, also play a role in the

entrainment of circadian rhythms, especially in light-insensitive peripheral clocks. Under conditions of continuous food availability, the SCN mediates the LD entrainment of peripheral clocks presumably through neural and humoral signals. However, restricted feeding schedules in conjunction with a regular LD cycle overrides SCN control and directly entrains peripheral clocks. In rodents, restricted feeding at a fixed time of day acts as an independent zeitgeber and entrains a distinct bout of activity that precedes the daily onset of food availability. For example, rats maintained on a restricted feeding schedule during the light phase of a standard LD 12:12 cycle show entrainment of food anticipatory activity (FAA) during the daytime along with LD-synchronized nocturnal activity in which daily onsets occur near lights-off (Mistlberger, 1994). Even during exposure to constant conditions, FAA entrainment to restricted feeding cycles persists whereas LD-entrainable activity free-runs with a circadian periodicity. Critical evidence for the role of food availability as a circadian zeitgeber is derived from observations indicating that FAA entrainment to restricted feeding schedules only occurs within a limited range when the period of the schedules is greater than 22 hours but less than 31 hours (Stephan, 1981). Importantly, the effects of restricted feeding schedules are mediated by a SCN-independent circadian oscillator or food-entrainable oscillator (FEO) because FAA entrainment persists following SCN lesions that abolish the circadian rhythm of light-entrainable activity (Krieger et al., 1977; Phillips and Mikulka, 1979).

Food-mediated entrainment of behavioral rhythmicity is accompanied by modulation of clock gene rhythms in peripheral tissues. Similar to the effects of a light pulse during the subjective night, restricted feeding during the daytime, but not nighttime, phase shifts

clock gene oscillations (e.g. *Per* and *Cry*). It is noteworthy that food-induced phase shifts occur more rapidly in the metabolically-active tissues such as the liver relative to those observed in heart and kidney (Damiola et al., 2000). However, restricted feeding cycles do not affect the molecular clockworks in the SCN (Stokkan et al., 2001), but differentially affect the phase and amplitude of clock gene rhythms in some brain regions (Verwey and Amir, 2011).

Nutrient availability also modulates circadian outputs such as rhythmic hormone release from peripheral tissues. For example, restricted feeding cycles shift the phase and increase the amplitude of the circadian rhythm in serum insulin levels (Rubin et al., 1988). In addition, daytime restricted feeding shifts the diurnal melatonin rhythm in the rat (Challet et al., 1997). The precise mechanism by which restricted feeding cycles modulate circadian rhythms is unknown, but these studies provided initial evidence for interactions between nutrient intake and circadian clock that may underscore the implications of circadian rhythm disruption in metabolic disorders.

Impact of Nutrient Metabolism on Circadian Clock

Consistent with the entraining effects of restricted feeding cycles, dietary composition and nutrient metabolism alter fundamental properties of circadian behavioral rhythms and the underlying molecular clockworks.

***Effects of high fat diet*:** High fat diet (HFD) has been shown to alter the circadian rhythm of wheel-running activity in mice such that the free-running period is increased

relative to animals fed a regular chow diet (Kohsaka et al., 2007a). Importantly, a small increase in the free-running period of the activity rhythm occurred immediately following the onset of the HFD treatment. HFD-fed mice also exhibited damped diurnal rhythms of feeding and locomotor behavior due to relative increases in food intake and total activity during the daytime. These data indicate that the systemic regulation of circadian behavior is modulated by dietary composition.

Since overt circadian behavior provides a reflection of the functional status of the core clock mechanism, it is not surprising that diet has a compatible impact on the molecular oscillations comprising clock feedback loops. In conjunction with its effects on circadian behavior, HFD altered clock gene oscillations in peripheral tissues (Kohsaka et al., 2007a). In HFD-fed mice, the amplitude of diurnal rhythms in *Clock*, *Bmal1*, *Per Rev-erbα* and *Rorα* mRNA was attenuated in liver and fat, but not in the SCN, after 6 weeks of treatment, indicating that dietary composition alters the molecular clock mechanism in tissue-specific manner.

The effects of HFD on circadian clock properties are not limited to changes in rhythm period and amplitude, but include differential shifts in the phase of clock gene oscillations in specific tissues. (Pendergast et al., 2013). HFD treatment for 7 days shifts the phase of the *Per2* rhythm in mice, inducing large advances in the liver and small delays in the spleen. However, HFD has no effect on the phase of *Per2* rhythm in the SCN. These diet-induced alterations in the phase control of circadian clocks in peripheral tissues mediating nutrient processing may represent contributing factors in the development of metabolic disorders.

***Effects of fatty Acids*:** Because fatty acids are the primary components distinguishing HFD and LFD, it will be necessary to differentiate the circadian effects of SFAs and polyunsaturated fatty acids due to their mutually antagonistic actions on inflammatory and metabolic signaling pathways. Palmitate is the most common SFA in animals, and dietary consumption of processed and fast foods is a major source of this putative proinflammatory fatty acid. Despite the well-characterized effects of palmitate on proinflammatory signaling and downstream metabolic processes, evidence for its impact on the molecular clockworks is limited to the finding that palmitate decreases the amplitude of the rhythms in *Per2* and *Rev-erb* mRNA expression in a hypothalamic cell line (Fick et al., 2011). Similarly, few studies have explored the influence of polyunsaturated fatty acids on circadian rhythms. In hamsters fed a polyunsaturated fatty acid-deficient diet, the nocturnal peak in pineal melatonin levels was attenuated relative to that observed in controls (Lavialle et al., 2008). These data suggest that the relative content of saturated and polyunsaturated fatty acids may determine how HFD modulates circadian clock properties.

***Effects of glucose*:** In addition to fatty acids, other nutrients such as glucose have a modulatory influence on the molecular clockworks. For instance, a low concentration of glucose in growth medium increases circadian period and decreases the amplitude of the rhythm in *Bmal1* expression in cultured mouse fibroblasts (Lamia et al., 2009). Furthermore, modulation of O-GlcNAcylation in the hexosamine biosynthetic pathway that mediates nutrient sensing through glucose alters the period of clock gene rhythms

(Kaasik et al., 2013; Li et al., 2013). Collectively, these data suggest that glucose sensing and metabolism may feedback to regulate the circadian clock machinery.

***Effects of metabolic hormones*:** Consistent with its modulation by specific nutrients such as glucose and fatty acids, fundamental properties of the clock mechanism are affected by humoral factors that mediate metabolic homeostasis and inflammatory responses. In streptozotocin-induced insulin-deficient diabetic rats, the rhythms of *Per, Cry, Bmal1, Rev-erb,* and *Ror* mRNA expression in liver are phase advanced relative to controls, and insulin treatment induces time-dependent phase shifts in the rhythm of *Dbp* mRNA levels in the liver (Yamajuku et al., 2012). In addition, leptin modulates the light-induced phase shifts of the circadian rhythm in mouse wheel-running activity such that pre-treatment with leptin amplifies the phase-advancing effects of light pulses administered during the late subjective night (Mendoza et al., 2011). Consistent with this modulation of light-induced phase shifts in circadian behavior, leptin treatment also potentiates the photic induction of *Per1* and *Per2* mRNA expression in the SCN during the late subjective night.

***Effects of inflammatory response*:** Nutrient-induced inflammatory responses also modulate circadian timekeeping. For example, prostaglandin E2, a pro-inflammatory compound derived from unsaturated cyclic fatty acids, has phase-resetting actions on peripheral clocks *in vivo* (Tsuchiya et al., 2005). Injections of prostaglandin E2 induce time-dependent shifts in the phase of the *Per1* mRNA rhythms in mouse liver, kidney and

heart, with advances during the early day and delays during the early night. Thus, dietary modulation of the circadian system is determined by the integrated influence of specific nutrients and humoral factors regulating metabolic processes and inflammatory responses.

Circadian Regulation of Nutrient Metabolism

The circadian system plays an important role in maintaining energy homeostasis through the regulation of many different physiological processes related to nutrient metabolism.

***Feeding cycle*:** The initial stage of clock involvement in energy homeostasis begins with circadian control of feeding cycles. In anticipation of daily changes in food availability (and susceptibility to predation), animal feeding behavior is rhythmically regulated such that food intake primarily occurs during the day in diurnal species and during the night in nocturnal species. The rhythm in feeding behavior is regulated by the circadian clock because complete lesions of the SCN abolish diurnal oscillations of food and water intake ((Nagai et al., 1978; Stephan and Zucker, 1972). Furthermore, genetic knockout or mutation of core clock genes also alters circadian rhythms of food intake. For example, both *Bmal1*$^{-/-}$- and *Cry1/Cry2*$^{-/-}$-deficient mice are characterized by the loss of feeding rhythms (Iijima et al., 2005; Storch and Weitz, 2009). In addition, Clock mutant mice also exhibit attenuated rhythm in feeding behavior due to hyperphagia (Pitts et al., 2003; Turek et al., 2005). Interestingly, all of these clock mutant or knockout mice show similar evidence of metabolic disorders in conjunction with the loss of rhythms in feeding

behavior. These data indicate that clock control of daily mealtimes is critical for its function in the regulation of energy homeostasis.

***Fatty acid metabolism*:** In addition to the timing of feeding behavior, circadian regulation of nutrient composition and quantity also has a major impact on energy homeostasis. Several studies indicate that the circadian clock regulates metabolism at many different levels, thus producing daily fluctuations of nutrient levels in peripheral tissues. In this way, free fatty acids in the serum fluctuate on a daily basis, with peak levels during the middle of the daytime (ZT6) (Shostak et al., 2013). However, $Clock^{\Delta 19}$ mutant and $Bmal1^{-/-}$-deficient mice are distinguished by low and arrhythmic free fatty acid content in the blood (Shostak et al., 2013), again suggesting that the rhythmic modulation of lipolysis rates and corresponding release of free fatty acids may be critical for clock function in the regulation of energy homeostasis. The precise modulation of free fatty acid levels through clock-controlled regulation of triglyceride hydrolysis is critical because excessive fatty acids can induce lipotoxicity or inflammation in peripheral tissues (Unger et al., 2010).

The circadian regulation of fatty acid metabolism including lipolysis and lipogenesis is largely mediated through the rhythmic expression or activation of enzymes in peripheral tissues. For example, the levels of fatty acids during nighttime fasting in diurnal animals are high due to increased lipolysis. Consistent with this observation, transcripts encoding lipolytic enzymes such as adipose triglyceride lipase (Atgl) and hormone sensitive lipase (Hsl) exhibit circadian oscillations with a peak at circadian time (CT)12 in adipose tissue

that are abolished in Clock$^{\Delta 19}$ and Bmal1 knockout mice (Shostak et al., 2013). In addition, *Acyl CoA synthetase (Acsl1)* mRNA, which is responsible for fatty acids biosynthesis, fluctuates rhythmically in adipose tissue peaking around CT6, and the phase of this rhythm is shifted by 12 hours in Clock$^{\Delta 19}$ mice (Shostak et al., 2013). Along with lysis and synthesis of nutrients, absorption is another regulatory mechanism that is critical for maintaining energy homeostasis in the body. Caveolin-2 is an important protein in fatty acid transport, and transcripts encoding this gene exhibit circadian variation with peak expression at CT12 in mouse adipose tissue. These studies indicate that the circadian regulation of metabolic enzymes and transport proteins is congruent with clock control of fatty acid metabolism.

***Glucose metabolism*:** The circadian clock also regulates various aspects of glucose metabolism in the mammals. Circadian variation is evident in plasma glucose levels, which peak during the daytime, and this oscillation is abolished in Clock mutant mice (Rudic et al., 2004). Furthermore, glucose tolerance responses, which reflect uptake of glucose, oscillate with a maximum during the nighttime and SCN ablation abolishes this diurnal rhythm by enhancing glucose tolerance during the daytime (la Fleur et al., 2001; Rubin et al., 1988). Diurnal oscillations in the physiological levels of glucose are a corollary of clock control of gluconeogenesis and glycolysis. Consistent with this observation, a key rate-limiting enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK), fluctuates on a daily basis in liver and aorta, and this diurnal rhythm was disrupted in Clock mutant mice (Rudic et al., 2004). Moreover, the conversion

of exogenous pyruvate to glucose, which provides an index of gluconeogenesis, was also greatly impaired in Bmal1-deficient ($Bmal1^{-/-}$) mice, indicating that clock disruption affects normal glucose metabolism. Because insulin is the principal regulator of glucose metabolism, circadian modulation of insulin release from pancreas beta cells is fundamental to clock function in maintaining energy homeostasis. The diurnal rhythm in plasma insulin concentration in rats is marked by a gradual increase near the end of the day and peak levels early in the night near the onset of activity (Yamamoto et al., 1987). SCN lesions eliminate this rhythmic variation in insulin secretion and hence the plasma glucose rhythm such that concentrations remain at basal levels. In addition to the circadian regulation of hormone secretion, insulin sensitivity varies during the daily cycle, with injections decreasing plasma glucose levels more rapidly during the nighttime or active phase than during the day (la Fleur et al., 2001).

***Other metabolic enzymes*:** Many metabolism-associated enzymes are clock-regulated. For example, the enzymatic activity of sirtuin, a NAD^+-dependent histone deacetylase that acts as a sensor of energy availability and modulates metabolism, fluctuates rhythmically in mouse liver with peak levels during the night (Bellet et al., 2011). Importantly, sirtuin also mutually interacts with other circadian-regulated metabolic enzymes, including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and AMPK to maintain energy homeostasis (Bellet et al., 2011). PGC-1α activates gluconeogenic gene transcription, but inhibits glycolytic gene transcription in the liver. In addition, PGC-1α stimulates mitochondrial biogenesis, which in turn promotes beta-

oxidation of fatty acids so as to reduce intracellular levels of fatty acids. PGC-1α expression in mouse liver and skeletal muscle exhibits diurnal rhythmicity with peak levels during the nighttime (Liu et al., 2007). AMPK is a nutrient sensing protein that increases beta-oxidation of fatty acids through PCG-1α-mediated mitochondria biogenesis and inhibits acetyl-CoA carboxylase, thus decreasing fatty acid production. *AMPK* mRNA expression and AMPK phosphorylation in mouse liver are marked by circadian rhythms that peak during the subjective day (Lamia et al., 2009). Consistent with these observations, we have observed circadian regulation of AMPK phosphorylation in mouse fibroblasts and this rhythm is abolished in fibroblasts with targeted disruption of *Per1* and *Per2*. In addition, microarray studies indicate that circadian gene expression in mouse fibroblasts is more prevalent among genes that regulate energetic processes such as glucose metabolism, mitochondrial energy transduction, lipid and fatty acid metabolism (e.g., stearoyl-coenzyme A desaturase 1 [Scd1], long chain fatty acyl CoA synthase [Facl], fatty acid synthase [Fasn]), and transporters of energy metabolites (Menger et al., 2007). Collectively, these observations indicate that circadian clock regulation of metabolic enzymes is important in the maintenance of energy homeostasis in peripheral tissues.

***Metabolites*:** Nutrient metabolism ultimately yields various metabolites, many of which oscillate on a circadian basis and depend on circadian clock function. Generally, the levels of amino acid metabolites are increased during the nighttime whereas carbohydrate, lipid, and nucleotide metabolites are increased during the daytime in nocturnal animals (Eckel-Mahan et al., 2012). In addition, the nucleotide metabolite,

nicotinamide adenine dinucleotide (NAD^+), is rhythmically expressed in mouse liver. Since NAD^+ is reduced to NADH during glycolysis, NAD^+ levels in the liver of fasted mice are elevated during the rest period, peaking near the middle of the subjective day (Peek et al., 2013). In ad lib-fed mice, NAD^+ exhibits a bimodal rhythm in liver with peaks in the middle of the day and middle of the night (Ramsey et al., 2009). In addition, nicotinamide phosphoribosyltransferase (NAMPT) mediates the NAD^+ oscillation because NAMPT directly regulates NAD^+ biosynthesis and its bimodal pattern in the liver is similar to that of NAD^+ (Ramsey et al., 2009). Importantly, both of these oscillations are clock-controlled because $Clock^{\Delta 19}$, $Bmal1^{-/-}$ or $Cry1/Cry2^{-/-}$ mutant mice display altered levels and/or arrhythmic profiles of NAMPT and NAD^+ in the liver (Nakahata et al., 2009; Ramsey et al., 2009). AMP is another rhythmically regulated nucleotide metabolite that is the byproduct of ATP degradation in response to energy demand. Similar to NAD^+, AMP levels in mouse liver are high during the day and low throughout the night (Eckel-Mahan et al., 2012). Cellular content of ATP fluctuates on a circadian basis in the rat SCN such that rhythmic levels peak during the middle of the subjective night (Yamazaki et al., 1994). Extracellular ATP accumulation similarly exhibits circadian oscillations in rat SCN *in vivo* and *in vitro*, with peak levels occurring during the latter half of the night (Womac et al., 2009). Furthermore, cAMP, a derivative of ATP, also oscillates in rat SCN with bimodal peaks during the late subjective day and late subjective night (Prosser and Gillette, 1991). These metabolites function as cofactors for various transcription factors and enzymes, therefore the circadian regulation of these metabolites is important to maintain energy homeostasis in peripheral tissues.

***Humoral factors*:** In conjunction with other processes regulating nutrient metabolism, the release of humoral factors plays a key role in fine-tuning energy homeostasis. Circadian oscillations in humoral factors such as adipokines contribute to the regulation of feeding cycles and nutrient metabolism by rhythmically modulating satiety or hunger. Adiponectin and leptin are two crucial adipokines released from adipose tissue in response to changes in systemic metabolic status. Adiponectin increases food intake whereas leptin has the converse effect. Interestingly, the peaks of circadian rhythms in serum levels of both adiponectin and leptin occur during the nighttime in rats. However, plasma levels of leptin are arrhythmic and constantly increased even during the daytime in $Clock^{\Delta 19}$ mice indicating that clock disruption causes metabolic disorders such as hyperleptinemia (Turek et al., 2005). In addition, hormones mediating local or systemic inflammation are associated with nutrient metabolism, especially in response to over-nutrition or high-fat diet. Recent evidence indicates that circadian clocks in peripheral tissues play an integral role in linking inflammatory and metabolic responses.

***Inflammatory response*:** Like other peripheral cells and tissues throughout the body, immune cells, including macrophages, B- and T-cells, contain cell-autonomous clocks (Keller et al., 2009). These endogenous clocks in immune cells provide for the circadian control of their abundance in the circulation, production of inflammatory factors, and functional responses to inflammatory challenge. For example, the circadian clock mediates the rhythmic regulation of: 1) the number of circulating immune cells in blood; 2) NF-κB, a key mediator of immune responses that modulates the expression of target

inflammatory genes (Bozek et al., 2009); 3) macrophage expression of toll-like receptor (TLR) 4 that regulates innate immunity and lipopolysaccharide (LPS) responsiveness; and 4) LPS-induced TNF-α and IL-6 release by macrophages (Keller et al., 2009). Importantly, the rhythmic regulation of immune system physiology and function is controlled by the circadian clock mechanism. For example, the circadian regulation of leukocyte abundance in the circulation is abolished in *Clock* mutant mice (Oishi et al., 2003; Oishi et al., 2006). In addition, disruption or deletion of *Per2* abolishes the daily rhythms in serum levels of the proinflammatory cytokine IFN-γ and the rhythm in susceptibility to LPS-induced inflammatory death (Arjona and Sarkar, 2006; Liu et al., 2006). Similarly, the circadian rhythm in TLR9 expression is altered in peritoneal macrophages from *Per2* mutant mice (Silver et al., 2012).

Thus, there is substantial evidence indicating that the circadian clock directly or indirectly regulates many different metabolic processes involved in the maintenance of energy homeostasis.

Disruption of the Circadian Clock and Metabolic Disorders

Over-nutrition has long been considered as the main cause of alterations in energy homeostasis that lead to metabolic disorders. However, recent studies suggest that dysregulation of circadian clock function may contribute to metabolic disorders based on their increased incidence in shift workers and individuals experiencing sleep loss or maintaining nocturnal lifestyles (Buxton et al., 2012). For example, shift work is associated with high levels of triglycerides and low levels of high-density lipoproteins in

the plasma, and elevated blood pressure, all of which are indicative of metabolic disorders (Karlsson et al., 2003). Shift-workers are also distinguished by increased risk of diabetes (Morikawa et al., 2005) and metabolic syndrome (obesity, high triglycerides, and low concentrations of HDL cholesterol) (Karlsson et al., 2003). In humans with nighttime eating syndrome (NES) in which frequent waking from sleep during the night is followed by food intake, metabolism- and feeding-related rhythms in circulating levels of insulin, leptin and ghrelin are phase shifted, and the circadian profile of plasma glucose levels is inverted relative to control subjects (Birketvedt et al., 1999; Goel et al., 2009). Studies using animal models of environmentally- and genetically-induced circadian rhythm disruption have similar implications for clock involvement in metabolic disorders including obesity and diabetes. Environmental dysregulation of circadian clock function in mice exposed to a 20hr LD cycle produces accelerated weight gain, hyperleptinemia, and elevated insulin secretion (Karatsoreos et al., 2011). Furthermore, circadian disruption in mice housed in constant light (LL) results in increased body weight gain and fat pad mass as well as impaired glucose tolerance (Fonken et al., 2010).

Consistent with the effects of environmental paradigms, genetic models with mutation or deletion of core clock genes correspondingly indicate that global and tissue-specific disruption of the circadian clock mechanism is an exacerbating factor in the development of metabolic disorders (Turek et al., 2005; Marcheva et al., 2010; Paschos et al., 2012). In conjunction with their altered circadian phenotype, Clock mutant mice exhibit many hallmarks of metabolic disease, including hyperphagia, obesity, glucose intolerance, and insulin resistance (Turek et al., 2005; Marcheva et al., 2010). Furthermore, global and

adipocyte-specific circadian disruption caused by deletion of Bmal1 produces a metabolic phenotype characterized by increased body weight and adipose tissue mass, adipocyte hypertrophy and reduced circulating levels of polyunsaturated fatty acids (Paschos et al., 2012). Similarly, pancreas-specific Bmal1 mutant mice are distinguished by impaired glucose tolerance and reduced insulin secretion (Marcheva et al., 2010). Glucose homeostasis is also altered in Cry-deficient (Cry1/Cry2) mice such that glucose tolerance is severely impaired but insulin sensitivity remains normal (Lamia et al., 2011; Zhang et al., 2010). Conversely, it is interesting that overexpression of Cry1 decreases blood glucose levels and improves glucose tolerance in insulin-resistant db/db mice (Zhang et al., 2010).

While these studies provide clear evidence that circadian clock dysregulation plays a key role in the development of metabolic diseases such as obesity and diabetes, the specific mechanisms underlying the link between circadian clock- and metabolic-dysfunctional phenotypes are unknown. Therefore, the **central hypothesis** of my dissertation research is that mutual interactions between circadian clocks and inflammatory signaling are critical in the mechanism by which HFD and saturated fats induce proinflammatory activation and metabolic dysregulation. To test this hypothesis, my dissertation experiments addressed the following specific aims:

***Specific Aim 1*:** Does high fat diet alter fundamental properties of SCN-driven and peripheral circadian rhythms? (**Chapter II)**;

***Specific Aim 2*:** Does environmental desynchronization of circadian rhythms recapitulate the effects of clock gene mutations in amplifying proinflammatory status? (**Chapter III)**;

***Specific Aim 3*:** Does inflammatory signaling mediate the differential effects of saturated and polyunsaturated fatty acids on circadian clocks? (**Chapter IV**);

***Specific Aim 4*:** Do proinflammatory cytokines and other mediators of inflammatory signaling govern the effects of high fat diet and saturated fatty acids on peripheral circadian clocks? **(Chapter V)**.

These studies will provide novel information on how mutual interactions between circadian clocks, inflammatory signaling pathways and metabolism are important both in metabolic homeostasis and the link between clock dysfunction and metabolic phenotypes in diet-associated obesity. These studies may ultimately lead to the development of chronotherapeutic (i.e., "timed") drug and/or even diet-specific strategies in the treatment/prevention of systemic metabolic disorders and inflammation-related pathologies (e.g., cardiovascular disease, stroke, arthritis).

CHAPTER II

HIGH FAT DIET MODULATES CIRCADIAN CLOCK PROPERTIES*

Introduction

Over-nutrition through consumption of high fat diet (HFD) is a major risk factor for metabolic disorders that have rapidly increased over the last few decades in association with elevated rates of obesity. HFD-induced obesity is highly associated with insulin resistance that contributes to the development of a wide variety of metabolic diseases including type 2 diabetes, fatty liver disease, and cardiovascular disease (Angulo, 2002; Hossain et al., 2007; Jensen et al., 2008). Current evidence indicates that HFD triggers chronic low-grade inflammation that is characterized by increased infiltration of immune cells, production of pro-inflammatory cytokines and activation of M1 macrophages (pro-inflammatory) in peripheral tissues such as liver, skeletal muscle and adipose tissue (Fujisaka et al., 2009; Lumeng et al., 2007; Weisberg et al., 2003a). Specifically, HFD initiates NF-κB mediated production of pro-inflammatory cytokines/chemokines and in turn, cytokines/chemokines induce immune cell infiltration and macrophage polarization toward M1. As such, HFD-induced inflammation is a key component in the pathogenesis of insulin resistance (Guo et al., 2010a; Kang et al., 2008b; Lumeng et al., 2007; Weisberg et al., 2003a).

*Reprinted from Xu H, Li H, Woo SL, Kim SM, Shende VR, Neuendorff N, Guo X, Guo T, Qi T, Pei Y, Zhao Y, Hu X, Zhao J, Chen L, Chen L, Ji JY, Alaniz RC, Earnest DJ, Wu C (2014) Myeloid cell-specific disruption of Period1 and Period2 exacerbates diet-induced inflammation and insulin resistance. J Biol Chem 289(23): 16374-88.

In addition to its effect on pro-inflammatory responses, HFD also modulates fundamental properties of circadian behavioral rhythms and the underlying molecular clockworks. For example, long term administration of HFD for 12 weeks increases the period of the activity rhythm and alters circadian oscillations in the expression of core clock components and clock-controlled genes that mediate nutrient metabolism in peripheral tissues in mice (Kohsaka et al., 2007a). In addition, short-term treatment with HFD for 1 week induces phase advance of the mPER2::LUC rhythm in the liver (Pendergast et al., 2013). However, both long- and short-term HFD treatment have little or no effect on SCN-driven behavioral rhythms and clock gene rhythms in the SCN relative to its effect on peripheral clocks. Collectively, this evidence indicates that HFD can veritably modulate circadian clock properties in peripheral tissues.

Circadian clocks in peripheral tissues and cells drive daily rhythms and coordinate many physiological processes including immune responses and metabolism. For example, NF-κB activation in response to immune challenge is rhythmically regulated with maximal activation at ZT6 in mice (Spengler et al., 2012), and is mediated by core clock components such as CLOCK and CRY (Narasimamurthy et al., 2012). Another key component of inflammatory signaling, c-Jun N-terminal kinase (JNK), is also phosphorylated rhythmically in rat-1 fibroblasts *in vitro* (Chansard et al., 2007). As key mediators of inflammation in obesity, macrophages contain cell-autonomous circadian clocks that rhythmically modulate LPS-induced secretion of proinflammatory cytokines such as TNFα and IL-6 in the spleen (Keller et al., 2009). The abundance of circulating immune cells is similarly clock-controlled. For example, leukocyte abundance in the

circulation fluctuates on a circadian basis and this rhythm is abolished in *Clock* mutant mice (Oishi et al., 2003; Oishi et al., 2006).

Recent observations suggest that circadian clocks play a key role in the regulation of metabolic homeostasis and that the disruption or even dysregulation of circadian timekeeping triggers the development of metabolic disease including obesity and diabetes. In mice with genetic mutation or deletion of core clock genes, global or adipocyte-specific disruption of circadian clock function has been shown to produce obesity or significant alterations in metabolism as well as exacerbated pro-inflammatory responses (Castanon-Cervantes et al., 2010; Marcheva et al., 2010; Paschos et al., 2012; Turek et al., 2005). However, the specific mechanism underlying the link between circadian clock- and metabolic-dysregulated phenotypes is unknown. As key components of inflammation in HFD induced obesity, macrophages contain cell-autonomous circadian clocks that have been shown to gate macrophage inflammatory responses including rhythms in LPS-induced cytokine secretion (Gibbs et al., 2012; Keller et al., 2009). Because HFD induces adipose tissue circadian clock dysregulation in conjunction with adipose tissue macrophage pro-inflammatory activation (Kohsaka et al., 2007a) environment-mediated circadian disruption amplifies macrophage pro-inflammatory responses (Castanon-Cervantes et al., 2010), our hypothesis is that over-nutrition causes circadian dysregulation in macrophage clocks, which in turn exacerbates pro-inflammatory activation and fat deposition in adipose tissue, thus leading to systemic insulin resistance. We initially tested this hypothesis by determining whether HFD induces circadian clock dysregulation in indigenous and adipose tissue macrophages.

Material and Methods

***Animals*:** Animals (male and female, 6 weeks old) used in this study were derived from breeding pairs of homozygous *mPer2*^{Luc} knock-in mice (C57BL/6J background; generously provided by Dr. Joseph Takahashi, UT Southwestern Medical School, Dallas, TX). In homozygous *mPer2*^{Luc} mice, a luciferase (Luc) gene was fused in-frame to the C-terminus of the endogenous mPER2 coding sequence so as to enable continuous recording of *Per2* oscillations via luciferase bioluminescence (Yoo et al., 2004a). All mice were maintained on a 12:12-h light-dark cycle (lights on at 06:00). All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

***Wheel running activity*:** To examine the dietary effect on *in vivo* circadian clock rhythmic in relation to obesity-associated metabolic phenotypes, *mPer2*^{Luc} mice were subjected to HFD (60% fat calories, 20% protein calories, and 20% carbohydrate calories, Table 1) feeding. Briefly, female homozygous *mPer2*^{Luc} mice, at 5 – 6 weeks of age, were fed an HFD for 12 weeks as previously described (Guo et al., 2010b; Huo et al., 2010a). Age- and gender-matched *mPer2*^{Luc} mice were fed a low-fat diet (LFD, 10% fat calories, 20% protein calories, and 70% carbohydrate calories) and used as controls. After the feeding regimen, both HFD-fed and LFD-fed *mPer2*^{Luc} mice were subjected to examination of wheel-running locomotor activity rhythms in constant darkness as previously described (Ko et al., 2011).

Table 1. Formula of high fat diet (from Research diets. Inc)

HFD		**gm%**	**kcal%**
Protein		26.2	20
Carbohydrate		26.3	20
Fat		34.9	60
	Total		100
	kcal/gm	5.24	

Ingredient	**gm**	**kcal**
Casein, 30 Mesh	200	800
L-Cystine	3	12
Corn Starch	0	0
Maltodextrin 10	125	500
Sucrose	68.8	275.2
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard	245	2205
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Blue Dye#1	0.05	0
Total	**773.85**	**4057**

***Isolation of stromal vascular cells from adipose tissue*:** Adipose tissue stromal vascular cells (SVC) were isolated using the collagenase digestion method as previously described (Huo et al., 2010a; Stienstra et al., 2008). After digestion and centrifugation, the pelleted cells were collected as SVC. Adipose tissue SVC from *mPer2*^{Luc} mice were subjected to bioluminescence analysis as previously described (Farnell et al., 2011).

***Macrophage differentiation and characterization*:** Bone marrow cells were isolated from the tibias and femurs of LFD- and/or HFD-fed *mPer2*^{Luc} mice as previously described (Odegaard et al., 2007a). After differentiation with Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum and 15% L929 culture supernatant for 8 days, bone marrow-derived macrophages (BMDM) were subjected to analysis of circadian rhythms of bioluminescence.

***Circadian properties of* mPer2^{Luc} *SVC and BMDM cells*:** SVC and BMDM prepared from LFD- and/or HFD-fed *mPer2*^{Luc} mice were suspended in supplemented Dulbecco's modified Eagle's medium (DMEM) (for SVC) or IMDM (for BMDM) and then plated on 35mm culture dishes (Corning). For both cell types, the medium was changed approximately 24 hr after plating so as to reduce the FBS concentration to 5% and about 12 hr later cultures were exposed to medium containing 50% adult horse serum for 2 hr. Thereafter, serum-shocked SVC and BMDM were maintained in serum-free recording medium containing 0.1mM beetle luciferin (Promega), 25 units/ml penicillin, and 25 ug/ml streptomycin for bioluminescence analysis as described previously (Ko et al., 2011).

Individual cultures were sealed airtight with sterile glass coverslips (VWR), and sterile silicon grease (Dow Corning). The temporal patterns of mPER2::LUC bioluminescence were analyzed using an automated 32-channel luminometer (LumiCycle; Actimetrics, Wilmette, IL, USA) that was maintained within a standard cell culture incubator at 35°C. Bioluminescence from individual cultures was continuously recorded with a photomultiplier tube (PMT) for ~70 sec at intervals of 10 minutes. Due to the transient induction of bioluminescence following the medium change at the initiation of this analysis, the first cycle was excluded from data analysis. Bioluminescence data was analyzed using the Lumicycle Analysis program (Actimetrics). For each raw data set, baseline drift was removed by fitting a polynomial curve with an order equal to one less than the number of recorded cycles. Rhythm parameters were determined from baseline-subtracted data using LM (damped Sine) Fit.

***Statistical methods*:** Numeric data are presented as means ± SE (standard error). Statistical significance was assessed by unpaired, two-tailed Student's *t* tests. Differences were considered significant at the two-tailed $P < 0.05$.

Results

***HFD induces a small, but significant, increase in the period of the circadian rhythm of mouse wheel running activity*:** To address a possible link between metabolic phenotype and alterations in circadian clock function, dietary effects on circadian rhythms *in vivo* were examined in homozygous *mPer2*^{Luc} knock-in mice (C57BL/6J background) that were

fed either a HFD or LFD for 12 weeks in constant darkness. During the 12-week treatment interval, both LFD- and HFD-fed mice exhibited clear free-running activity rhythms in DD. Although the general rhythmic patterns absolute levels of wheel-running activity in DD were comparable in both treatment groups, the free-running period of the activity rhythm in HFD-fed *mPer2*^{Luc} mice was significantly increased (p<0.001) by ≈0.4hr relative to that observed in LFD-fed mice (Figure 1; mean $\tau = 24.2 \pm 0.1$ vs 23.8 ± 0.1 hr, respectively).

***HFD feeding lengthens the period of clock gene oscillations in mouse adipose tissue stromal vascular cells and bone marrow-derived macrophages*:** Given the role of macrophages in inflammation (Huo et al., 2010a; Lumeng et al., 2007), we analyzed circadian oscillations of the clock gene *Per2* in adipose tissue SVC, the immune cell-containing fraction of collagenase-digested visceral fat that has been widely used to examine the inflammatory status of adipose tissue macrophages (Lumeng et al., 2007; Odegaard et al., 2007a), and BMDM derived from *mPer2*^{Luc} mice. SVC cultures from LFD-fed mice exhibited robust mPER2::LUC rhythms with a circadian period of 25.06 ± 0.4 hrs (Figure 2A). However, SVC cultures from HFD-fed mice were characterized by mPER2::LUC rhythms in which the period was significantly increased (p<0.05) by ~ 9 hrs (Figure 2A). Similarly, the period of BMDM rhythms in mPER2::LUC bioluminescence was significantly increased (p<0.05) in cultures from HFD-fed *mPer2*^{Luc} mice relative to those from LFD-fed mice (Figure 2B). BMDM cultures from HFD-fed

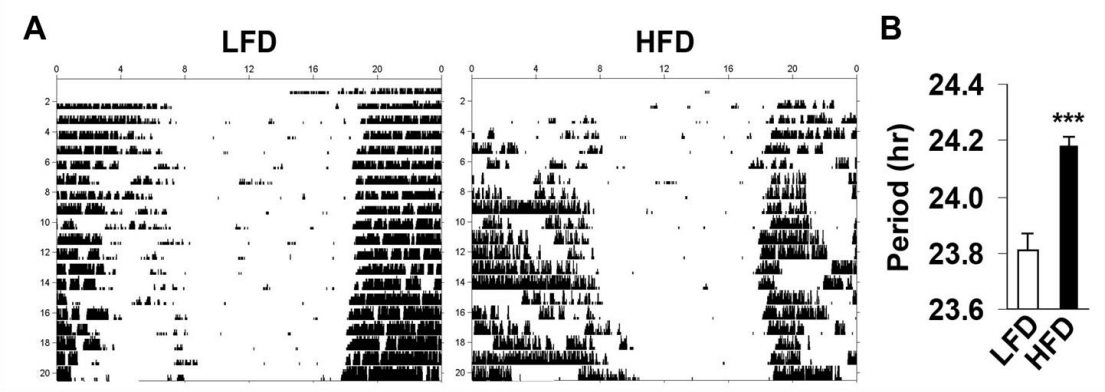

Figure 1: HFD alters the free-running period of the mouse activity rhythm. **(A)** Representative circadian rhythms of wheel-running activity in mice (5 – 6 weeks of age) that were fed a low-fat diet (LFD) (left panel) or high-fat diet (HFD) for 12 weeks (n = 5 – 10) and exposed to constant darkness. **(B)** Bar graph depicts differences in the free-running period of the activity rhythm between treatment groups. Data are means ± SEM in bar graph. ***, $p < 0.001$, HFD vs. LFD.

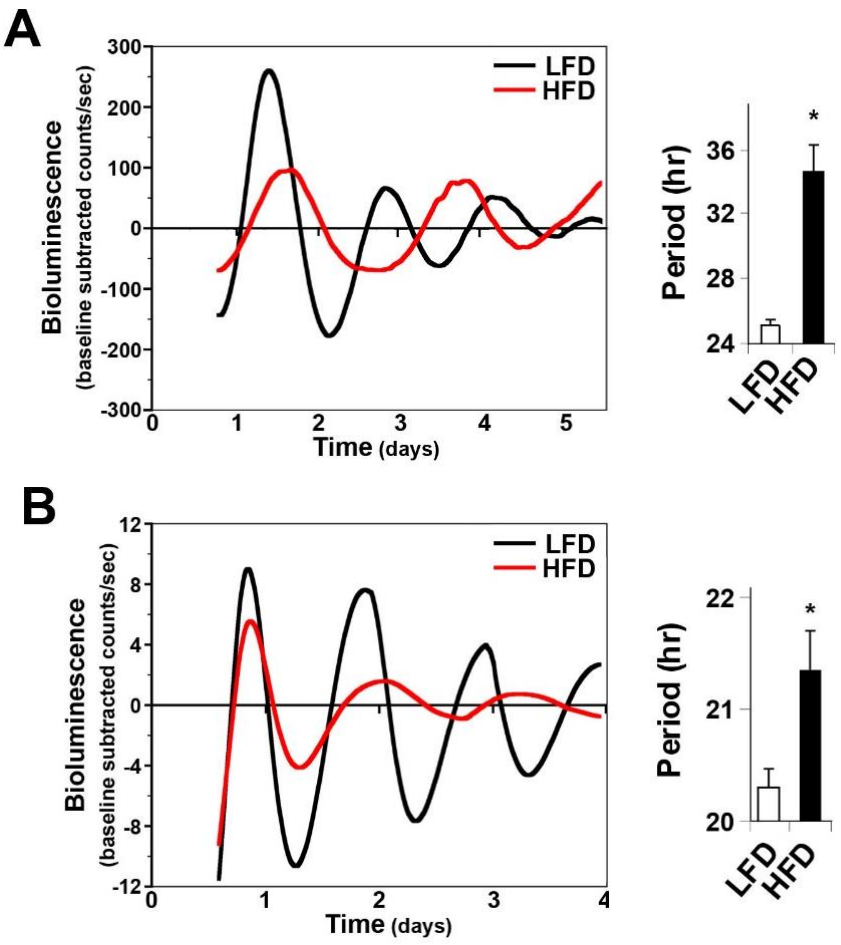

Figure 2: HFD lengthens the period of clock gene oscillations in adipose tissue SVC and in macrophages. Representative temporal patterns of ensemble PER2::LUC bioluminescence (expressed as detrended baseline-subtracted counts per second) in cultures of **(A)** adipose tissue stromal vascular cells (SVC) or **(B)** bone marrow-derived macrophages (BMDM) derived from *mPer2*^{Luc} mice (5 – 6 weeks of age) that were fed a LFD or HFD for 12 weeks (n = 5 – 6). Bar graphs depict group differences in PER2::LUC rhythm period between treatment groups. For bar graphs (A and B), data are means ± SEM. *, $P < 0.05$ and **, $P < 0.01$ HFD vs. LFD (A and B).

mPer2^{Luc} mice were also distinguished by robust decreases in the amplitude of the mPER2::LUC rhythm.

Discussion

During obesity, macrophage pro-inflammatory activation initiates or aggravates inflammation in key metabolic organs including adipose and liver tissues, thereby leading to systemic insulin resistance. However, it remains to be elucidated exactly how over-nutrition induces pro-inflammatory activation of macrophages, particularly in those found in adipose tissue where increased inflammation plays a causal role in the pathogenesis of insulin resistance (Guo et al., 2010b; Huo et al., 2010a; Kang et al., 2008a; Lumeng et al., 2007; Weisberg et al., 2003b). In parallel with this inductive effect on macrophage pro-inflammatory activation (Lumeng et al., 2007), HFD feeding has been shown to decrease the amplitude of adipose tissue clock gene rhythms in *Bmal1*, *Clock*, and *Per 2* expression (Kohsaka et al., 2007b) and to lengthen the period of the *Per2* rhythm in cultured adipose tissue (Shi et al., 2013a). Taken together, these observations suggest that dysregulation of peripheral circadian clocks may link diet-associated obesity to adipose tissue inflammation. In support of this putative association, the present study demonstrates that in HFD-fed mice where minor increases in period of the activity of rhythm were similar to those observed previously (Kohsaka et al., 2007b), over-nutrition caused dysregulation of peripheral circadian clocks (i.e., substantial increases in the period of *Per 2* oscillations) specifically within key mediators of adipose tissue inflammation, macrophages in stromal vascular cells and BMDM.

Importantly, the results of the current study are consistent with previous observations indicating that HFD-induced alterations in the circadian timekeeping properties of peripheral clocks are considerably greater in comparison with its impact on SCN clock gene oscillations and SCN-driven rhythms. In this regard, Kohsaka and colleagues (Kohsaka et al., 2007a) have reported that HFD treatment for 6 weeks similarly has little or no effects on the free-running period of the mouse activity rhythm and hypothalamic (near SCN) clock gene oscillations but induces severe damping of the same rhythms in liver and fat. Similarly, short-term HFD treatment (7days) advanced the phase of clock gene rhythms in mouse liver by about 5hr, but the phase of SCN clock gene oscillations was not affected (Pendergast et al., 2013). At present, the basis for the differential effects of HFD on SCN and peripheral clocks is unclear. Because HFD activates inflammatory signaling pathways and increases immune cell infiltration into tissue (Lumeng et al., 2007; Westfall et al., 2013; Yoshida et al., 2013), differences in the trafficking of immune cells and other mediators of inflammation may govern the extent to which HFD affects peripheral versus central clocks. The central nervous system (CNS) is distinguished by immune privilege so as to protect neuronal cells from damage caused by inflammation (Wenkel et al., 2000). The blood-brain barrier shields the CNS against this type of insult by restricting the migration and infiltration of peripheral immune cells into the brain (Engelhardt, 2008). Consequently, the brain is protected under normal circumstances from HFD-induced activation of peripheral macrophages and proinflammatory signaling and this may account for the predominant impact of HFD on peripheral clocks.

Because adipose tissue inflammatory responses and corresponding insulin sensitivity in over-nutrition-related obesity are differentially engaged by specific types of fatty acids found in HFDs, future studies are warranted to compare the effects of saturated and polyunsaturated fatty acids on macrophage and adipocyte circadian clocks. Thus, it will be important to determine whether saturated fatty acids that activate pro-inflammatory signaling pathways and lead to impaired insulin sensitivity (e.g., palmitate) also modulate the circadian timekeeping function of macrophage and adipocyte clocks and whether this circadian clock dysregulation is blocked or abated by polyunsaturated fatty acids that repress adipose tissue inflammatory responses and have a beneficial impact on insulin sensitivity.

CHAPTER III

ENVIRONMENTAL DESYNCHRONIZATION OF CIRCADIAN RHYTHMS AMPLIFIES DIET-INDUCED INFLAMMATION IN CONCERT WITH SYSTEMIC METABOLIC DYSREGULATION

Introduction

Circadian clocks located in peripheral tissues and cells throughout the body regulate daily rhythms that provide for the temporal organization of many local physiological processes including inflammation and metabolism. Functional properties of immune cells are rhythmically regulated by circadian clock machinery. For example, monocytes and macrophages exhibit rhythms in cytokine release during exposure to light-dark cycles and constant environmental conditions (Born et al., 1997; Rahman et al., 2015). In addition, TLR9 expression in macrophages is clock-controlled, thus mediating rhythmic responses to immunization in mice (Silver et al., 2012b). In mammals, robust circadian rhythms are also observed in metabolic processes such as lipid levels in the circulation which peak rhythmically in the morning (Dallmann et al., 2012). Thus, this precise temporal coordination of inflammatory responses and metabolism is thought to be critical for the maintenance of physiological homeostasis.

As indicated previously, dysregulation or disruption of circadian timekeeping contributes to the development of metabolic disorders including obesity and diabetes. In this regard, most studies have used mice with genetic mutation or deletion of core clock genes to demonstrate that global, or adipocyte-specific, disruption of circadian clock

function produces obesity or other signs of metabolic dysregulation (Paschos et al., 2012; Shi et al., 2013b). Similarly, our published studies using mice with targeted disruption of the *Per1* and *Per2* genes demonstrate that clock disruption exacerbates high fat diet-induced inflammation, resulting in systemic insulin resistance. This observation suggests that clock malfunction may play a key role in the mechanism by which diet-induced obesity triggers macrophage proinflammatory activation, adipose tissue inflammation, and insulin resistance.

Because many of the clock genes targeted in our and other studies function as transcription factors that regulate important mediators of inflammatory responses (e.g., Toll-like receptor [TLR], CCL2, IL-6, PPARγ) (Curtis et al., 2014; Lam et al., 2013; Nguyen et al., 2013; Silver et al., 2012a), it is possible that the altered inflammatory and metabolic phenotypes observed using genetic models are due to the disruption of non-clock, rather than circadian timekeeping, functions of core clock genes. To address this possibility and the implications of non-clock functions of core clock genes in interpreting results obtained with genetic models, many studies have resorted to environmental disruption of circadian rhythms using chronic jet lag or shift work paradigms. Interestingly, animals used in these studies display similar metabolic phenotypes as those found in genetic models. For example, mice exposed to chronic jet lag paradigms gain more body weight and show higher lipid levels in the circulation and increased adiposity relative to controls (Casiraghi et al., 2016). Therefore, the objective of this study was to determine whether environmental desynchronization of circadian rhythms in response to chronic shifts in the light-dark cycle (12hr every 5 days) recapitulates the effects of clock gene

deletions or mutations on HFD-induced macrophage polarization and infiltration, proinflammatory signaling, as well as glucose and insulin tolerance.

Material and Methods

Animals: Animals (male and female, 6 weeks old) used in this study were derived from breeding pairs of homozygous *mPer2*^{Luc} knock-in mice (C57BL/6J background) or WT mice (129J background) obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Homozygous *mPer2*^{Luc} mice are characterized by a *luciferase* (*Luc*) gene fused in-frame to the C-terminus of the endogenous mPER2 coding sequence so as to enable real-time recording of circadian oscillations in mPER2-driven bioluminescence (Yoo et al., 2004a). All animal procedures used in this study were conducted in compliance with Animal Use Protocol #2014-0248 as reviewed and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

***Wheel running activity*:** To examine the effect of environmental disruption of circadian rhythms *in vivo* circadian clock rhythmicity, *mPer2*^{Luc} mice were maintained on a 12:12-h light-dark cycle (lights on at 07:00) for about 2 weeks, and then randomly divided into 2 groups exposed either to this "fixed" (control) LD 12:12 cycle or to a "shifted" LD cycle. In shifted LD cycle, lights on was advanced by 12 hours every 5 days for about 10 weeks. In all fixed- or shifted-LD mice, wheel-running activity was continuously recorded, stored in 10-minute bins, graphically depicted in actograms and analyzed using ClockLab data collection and analysis software (ActiMetrics) as

previously described (Ko et al., 2011). Entrainment and qualitative parameters of the activity rhythm were measured over the same interval for all animals. Chi-square periodogram analysis was used to determine the period of the activity rhythm during exposure to the fixed or shifted LD cycles.

***Glucose and Insulin tolerance tests*:** To examine the effect of environmental disruption of circadian rhythms on obesity-associated metabolic phenotypes *in vivo*, 5-6 week old *mPer2*^{Luc} mice exposed to fixed or shifted LD cycles were fed a high-fat diet (60% fat calories, 20% protein calories, and 20% carbohydrate calories) for approximately 8 weeks as described previously (Guo et al., 2010b; Huo et al., 2010a). Body weight was recorded every 10 days during the HFD feeding regimen. All metabolic testing was conducted so as to occur at similar times during the circadian cycle in both fixed- or shifted-LD mice. For glucose tolerance and insulin resistance testing, mice were fasted for 12 h and 6 h respectively. Glucose tolerance tests were initiated at ZT 2 (9am) by intraperitoneal injection of D-glucose (2g/kg body weight) and collecting blood samples from the tail vein immediately before and at 15, 30, 60, and 120 min afterward. Insulin resistance tests were initiated at ZT 7 (2pm) intraperitoneal injection of insulin (1 unit/kg body weight) and collecting blood samples from the tail vein immediately before and at 30, 60, and 120 min afterward.

***Isolation of stromal vascular cells from adipose tissue*:** Epididymal adipose tissue was collected from HFD-fed *mPer2*^{Luc} mice exposed to fixed or shifted LD cycles and

stromal vascular cells (SVC) were isolated using the collagenase digestion method as previously described (Huo et al., 2010a; Stienstra et al., 2008). After digestion and centrifugation, the pelleted adipose tissue SVC cells were cultured for 7 days, and then independently subjected to FACS analysis, real-time PCR analysis of inflammatory cytokines and bioluminescence analysis of clock gene rhythms as previously described (Farnell et al., 2011; Xu et al., 2014).

***Macrophage differentiation and characterization*:** Bone marrow cells were isolated from the tibias and femurs of HFD-fed *mPer2^{Luc}* mice exposed to fixed or shifted LD cycles as previously described (Odegaard et al., 2007a). After differentiation with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 10ng/ml monocyte-colony stimulating factor (M-CSF) for 7 days, bone marrow-derived macrophages (BMDM) were independently subjected to bioluminescence analysis of clock gene rhythms, FACS analysis and real-time PCR analysis of inflammatory cytokines.

***Circadian properties of* mPer2^{Luc} *SVC and BMDM cells*:** SVC and BMDM prepared from HFD-fed *mPer2^{Luc}* mice exposed to fixed or shifted LD cycles were cultured in supplemented Dulbecco's modified Eagle's medium (DMEM) and then maintained in serum-free recording medium containing 1uM forskolin, 25mM HEPES, 292μg/ml L-glutamine, 100units/ml penicillin, 100μg/ml streptomycin and 10uM luciferin (Promega) for bioluminescence analysis as described previously (Ko et al., 2011). Individual cultures

were sealed airtight with sterile glass coverslips (VWR), and sterile silicon grease (Dow Corning). The temporal patterns of mPER2::LUC bioluminescence were analyzed using an automated 32-channel luminometer (LumiCycle; Actimetrics, Wilmette, IL, USA) that was maintained within a standard cell culture incubator at 32°C. Bioluminescence from individual cultures was continuously recorded with a photomultiplier tube (PMT) for ~70 sec at intervals of 10 minutes. Due to the transient induction of bioluminescence following the medium change at the initiation of this analysis, the first cycle was excluded from data analysis. Bioluminescence data was analyzed using the Lumicycle Analysis program (Actimetrics). For each raw data set, baseline drift was removed by fitting a polynomial curve with an order equal to one less than the number of recorded cycles. Rhythm parameters were determined from baseline-subtracted data using Levenberg-Marquardt (damped Sine) Fit.

***Flow cytometry analysis*:** SVC and BMDM derived from HFD-fed *mPer2*^{Luc} exposed to fixed or shifted LD cycles were stained with fluorescence-tagged antibodies: anti-F4/80, anti-CD11b for macrophages, and anti-CD11c and anti-CD206 for macrophage activation as previously described (Prieur et al., 2011), and subjected to FACS analyses using Accuri flow cytometer (BD Biosciences, San Jose, California, USA). Briefly, the harvested cells were analyzed based on FSC-A and SSC-A. Live cells were then examined for F4/80 (FITC) and CD11b (PerCP/Cy5.5) expression. Mature macrophages (F4/80^{+} CD11b^{+} cells) were then gated for CD11c (PE/Cy7) and CD206 (PE) expression (macrophage

polarization). Mature macrophages that were positive for CD11c but negative for CD206 were considered as M1 macrophages ($F4/80^+$ $CD11b^+$ $CD11c^+$ $CD206^-$ cells).

***RNA isolation, reverse transcription, and real-time PCR*:** To examine macrophage pro-inflammatory activation and signaling, SVC and BMDM were treated with PBS or LPS (10 ng/ml) for 30 min prior to cell harvest. Total cellular RNA Total cellular RNA was later extracted from individual cultures of SVC and BMDM cells using PureLink RNA mini kit (Ambion, Waltham, MA) according to the manufacturer's protocols. Relative quantification of interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) mRNA abundance was performed using SYBR-Green PCR technology [Applied Biosystems Inc. (ABI)] as described previously (Farnell et al., 2011; Xu et al., 2014). For each sample, real-time PCR analysis of IL-6 or TNFα mRNA levels was performed on duplicate aliquots using the cDNA equivalent of 1ng of total RNA. To control for differences in sample RNA content, β-actin mRNA was amplified with the cDNA equivalent of 1ng total RNA from the same samples. The comparative CT method was utilized to calculate the relative abundance for a given cytokine mRNA by normalization to corresponding β-actin levels in each sample and to a calibrator consisting of pooled cDNA from multiple samples. Results were normalized to β-actin mRNA and adjusted relative to the averages of fixed LD controls, which were arbitrarily set as 100%.

***Statistical methods*:** Statistical significance was assessed by pooled Student's *t* tests. Differences between fixed and shifted LD groups were considered significant at $p < 0.05$.

Results

***Shifted LD cycles disrupt circadian entrainment of the rhythm in mouse wheel-running activity*:** To determine whether environmental manipulations simulating shift work-type schedules induce circadian rhythm disturbances or desynchronization, wheel running activity was examined in mice exposed to either fixed or shifted LD cycles for about 10 weeks. Throughout the 10 weeks of analysis, all mice maintained on a fixed LD cycle showed stable entrainment (Figure 3) such that their daily onsets of activity consistently occurred shortly after lights off (at 1900hr). However, mice exposed to shifted LD cycles were distinguished by desynchronized patterns of wheel-running behavior in which the timing of activity onsets relative to lights-off was highly variable from day to day. Consistent with their desynchronized activity patterns, the mean period of the activity rhythm in shifted LD mice was considerably greater than 24hr ($\tau = 25.5 \pm 0.5$ hr).

***Effect of shifted LD cycles on clock gene rhythms in lymphoid tissues and cells*:** Lymphoid tissues and cells play an important role in inflammation and metabolism. In order to understand whether chronic shifts of the LD cycle alter clock gene rhythms in lymphoid tissues/cells, circadian oscillations of the peripheral clock gene *Per2* were analyzed in spleen, adipose tissue SVC and BMDM isolated from *mPer2*^{Luc} mice. The spleen, which plays a role in inflammation as well as lipid metabolism in response to HFD (Fatouros et al., 1995), of HFD-fed mice maintained on fixed LD cycles displayed robust mPER2::LUC rhythms with a circadian period of 23.18 ± 0.3 hrs. However, exposure to

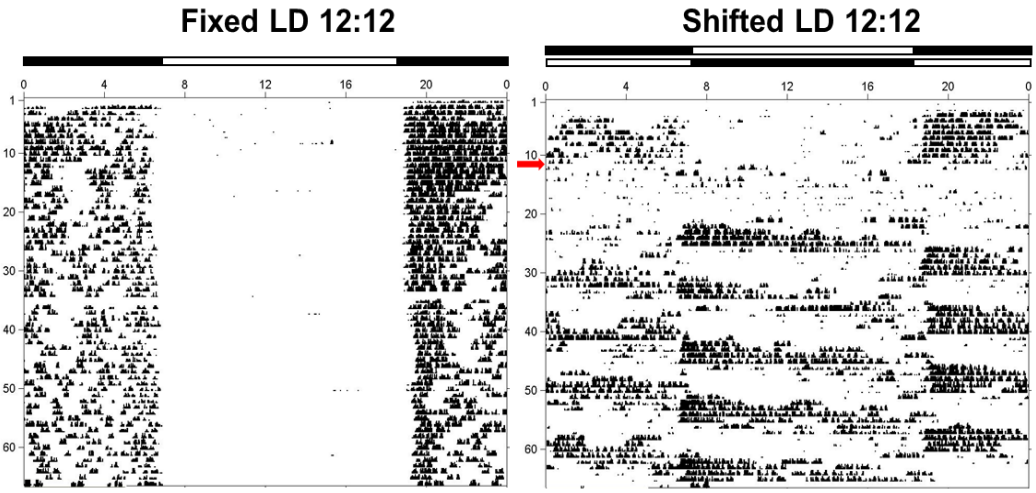

Figure 3: Shifted LD cycles disrupt circadian entrainment of the rhythm in mouse wheel-running activity. Representative records of wheel-running activity in mice (5 – 6 weeks of age) that were maintained in a fixed LD 12:12 cycle (left) or exposed to a shifted (12h advance every 5 days) LD 12:12 cycle (right). The open and closed bars at the top respectively signify the timing of the light and dark phase in the fixed and shifted LD 12:12 cycles.

shifted LD cycles significantly decreased ($p<0.05$) by about 1hr the period of mPER2::LUC rhythms in spleen cultures from HFD-fed mice (Figure 4A). Similarly, the period of PER2::LUC rhythms in SVC cultures isolated from HFD-fed mice on shifted LD cycles was significantly decreased ($p<0.05$) relative to the SVC rhythms observed in HFD-fed mice on fixed LD cycles (22.49 $\pm$ 0.4 hrs in the fixed LD cycles vs. 21.47 $\pm$ 0.2 hrs in the shifted LD cycles) (Figure 4B). In BMDM cultures from HFD-fed mice, there was no significant difference in the period of the PER2::LUC rhythms in the fixed and shifted LD groups (Figure 4C).

***Circadian desynchronization by shifted LD cycles increases macrophage pro-inflammatory activation*:** Since the circadian clock is deeply involved in regulating immune system and macrophages play a critical role in inflammation (Huo et al., 2010a; Lumeng et al., 2007), we examined macrophage pro-inflammatory activation and immune response in SVC, the immune cell-containing fraction of adipose tissue, and BMDM derived from HFD-fed *mPer2*^{Luc} mice kept either in fixed or shifted LD cycles. Similar to our observations based on the genetic disruption of myeloid cell-specific circadian clock function (Xu et al., 2014), circadian rhythm desynchronization during exposure to shifted LD cycles increased adipose tissue infiltration of pro-inflammatory macrophages. Following HFD treatment, the SVC of mice exposed to shifted LD cycles were distinguished by a significant ($p<0.05$) increase in mature macrophages (F4/80$^+$ CD11b$^+$ cells) relative to those found in fixed LD controls (Figure 5). Further analysis of the inflammatory status of the mature macrophages revealed that adipose tissue SVC from

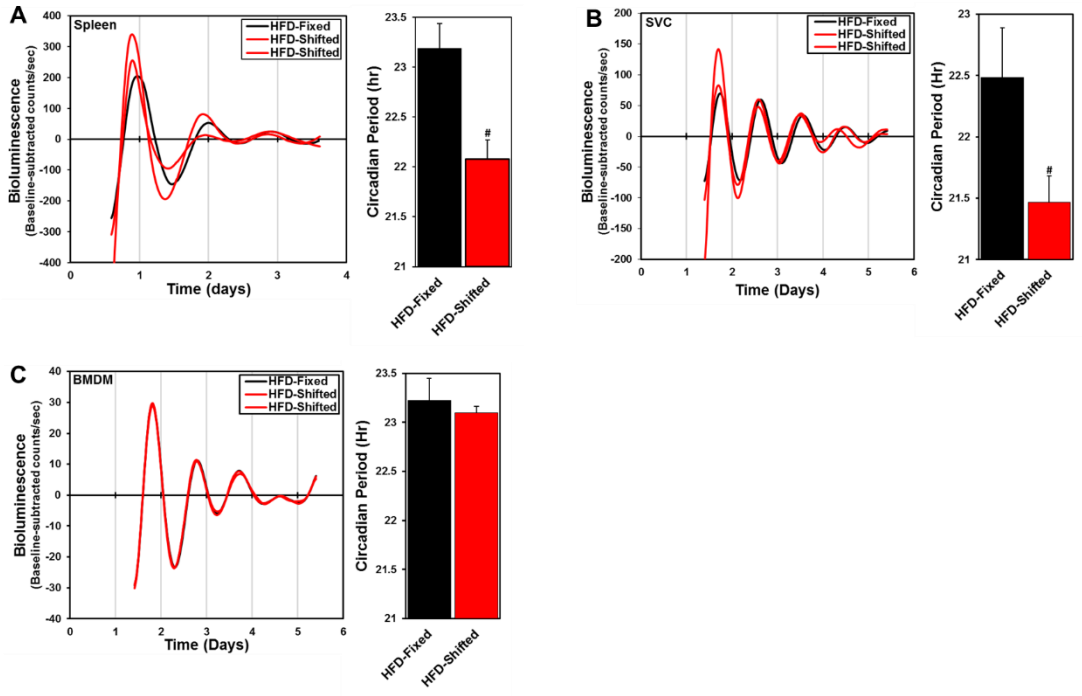

Figure 4: Effect of shifted LD cycles on the period of clock gene oscillations in lymphoid tissues and cells. Representative temporal patterns of ensemble PER2::LUC bioluminescence (expressed as detrended baseline-subtracted counts per second) in cultures of **(A)** spleen, **(B)** adipose tissue stromal vascular cells (SVC) or **(C)** bone marrow-derived macrophages (BMDM) from HFD-fed *mPer2*^{Luc} mice that were maintained in a fixed LD 12:12 cycle (black) or exposed to a shifted (12h advance every 5 days) LD 12:12 cycle (red). Bar graphs depict comparisons of the circadian period (mean + SEM) of the PER2::LUC rhythms in cultures from the fixed and shifted LD groups. Asterisk indicates that the period of the PER2::LUC rhythms in cultures from fixed LD controls was significantly greater ($p<0.05$) than that in tissue or cells from shifted LD mice.

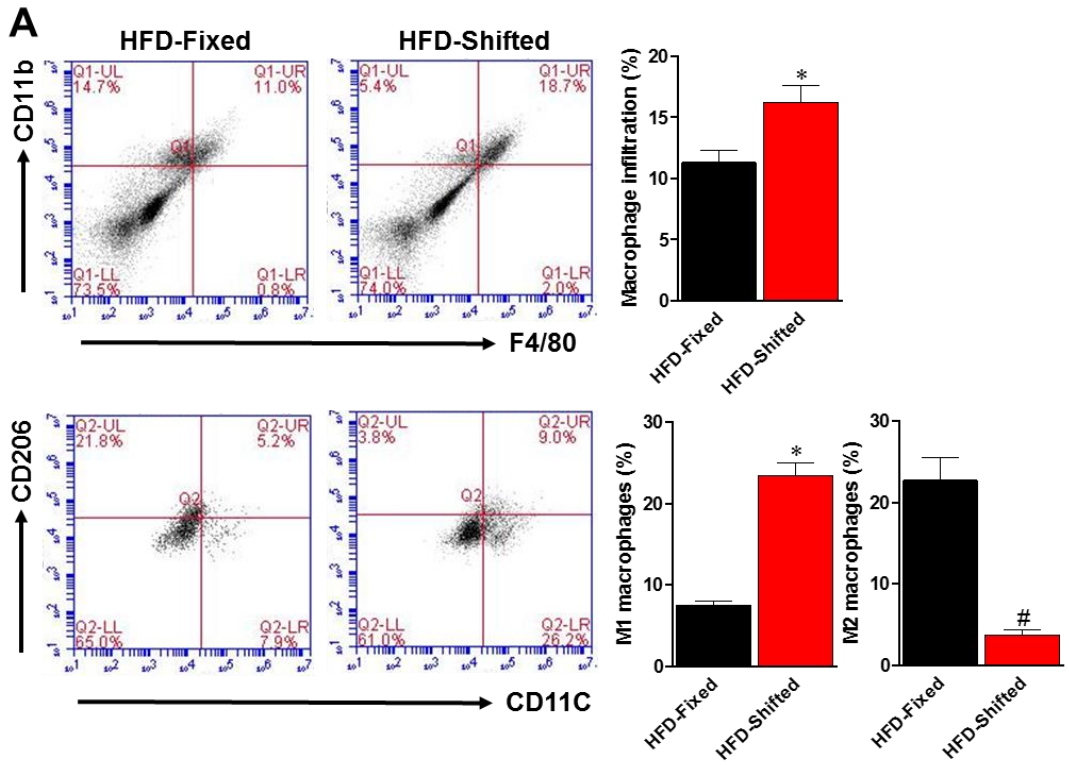

Figure 5: Environmental desynchronization of circadian rhythms exacerbates diet-induced adipose tissue inflammation. FACS analyses of mature macrophages ($CD11b^+$ and $F4/80^+$ cells, upper right corner on the scatter plot) in adipose tissue SVC from HFD-fed mice that were maintained in a fixed LD 12:12 cycle (left scatter plot) or exposed to a shifted (12h advance every 5 days) LD 12:12 cycle (right scatter plot). Bar graphs depict quantification of the percentages (mean ± SEM) of macrophage infiltration, proinflammatory M1 macrophages (F4/80+ CD11b+ CD11c+ CD206− cells) and anti-inflammatory M2 macrophages (F4/80+ CD11b+ CD11c− CD206+ cells) in SVC isolated from HFD-fed mice in the fixed and shifted LD groups. *, #, $p < 0.05$, Fixed vs. Shifted.

shifted LD showed a significant increase in pro-inflammatory M1 macrophages ($F4/80^+$ $CD11b^+$ $CD11c^+$ $CD206^-$ cells) and decrease in anti-inflammatory M2 macrophages ($F4/80^+$ $CD11b^+$ $CD11c^-$ $CD206^+$ cells) compared to the observed percentages in fixed LD controls (Figure 5). Furthermore, BMDM from mice exposed to shifted LD cycles similarly showed a significantly ($p<0.05$) higher percentage of M1 macrophages relative to that observed in fixed LD BMDM (Figure 6).

Consistent with the increases in M1 macrophages associated with circadian rhythm desynchronization, LPS-induced increases in BMDM expression of pro-inflammatory cytokines were much greater in shifted LD mice than in fixed LD controls. In response to LPS treatment, IL-6, IL-1β and TNFα mRNA levels in BMDM from HFD-fed mice on shifted LD cycles were significantly increased ($p<0.05$) relative to their respective levels in BMDM from HFD-fixed LD-exposed mice (Figure 7).

***Circadian desynchronization by shifted LD cycles exacerbates HFD-induced insulin resistance and glucose intolerance*:** High fat diet increases macrophage pro-inflammatory activation (Xu et al., 2014) and the corresponding induction of inflammatory signaling contributes to the development of insulin resistance and glucose intolerance (Han et al., 2013; Solinas et al., 2007), which are major phenotypes of metabolic disorders. Therefore, we next determined whether the changes in inflammatory status observed in shifted LD mice were accompanied by corresponding indices of metabolic dysregulation. During HFD feeding for 8 weeks, LD shifted mice exhibited a progressive increase in body weight

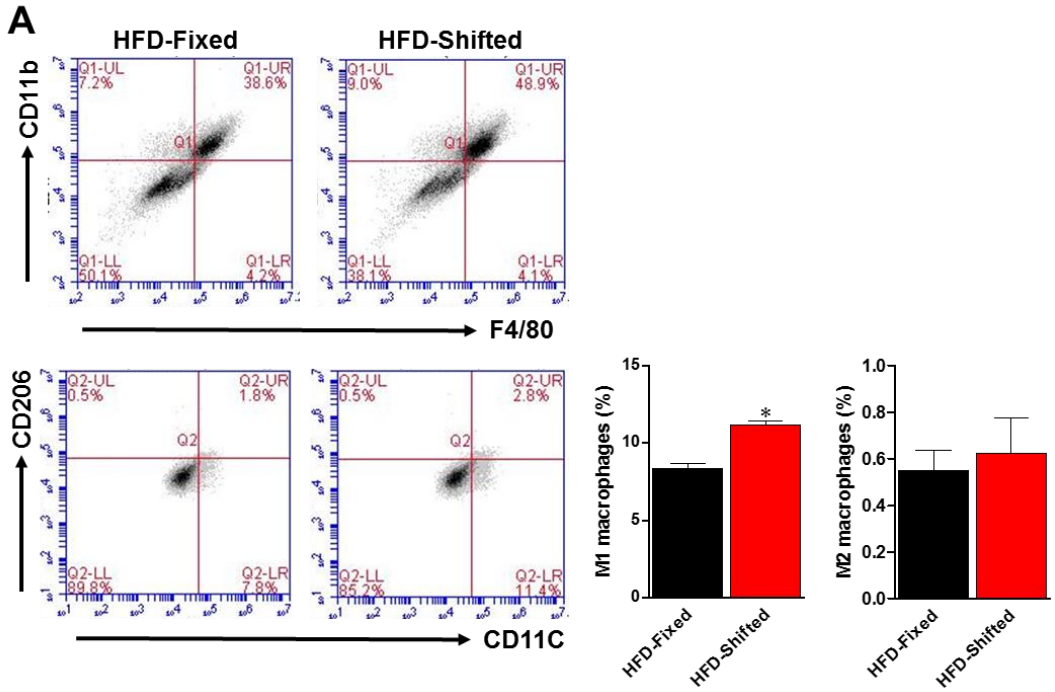

Figure 6: Environmental desynchronization of circadian rhythms increases diet-induced macrophage proinflammatory activation. FACS analyses of proinflammatory M1 macrophage (F4/80+ CD11b+ CD11c+ CD206− cells, upper left corner on the scatter plot) and anti-inflammatory M2 macrophages (F4/80+ CD11b+ CD11c− CD206+ cells, lower right corner on the scatter plot) in BMDM of HFD-fed mice that were maintained in a fixed LD 12:12 cycle (left scatter plot) or exposed to a shifted (12h advance every 5 days) LD 12:12 cycle (right scatter plot). Bar graphs depict quantification of proinflammatory M1 macrophages and anti-inflammatory M2 macrophages in cultured BMDM obtained from mice in the fixed and shifted LD groups. The depicted data are means ± SEM. *, $p < 0.05$, Fixed vs. Shifted.

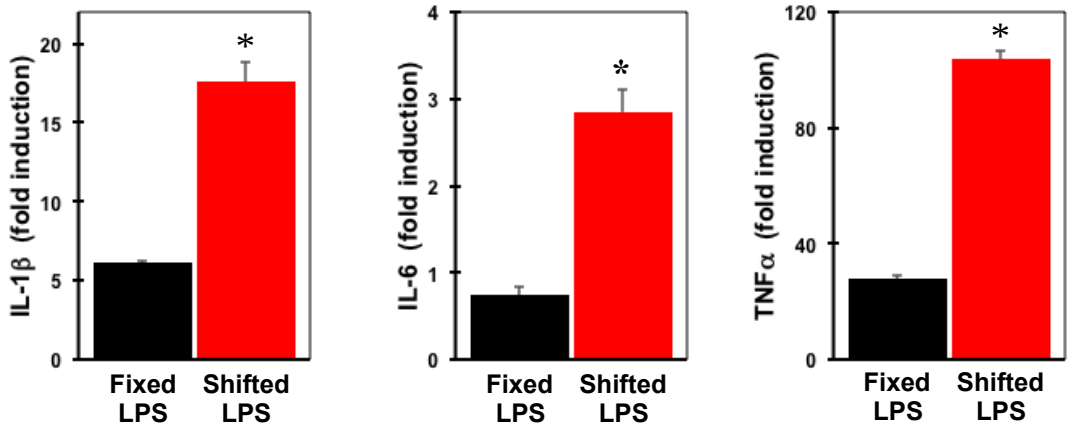

Figure 7: Environmental desynchronization of circadian rhythms increases expression of proinflammatory cytokines in response to LPS stimulation in BMDM. BMDM cultures were treated with LPS (10 ng/ml) or PBS for 30 mins before cell harvest. The mRNA levels of IL-1β, IL-6, and TNFα were quantified using real-time PCR and plotted as fold induction relative to PBS control group. For bar graphs, data are the means ± SEM. *, $p < 0.05$, Fixed vs. Shifted

compared to those maintained on a fixed LD cycle. Before HFD feeding, the body weight of shifted LD mice did not differ from that observed in LD controls. However, the HFD-induced increase in body weight of shifted LD mice was significantly ($p < 0.05$) greater than that found in fixed LD controls. Importantly, the observed effect of shifted LD on the body weight was not associated with a corresponding change in food intake because total daily consumption of HFD was similar in the fixed and shifted LD groups (Figure 8A). However, it is interesting that food consumption in LD shifted mice was almost equally distributed between the day and night whereas fixed LD mice consumed significantly ($p<0.05$) more food during night than day (Figure 8A). As a result of these treatment differences in the diurnal distribution of food intake, daytime HFD consumption was significantly ($p<0.05$) greater in shifted LD mice than fixed LD controls. Consistent with our reported findings on the metabolic dysregulation induced by myeloid cell-specific *Per1/2* disruption (Xu et al., 2014), HFD-fed mice on shifted LD cycles displayed increases in the severity of systemic glucose intolerance and insulin resistance, such that plasma glucose levels at 30, 60 and 120min after initiation of these metabolic tests were significantly ($p<0.05$) higher in shifted LD mice than fixed LD controls (Figure 8B). Thus, environmental desynchronization of circadian rhythms is sufficient to recapitulate the effects of genetic disruption of circadian clock function in amplifying HFD-induced inflammation in concert with systemic metabolic dysregulation.

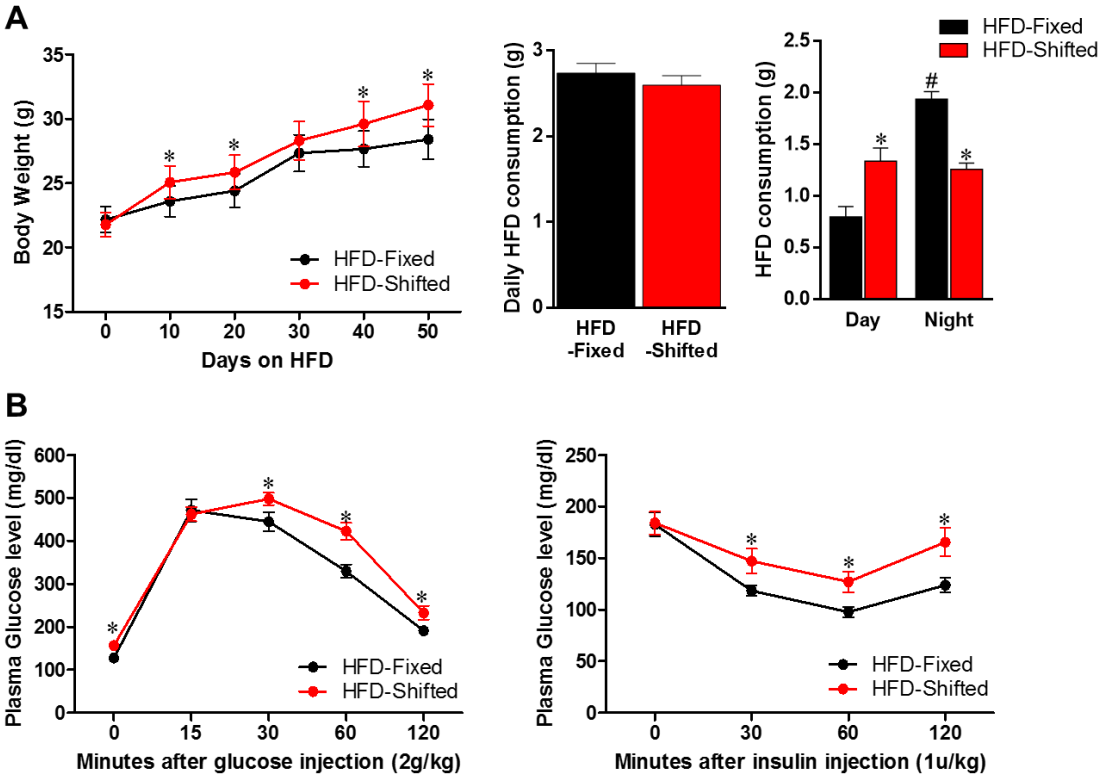

Figure 8: Environmental desynchronization of circadian rhythms exacerbates diet-induced insulin resistance and glucose intolerance. Graphs depict determinations (means ± SEM) of: **(A)** body weight (every 10 days), average daily food intake, and day-night distribution of HFD consumption; and **(B)** plasma glucose levels at timed intervals following intraperitoneal injection of (left panel) glucose (2 g/kg; glucose tolerance test) or (right panel) insulin (1 U/kg; insulin tolerance test) in mice that were fed an HFD for 8 weeks and exposed to fixed or shifted LD cycles (n = 12), For A – B, *, $p<0.05$ fixed vs. shifted LD groups.

Discussion

The link between circadian rhythm disruption and metabolic phenotypes is well established by studies using transgenic mice with genetic mutation or deletion of core clock genes. For example, hyperlipidemia, hyperglycemia, and hypoinsulinemia were observed in *Clock*$^{\Delta 19/\Delta 19}$ and *Bmal1*$^{-/-}$ mice. In addition, pancreas specific clock mutant mice also displayed glucose intolerance and low level of insulin secretion associated with impaired β-cell function (Marcheva et al., 2010; Turek et al., 2005). Similar to the effects of global arrhythmicity observed in *Clock* mutant and *Bmal1*-deficient mice, our previous studies demonstrate that transplantation of *Per1/2*-disrupted bone marrow cells into irradiated wild-type mice amplifies the severity of HFD-induced metabolic dysregulation (Xu et al., 2014). However, it is unclear whether the metabolic-dysregulated phenotypes are caused by the disruption of the circadian timekeeping function or transcriptional regulatory activity of these core clock genes. In the present experiments, chronic 12-hour advances in the LD cycle were observed to disrupt photic entrainment of circadian behavior, modulate clock gene rhythms in adipose tissue macrophages and similarly exacerbate HFD-induced increases in body weight, glucose intolerance and insulin resistance. Importantly, these findings are consistent with other rodent studies examining the effects of various chronic jet lag or shift work paradigms on diet-induced metabolic pathophysiology (Iwamoto et al., 2014; Oike et al., 2015), indicating that environmental desynchronization of circadian rhythms is sufficient to disrupt metabolic homeostasis. Interestingly, increased daytime HFD consumption, but not total daily food intake, in shifted LD mice may contribute to the observed increases in body weight, glucose

intolerance and insulin resistance because the coincidence of HFD feeding during the daytime or inactive phase in rodents has been identified as a determining factor in diet-mediated metabolic dysfunction (Arble et al., 2009; Hatori et al., 2012). Collectively, these observations have important implications in the interpretation of previous studies using genetic models, suggesting that the systemic metabolic dysregulation is a consequence of the disruption of the circadian timekeeping function of core clock genes, not their role as transcription factors that regulate downstream signaling pathways.

Chronic inflammation is thought to play an important role in diet-associated obesity and metabolic syndrome. In particular, studies on human obesity suggest that chronic activation of proinflammatory signaling pathways induces low grade inflammation, which in turn leads to decreased insulin sensitivity (Hotamisligil et al., 1995; Sartipy and Loskutoff, 2003). As such, inflammation may provide a critical link between circadian clock- and metabolic-dysregulated phenotypes. Circadian disruption using a chronic jet lag paradigm is characterized by increased inflammation and proinflammatory responses of the innate immune system (Castanon-Cervantes et al., 2010; Xu et al., 2014). Furthermore, we have shown that macrophages from mice with targeted disruption of *Per1* and *Per2* are distinguished by enhanced proinflammatory activation and that myeloid cell-specific $Per1^{ldc/}Per2^{ldc}$ disruption exacerbates HFD-induced inflammation and systemic metabolic dysregulation in chimeric mice repopulated with only mutant bone marrow cells (Xu et al., 2014). In our current study, circadian desynchronization in HFD-fed mice exposed to shifted LD cycles essentially recapitulates the effects of genetic disruption of the core clock mechanism, inducing robust increases in macrophage

infiltration and proinflammatory activation in close correspondence with the intensification of metabolic phenotypes in diet-induced obesity. Collectively, these findings suggest that macrophage infiltration, proinflammatory activation and inflammatory signaling may be key processes in the mechanism by which genetic disruption and environmental desynchronization of circadian rhythms hinder metabolic homeostasis.

Although our study and other literature indicate that increased proinflammation is a major feature found in genetic disruption and environmental desynchronization of circadian rhythms, how circadian disruption specifically triggers proinflammatory macrophage activation and inflammatory signaling cascades that lead to systemic metabolic dysregulation remains to be determined. Because chronic jet lag and shift work paradigms alter clock gene expression as well as phase alignment and amplitude of their rhythmic profiles (Filipski et al., 2004; Reddy et al., 2002; Yan, 2011), it is possible that core or auxiliary feedback loops comprising the circadian clockworks directly mediate the effects of circadian disruption on key elements of inflammatory signaling cascades such as NF-κB, IL-6 and Ccl2 (Curtis et al., 2014). This speculation is supported by our previous observations indicating that myeloid cell-specific *Per1* and *Per2* disruption increased NF-κB and JNK1 phosphorylation as well as IL-6 mRNA expression and decreased levels of PPARγ, which promotes anti-inflammatory M2 activation, in macrophages isolated from HFD-fed mice (Xu et al., 2014). Direct involvement of core or auxiliary clock genes in linking circadian clock dysregulation with the potentiation of diet-induced inflammation is further suggested by studies demonstrating that: 1) CLOCK

upregulates NF-κB activity (Spengler et al., 2012); and 2) REV-ERBα modulates macrophage Toll-like receptor (TLR) signaling (Fontaine et al., 2008) and inhibits proinflammatory IL-6 release (Gibbs et al., 2012). Thus, it will be important in future studies to determine whether blockade of inflammatory signaling via NF-κB, IL-6 or downregulation of PPARγ ameliorate the inductive effects of circadian disruption on diet-associated metabolic dysregulation. Nevertheless, the present data provides primary evidence for the link between circadian clock dysregulation and macrophage proinflammatory activation in diet-induced metabolic dysfunction.

CHAPTER IV

ROLE OF INFLAMMATORY SIGNALING IN THE DIFFERENTIAL EFFECTS OF SATURATED AND POLYUNSATURATED FATTY ACIDS ON PERIPHERAL CIRCADIAN CLOCKS*

Introduction

Over-nutrition, especially through the consumption of a HFD, is a critical factor in the rapidly increasing incidence of obesity. HFD-induced obesity is associated with systemic insulin resistance and the corresponding development of metabolic disorders such as type 2 diabetes, cardiovascular disease and non-alcoholic fatty liver disease (Angulo, 2002; Hossain, Kawar, and El Nahas, 2007; Jensen et al., 2008). Increasing evidence indicates that chronic low-grade inflammation in peripheral tissues contributes to HFD-mediated obesity and insulin resistance. HFD increases plasma levels of saturated fatty acids, which can trigger pro-inflammatory responses through the induction of NF-κB and JNK signaling. Thus, the precise modulation of free fatty acid levels and inflammatory signaling are vital for the maintenance of metabolic homeostasis.

Cell-autonomous circadian clocks in peripheral tissues are involved in the regulation of inflammatory responses and metabolic homeostasis. Endogenous clocks in immune cells provide for the circadian control of their abundance in the circulation, production of

* Part of this chapter has been reprinted from Kim SM, Neuendorff N, Chapkin RS, Earnest DJ (2016) Role of Inflammatory Signaling in the Differential Effects of Saturated and Poly-unsaturated Fatty Acids on Peripheral Circadian Clocks. EBioMedicine 7: 100-11.

inflammatory factors, and functional responses to inflammatory challenge (Keller et al., 2009; Lange et al., 2010). Tissue- and cell-specific clocks also provide for the local coordination of circadian rhythms in the metabolism of fatty acids (Stenvers et al., 2012). For example, circulating levels of free fatty acids and adipose tissue expression of genes mediating lipolysis and fatty acid biosynthesis or transport are characterized by clock-controlled rhythmicity (Shostak et al., 2013). Moreover, genetic or environmental disruption of circadian clock function has been shown to potentiate inflammatory responses and to produce obesity and other metabolic disorders (Turek et al., 2005; Marcheva et al., 2010; Gibbs et al., 2012; Paschos et al., 2012). Our recent findings demonstrate that circadian clock disruption in bone marrow cells exacerbates HFD-induced tissue inflammation, adiposity, and systemic insulin dysregulation (Xu et al., 2014).

While the hierarchy of circadian clocks throughout the body is clearly involved in regulating inflammatory and metabolic processes, this interaction is not strictly unidirectional as inflammatory signals and fatty acid metabolism have been conversely implicated in the feedback modulation of the circadian clock mechanism. In this regard, the cytokines TNF-α and IL-1β induce phase delays and HFD increases the free-running period of the activity rhythm in mice (Kohsaka et al., 2007; Leone et al., 2012; Xu et al., 2014). Thus, mutual interactions between circadian clocks and key mediators of inflammation may be important in maintaining metabolic homeostasis and in linking clock dysregulation and metabolic phenotypes in diet-associated obesity. Because inflammatory responses and corresponding metabolic disturbances in diet-induced obesity are

differentially engaged by specific types of fatty acids found in HFDs, we compared the differential effects of palmitate, the prevalent pro-inflammatory SFA in HFD versus the anti-inflammatory, PUFA DHA on circadian clock function. The extent of the coupling between diet-mediated inflammatory responses and alterations in fundamental clock properties was examined by analyzing the phase-shifting effects of acute palmitate and DHA treatment at different times throughout the circadian cycle and then determining their coincidence with fatty acid-mediated induction of inflammatory signaling. To examine the role of inflammatory signaling in the mechanism by which SFAs modulate clock properties, experiments were also conducted to determine whether inhibition of pro-inflammatory responses blocks palmitate-induced phase shifts of peripheral circadian clocks.

Material and Methods

***Cell culture*:** Mouse *Bmal1-dLuc* fibroblasts (Dr. Andrew Liu, University of Memphis, Memphis, TN; Ramanathan et al., 2012) were propagated on 60mm dishes in Dulbeco's Modified Eagle Medium (DMEM; HyClone) containing 292ug/mL L-glutamine, 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin and maintained at 37°C and 5% CO2. Medium was replaced every 48hr and cultures were split 1:4 every 3 days. As established previously (Huo et al., 2010, 2012), adipocytes were differentiated from *Bmal1-dLuc* fibroblasts maintained in DMEM containing 10µg/ml insulin, 1µM dexamethasone, and 0.5mM 3-isobutyl-1-methylxanthine for 48hr, and then incubated for 4 additional days in medium

supplemented with 10μg/ml insulin. Following differentiation, adipocytes were maintained for 2 days in normal growth medium prior to experimentation. Cell differentiation into adipocytes was verified by positive staining with oil red o (Figure 9A) and by upregulated expression of PPARγ and adiponectin (Figure 9B). While our analysis of these phenotypic markers indicates that *Bmal1-dLuc* fibroblasts exhibit many adipocyte-like properties following differentiation, it is unclear whether the cells are fully differentiated into mature adipocytes, thus warranting their subsequent designation as 'differentiated adipocytes'.

***Fatty acid/drug preparation and treatment*:** Palmitate (Sigma) and DHA (Nu-Chek-Prep, Inc.) were dissolved in ethanol and then diluted (1:5.4 ratio) with 10% BSA (fatty acid-free and low endotoxin, Sigma A6003) diluted in 0.1M phosphate-buffered saline (PBS). Palmitate and DHA treatment in these studies was based on physiological concentrations that have been previously observed *in vivo* or used for *in vitro* studies (Ajuwon and Spurlock, 2005; Han and Liu, 2010; Puri et al., 2009; Weldon et al., 2007). Controls for fatty acid treatment contained BSA diluted in PBS with an equivalent ratio of ethanol.

AICAR (Tocris) and cardamonin (Tocris) were dissolved in DMSO and then diluted 1:400 and 1:10000 in culture medium to achieve final concentrations of 500uM or 5μM, respectively. Vehicle controls for AICAR and cardamonin treatment consisted of cultures in which an equivalent amount of DMSO was added to the medium.

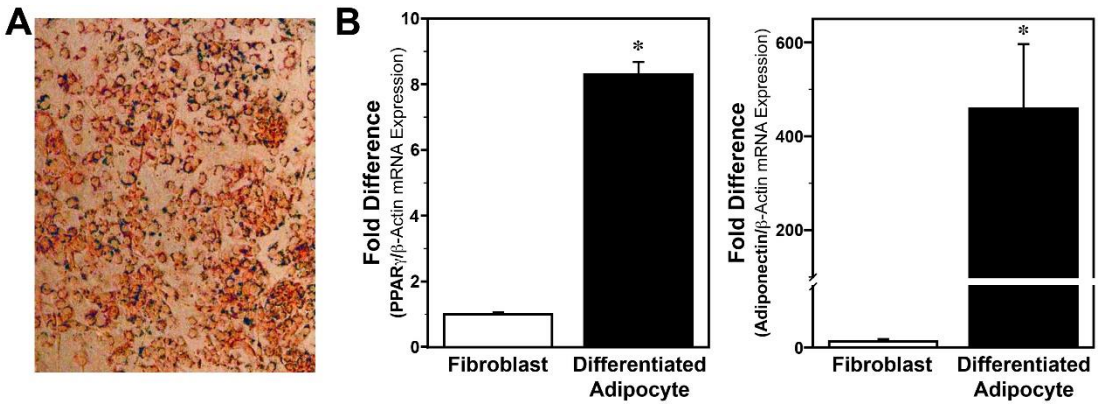

Figure 9: Characterization of adipocyte phenotypes following differentiation of *Bmal1-dLuc* fibroblasts. **(A)** Representative microscopic image illustrating the cytoplasmic accumulation of lipids in *Bmal1-dLuc* cells stained with oil red o staining after differentiation using the methods of Huo and colleagues (Huo et al., 2010b; Huo et al., 2012). **(B)** Real-time PCR determinations (mean ± SEM) of PPARγ and adiponectin mRNA expression in *Bmal1-dLuc* fibroblast and differentiated adipocyte cultures. PPARγ (left panel) and adiponectin (right panel) mRNA signal were normalized to β-actin mRNA levels in each sample and the fold difference was determined by adjusting these values in relation to the average of undifferentiated fibroblast cultures, which was set at 1. The relative levels of PPARγ and adiponectin mRNA in differentiated adipocyte cultures were increased by 8-fold and 450-fold, respectively and were significantly greater ($p<0.05$) than that found in undifferentiated *Bmal1-dLuc* fibroblasts.

***Effect of prolonged fatty acid treatment on circadian period*:** *Bmal1-dLuc* fibroblasts were plated onto 35mm dishes and ≈24hr later treated with either vehicle (EtOH with 10% BSA), palmitate (150µM), or DHA (150µM) for 48hr. Following fatty acid treatment, cultures were rinsed and then maintained in recording media for 6-7 days during real-time analysis of *Bmal1-dLuc* bioluminescence.

The effects of prolonged palmitate treatment were also examined *in vivo*. Homozygous *mPer2*^{Luc} knock-in mice (C57BL/6J background, male and female, 6 weeks old) were initially maintained on a 12:12-h light-dark cycle (lights on at 06:00) and fed *ad libitum*. To examine the effects of palmitate on circadian rhythms *in vivo* mice (5 – 6 weeks old) were anesthetized (ketamine -100mg/kg; /xylazine - 10 mg/kg body weight) and then an osmotic mini-pump (Alzet) containing BSA or 600mM palmitate was implanted subcutaneously posterior to the scapulae. Following mini-pump implantation, mice were maintained in constant darkness and the effects of palmitate infusion (0.15ul/hr-600mM) on the free-running period of the activity were analyzed for 6 weeks as previously described (Ko et al., 2011).

***Time-dependent variation in the phase shifting and pro-inflammatory effects of acute fatty acid treatment*:** *Bmal1-dLuc* fibroblast cultures on 35mm dishes were exposed for 2hr to medium containing 15uM forskolin to facilitate circadian oscillation synchronization across cultures (Menger et al., 2007) and then treated for 4hr with BSA (10% in EtOH), palmitate (250µM) or DHA (250µM) at 6hr intervals throughout the circadian cycle. Cultures were subjected to control or fatty acid treatments at 6, 12, 18 or

24hr after forskolin administration and then placed in recording media for bioluminescence analysis of treatment-induced phase shifts of *Bmal1-dLuc* oscillations.

For parallel analyses of inflammatory responses to acute fatty acid treatment, confluent cultures of *Bmal1-dLuc* fibroblasts on 6-well plates were exposed for 2hr to 15uM forskolin and then 6, 12, 18 or 24hr later treated with BSA, palmitate (250μM) or DHA (250μM) for 4hr. After treatment, cells were rinsed, collected, frozen in liquid nitrogen and stored at -80°C for subsequent analyses of NF-κB activation or cytokine mRNA expression.

The effects of acute palmitate treatment were also examined *in vivo*. Homozygous *mPer2*^{Luc} knock-in mice (C57BL/6J background) were maintained under constant darkness and fed *ad libitum*. To examine the phase shifting effects of acute palmitate on circadian rhythms *in vivo* mice (5 – 10 weeks old) received intraperitoneal (i.p.) injection of either BSA or palmitate (100mg/kg) at CT 6, 12, 18 and 24. Following i.p. injection of BSA or palmitate, mice were maintained for 2 weeks in constant darkness until data analysis. Phase shifts of locomotor activity were determined by comparing the phase angles between pre-treatment and post-treatment of BSA or palmitate. Activity onset was used as phase reference point.

***Effect of inflammatory signaling inhibitors on fatty acid-induced phase shifts of the circadian clock*:** Real-time analysis of cells transfected with an inducible NF-kB-responsive GFP construct was used to test whether treatment with DHA, AICAR, or cardamonin, a chalcone with anti-inflammatory activity (Ahmad et al., 2006), modulates

palmitate-induced inflammatory signaling when its phase-shifting effects are maximal. GFP-reported NF-κB activity was quantified in cells that were treated with: 1) DHA (50uM) for 12hr in advance and during exposure to palmitate (250μM) for 4hr; or 2) AICAR (500uM) or cardamonin (5μM) in conjunction with palmitate (250μM) administration for 4hr. Effects of these anti-inflammatory treatments on peak phase-shifting responses of the *Bmal1-dLuc* rhythm were examined in parallel cultures that were similarly treated with DHA, AICAR or cardamonin relative to palmitate exposure at hour 12. Following treatment, cultures were placed in recording media for bioluminescence analysis of treatment effects on palmitate-induced phase shifts of *Bmal1-dLuc* oscillations.

***Protein extraction and Western blot analysis of BMAL1 and NF-κB activation*:** *Bmal1-dLuc* fibroblasts were lysed in mammalian protein extraction reagent (Pierce Biotechnology) containing protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein content in cell homogenates was measured using the bicinchoninic acid method. BMAL1, ACTIN, NF-κB and phospho-NF-κB in cell lysates (10-30ug protein/lane) were assessed by Western blot analysis using 10% Tris-Glycine gels as described previously (Shende et al., 2013). Membranes were probed with antibodies against BMAL1 (Cell Signaling Technology), ACTIN (Sigma), phospho-NF-κB (p-p65; Cell Signaling Technology) or NF-κB (p-65; Cell Signaling Technology) followed by incubation with horse-radish peroxidase-conjugated goat anti-rabbit IgG (Biorad). Densitometric analyses of size-appropriate immunoreactive bands were performed using NIH ImageJ software. To control for inter-sample differences in protein content, BMAL1

and p-NF-κB signal intensity were normalized to NF-κB and ACTIN values in each sample respectively.

***Real-time reporter assay of NF-κB activity*:** The Cignal NF-κB Reporter Assay Kit (Qiagen) was used to quantify treatment-induced changes in the transcriptional regulatory activity of NF-κB. Cultures of *Bmal1-dLuc* fibroblasts (70-80% confluent) on opaque 96-well plates were transfected for 48hr with an inducible NF-kB-responsive GFP reporter construct (Qiagen) complexed with Lipofectamine® 3000 (Invitrogen). Approximately 24hr after transfection, cells were exposed to medium containing 15uM forskolin and 12hr later treated with BSA (control) or palmitate for 4hr. Experimental groups included cultures treated with: 1) DHA (50uM) for 12hr in advance and during exposure to palmitate; or 2) AICAR (500uM) or cardamonin (5μM) in conjunction with palmitate administration. NF-κB signaling activity was assessed through quantitative measurement of GFP fluorescence and then compared relative to negative controls.

***RNA extractions and real-time PCR*:** Total RNA was extracted from all cell lysates and relative quantification of interleukin-6 (IL-6) mRNA abundance was performed using SYBR-Green PCR technology [Applied Biosystems Inc. (ABI)] as described previously (Farnell et al., 2011; Xu et al., 2014). For each sample, real-time PCR analysis of IL-6 mRNA was performed on duplicate aliquots using the cDNA equivalent of 1ng of total RNA. To control for differences in sample RNA content, β-actin mRNA was amplified with the cDNA equivalent of 1ng total RNA from the same samples. Consistent with our

previous studies (Xu et al., 2014), β-actin mRNA showed no sign of circadian variation. The comparative CT method was utilized to calculate the relative abundance for a given cytokine mRNA by normalization to corresponding β-actin levels in each sample and to a calibrator consisting of pooled cDNA from multiple samples.

***Real-time analysis of Bmal1-dLuc rhythms*:** Prior to bioluminescence analysis, growth medium was removed and cultures were placed in DMEM recording medium containing 1uM forskolin, 25mM HEPES, 292µg/ml L-glutamine, 100units/ml penicillin, 100µg/ml streptomycin and 10uM luciferin (Promega). Cultures were sealed airtight with sterile glass coverslips, and sterile silicon grease. The temporal patterns of *Bmal1-dLuc* bioluminescence were analyzed using an automated 32-channel luminometer (LumiCycle; Actimetrics) housed in a standard culture incubator at 32°C. Bioluminescence from individual cultures was continuously recorded for ~70sec at intervals of 10min. Rhythm parameters were determined from baseline-subtracted data using the damped sine fit and Levenberg–Marquardt algorithm. The amplitude of phase shifts in response to fatty acid treatment was determined by measuring the time difference between the peaks of the *Bmal1-dLuc* rhythms during the third cycle in BSA or BSA/vehicle (DMSO) control and experimental treatment groups.

***Statistical analysis*:** Independent t-tests were performed to determine the significance of treatment effects (DHA, palmitate, AICAR, or cardamonin relative to vehicle controls) on NF-κB activity, IL-6 expression and palmitate-induced phase shifts. In each case, the

p-value was set at 0.05. Time-dependent differences in phase-shifting effects of DHA or palmitate on the *Bmal1-dLuc* rhythm were first analyzed by one-way analysis of variance (ANOVA). Paired comparisons between peak phase-shifting responses and the corresponding minima were analyzed post hoc for statistical differences using the Student Newman-Keuls sequential range test. The p-value was set at 0.05 for these post hoc analyses.

Results

***Effect of prolonged fatty acid treatment on circadian period*:** Given that HFD feeding induces pronounced alterations in circadian timekeeping in peripheral clocks (Xu et al., 2014), we first determined whether prolonged treatment with palmitate, the prevalent SFA in HFD, similarly affects clock gene oscillations in *Bmal1-dLuc* fibroblasts in vitro. All BSA-, DHA- and palmitate-treated fibroblast cultures exhibited circadian rhythms of Bmal1-dLuc bioluminescence that persisted at least 4-5 cycles (Figure 10). The period of the *Bmal1-dLuc* rhythms in cultures exposed to DHA for 48hr was greater (by ≈0.4hr) but not significantly different ($p=0.14$) relative to that observed in BSA-treated controls (Figure 10A). In contrast, palmitate-treated fibroblast cultures were distinguished by *Bmal1-dLuc* rhythms in which the period was significantly increased ($p>0.05$) by ≈3 hours in comparison to BSA controls (Figure 10B). Palmitate treatment also had a distinct effect in reducing rhythm amplitude to ≈40% of control values.

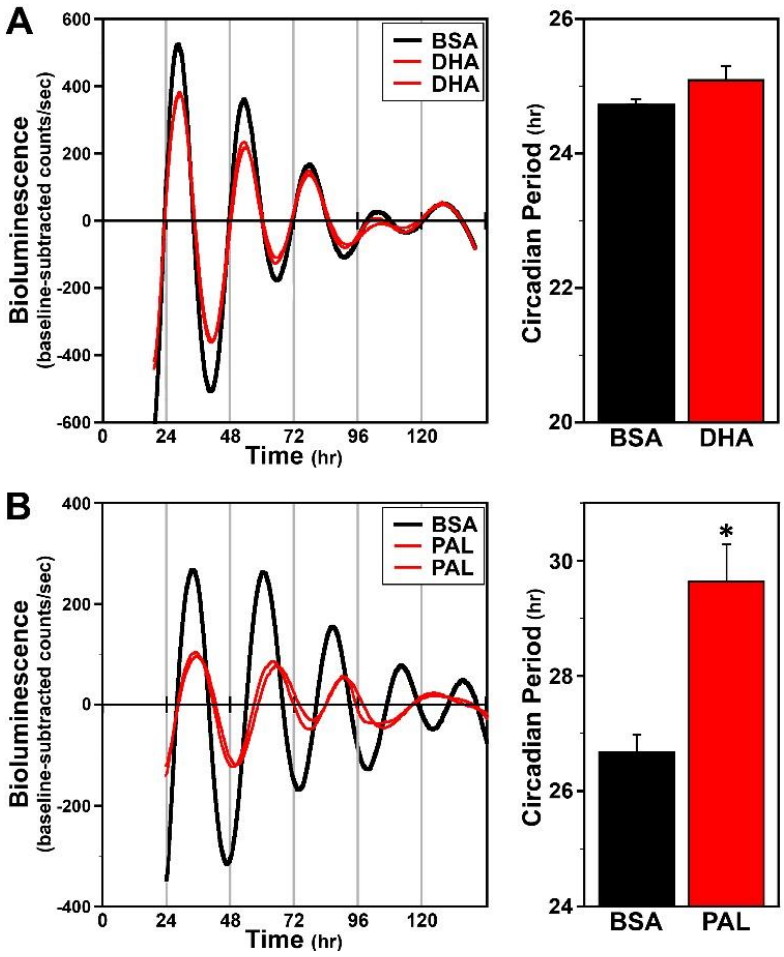

Figure 10: Effects of prolonged PUFA and SFA treatment on ensemble *Bmal1-dLuc* rhythms in cultured fibroblasts. Individual recordings of ensemble bioluminescence (expressed as detrended baseline-subtracted counts per second) from representative cultures of *Bmal1-dLuc* fibroblasts treated with: **(A)** BSA (n=6) or 150µM DHA (n=8), and **(B)** BSA (n=6) or 150µM palmitate (n=8). Bar graphs depict comparisons of the circadian period (mean + SEM) of the *Bmal1-dLuc* rhythms in BSA control and DHA- or palmitate-treated fibroblasts. Asterisk indicates that the period of the *Bmal1-dLuc* rhythms in palmitate-treated cultures was significantly greater ($p<0.05$) than that in BSA controls.

Prolonged palmitate treatment *in* vivo had a similar effect on the circadian period of the rhythm in mouse wheel-running behavior, except its amplitude was much smaller than that observed on fibroblast *Bmal1-dLuc* rhythms. During palmitate infusion via osmotic mini pump (0.15ul per hour, 600mM) for 6 weeks, the free-running period of the activity rhythm was significantly increased ($p>0.05$) by ≈0.5hr in comparison with BSA-treated control mice (Figure 11A). Importantly, this palmitate-induced increase in circadian period *in vivo* was accompanied by corresponding increases in circulating levels of free fatty acids (Figure 11B). In palmitate-infused mice, serum levels of free fatty acids were significantly elevated ($p>0.05$) by about 1.7 fold relative to BSA controls.

***Time-dependent variation in the phase shifting and pro-inflammatory effects of acute fatty acid treatment*:** In response to acute (4hr) treatment at hour 6, 12, 18 and 24 with 250µM DHA or palmitate, the period of the *Bmal1-dLuc* rhythms in cultures of undifferentiated fibroblasts was not significantly different from that observed in experiment-matched BSA controls. Phase shifting analysis revealed that DHA had negligible effects on the *Bmal1-dLuc* rhythm at hour 6 and 24 whereas treatment at hour 12 and 18 induced small phase delays of about 0.5hr (Figure 12). Following bioluminescence analysis, no differences in cell viability were evident in cultures treated with 250µM DHA. Similar to DHA, acute palmitate treatment shifted the phase of fibroblast *Bmal1-dLuc* rhythms in a time-dependent manner, but the directionality of these shifts was different and their amplitude was much larger (Figure 13). At hour 12 and 18

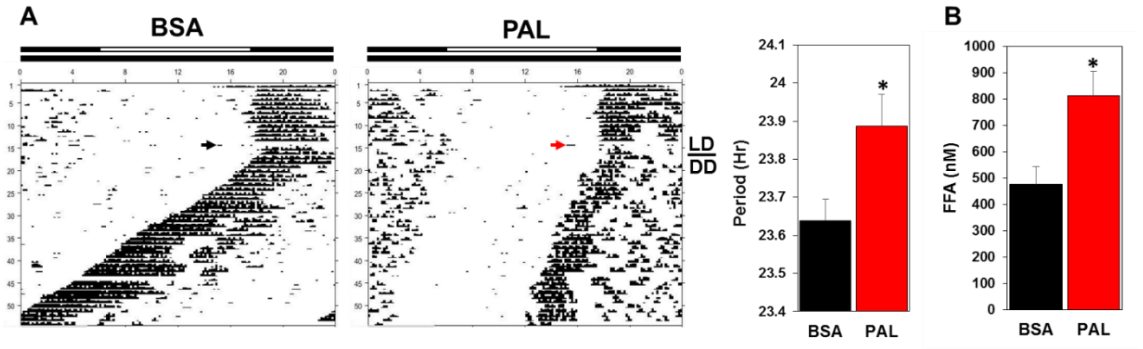

Figure 11: Effect of prolonged SFA treatment on the free-running rhythm of locomotor activity *in vivo*. **(A)** Representative records of wheel-running activity in BSA- or PAL-treated mice. Actograms are plotted over a 24-hour period. The open and closed bars at the top respectively signify the timing of the light and dark phase during initial entrainment to LD 12:12. At the arrow on the actograms, animals were implanted with an osmotic mini-pump infusing BSA or palmitate (0.15ul/hr-600mM) and then exposed to constant darkness. Bar graph depicts average free-running period of the activity rhythm (±SEM) in BSA- and PAL-treated mice during exposure to constant darkness. *, $P < 0.05$, BSA vs. PAL **(B)** Serum levels of free fatty acids in mice during BSA or PAL infusion. Bars represent mean values (± SEM). *, $P < 0.05$, BSA vs. PAL.

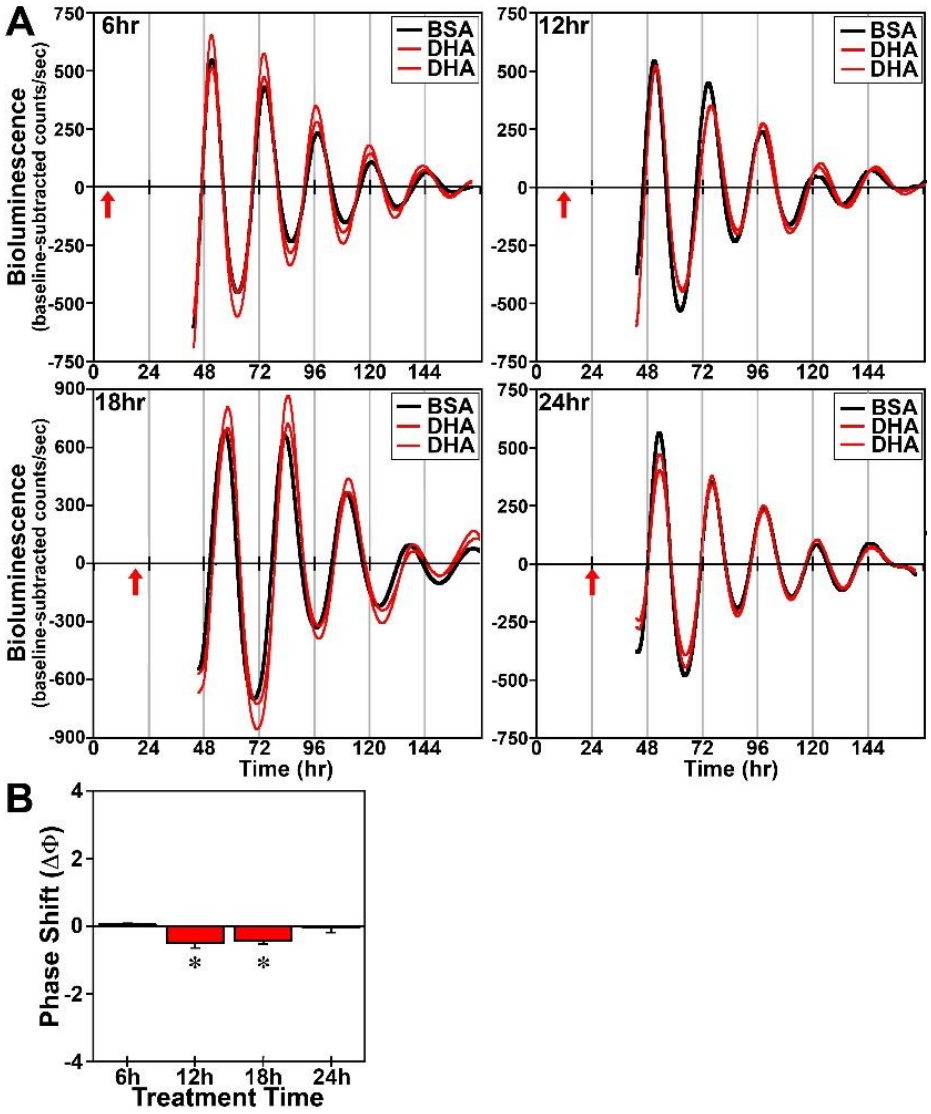

Figure 12: Phase-shifting effects of acute DHA treatment on ensemble *Bmal1-dLuc* rhythms in cultured fibroblasts. **(A)** Representative recordings of ensemble bioluminescence from individual cultures of *Bmal1-dLuc* fibroblasts treated for 4hr with BSA (n=4) or 250μM DHA (n=8) at hour 6, 12, 18 and 24. Red arrows indicate time of BSA or DHA treatment. **(B)** Time-dependent phase shifts of fibroblast *Bmal1-dLuc* rhythms in response to DHA treatment for 4hr. Mean (+SEM) phase shifts (ΔΦ) in hours are plotted as a function of the timing of DHA treatment (at hour 6, 12, 18 and 24). Phase delays of the fibroblast *Bmal1-dLuc* rhythm are indicated by negative values and advances are denoted by positive values. Asterisks indicate that phase-shifting responses to DHA at hour 12 and 18 were significantly greater ($p<0.05$) than those observed during the corresponding minima.

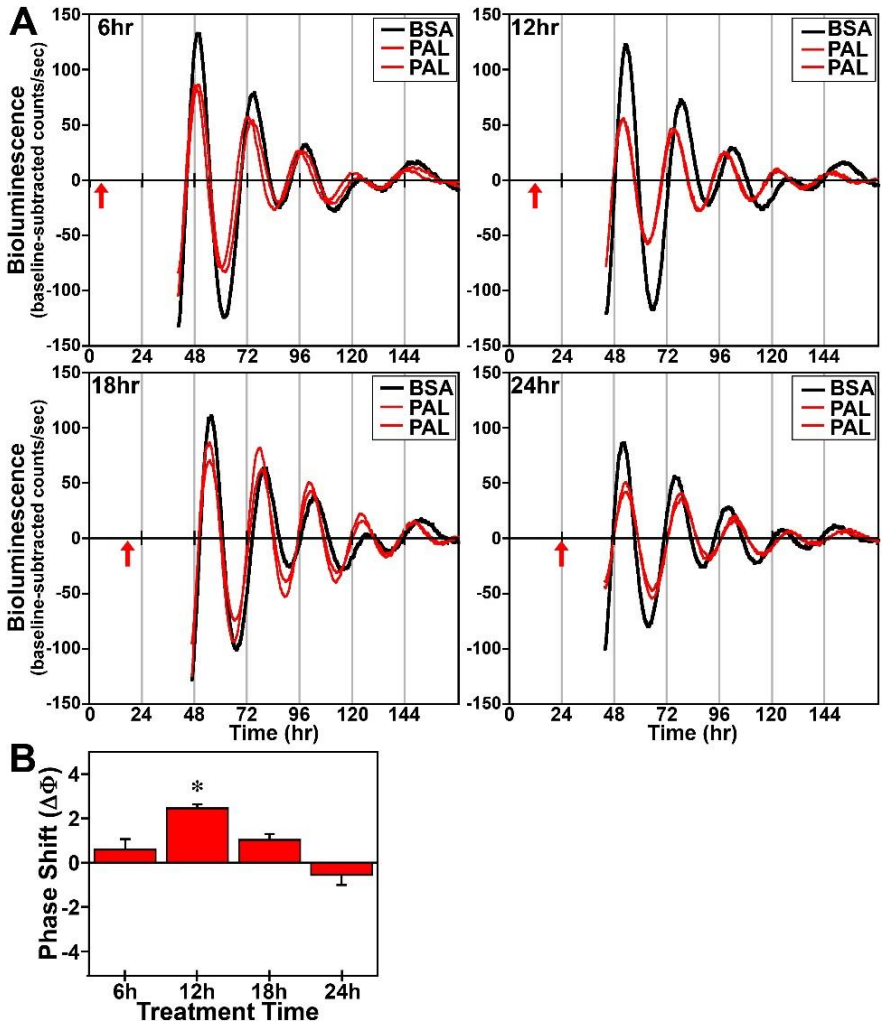

Figure 13: Phase-shifting effects of acute palmitate (PAL) treatment on ensemble *Bmal1-dLuc* rhythms in cultured fibroblasts. **(A)** Representative recordings of ensemble bioluminescence from individual cultures of *Bmal1-dLuc* fibroblasts treated for 4hr with BSA (n=5-6) or 250μM PAL (n=5-6) at hour 6, 12, 18 and 24. Red arrows indicate time of BSA or PAL treatment. **(B)** Time-dependent phase shifts (mean ± SEM) of fibroblast *Bmal1-dLuc* rhythms in response to PAL treatment at hour 6, 12, 18 and 24. The asterisk indicates that peak phase-shifting responses to PAL at hour 12 were significantly greater ($p<0.05$) than those observed during the corresponding minima.

when DHA induced small phase delays, palmitate produced the opposite effect: phase advances of fibroblast *Bmal1-dLuc* rhythms. Peak phase-shifting responses to palmitate were observed at hour 12 when treatment induced large phase advances of ≈2.5 hours. Palmitate treatment at hour 18 also produced clear phase advances of the *Bmal1-dLuc* rhythm although the amplitude (≈1hr) was reduced relative to its phase-shifting effects at hour 12. Palmitate-induced phase shifts were minimal at hour 6 and 24, with treatment inducing small advances and delays of ≈0.5hr respectively at these timepoints. Unlike prolonged exposure, acute treatment with DHA or palmitate had little or no consistent effect on the amplitude of the *Bmal1-dLuc* rhythm.

To determine whether DHA or palmitate phase shift clock gene rhythms in other peripheral cell types and whether the time-dependent nature of phase-shifting responses differs among peripheral circadian clocks, parallel analysis was performed on adipocytes differentiated from *Bmal1-dLuc* fibroblasts. Irrespective of treatment time, DHA had little or no phase-shifting effects on the *Bmal1-dLuc* rhythm in differentiated adipocytes (Figure 14). DHA produced small phase advances (≈0.25hr) at hour 6 and phase delays (≈0.2hr) at hour 12 whereas phase-shifting effects of this PUFA were marginal (<0.1hr) at hour 18 and 24. Similar to its effects on undifferentiated fibroblasts, acute palmitate treatment shifted the phase of adipocyte *Bmal1-dLuc* rhythms (Figure 15). However, the time-dependent variation in adipocyte phase-shifting responses to palmitate was markedly different from the pattern in undifferentiated fibroblasts. Palmitate-induced phase shifts in differentiated adipocytes were maximal at hour 6, when treatment had negligible effects on the phase of undifferentiated fibroblast *Bmal1-dLuc* rhythms. At hour 6, palmitate

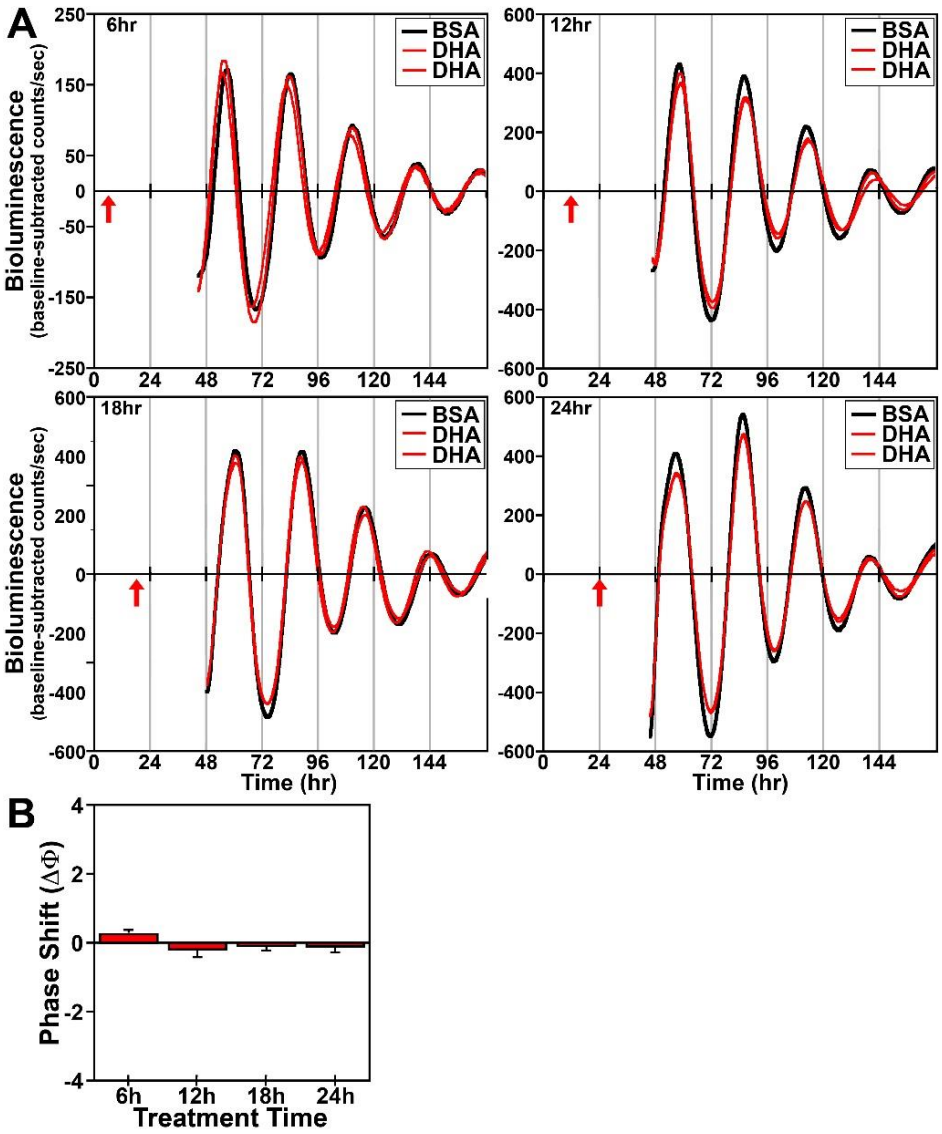

Figure 14: Phase-shifting effects of acute DHA treatment on ensemble *Bmal1-dLuc* rhythms in cultures of differentiated adipocytes. **(A)** Representative recordings of ensemble bioluminescence from individual cultures of differentiated *Bmal1-dLuc* adipocytes treated for 4 hours with BSA (n=4) or 250μM DHA (n=5-10) at hour 6, 12, 18 and 24. Red arrows indicate the approximate time at which cultures were exposed to BSA or DHA. **(B)** Time-dependent phase shifts (mean ± SEM) of adipocyte *Bmal1-dLuc* rhythms in response to DHA treatment at hour 6, 12, 18 and 24.

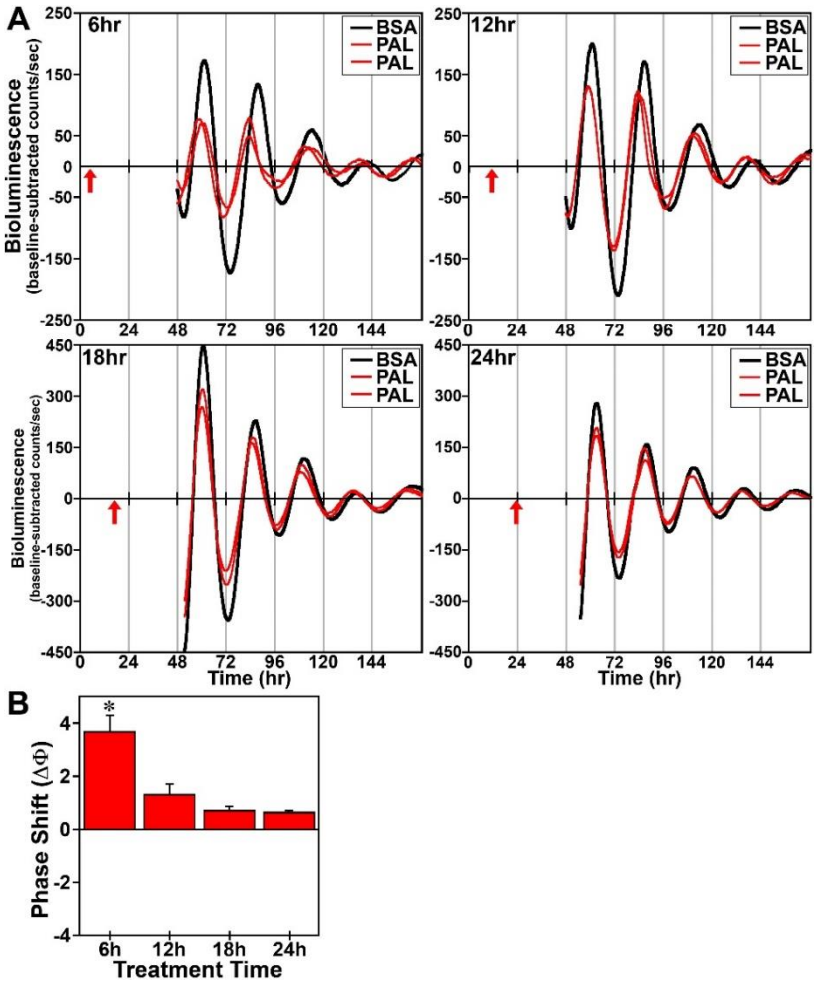

Figure 15: Phase-shifting effects of acute palmitate (PAL) treatment on ensemble *Bmal1-dLuc* rhythms in cultures of differentiated adipocytes. **(A)** Representative recordings of ensemble bioluminescence from individual cultures of differentiated *Bmal1-dLuc* adipocytes treated for 4hr with BSA (n=6) or 250µM PAL (n=6) at hour 6, 12, 18 and 24. Red arrows indicate time of BSA or PAL treatment. **(B)** Time-dependent phase shifts (mean ± SEM) of adipocyte *Bmal1-dLuc* rhythms in response to PAL treatment for at hour 6, 12, 18 and 24. The asterisk indicates that peak phase-shifting responses to PAL at hour 6 were significantly greater ($p<0.05$) than those observed during the corresponding minima.

advanced the phase of adipocyte *Bmal1-dLuc* rhythms by ≈4hr. In differentiated adipocyte cultures, palmitate also induced small phase advances (≈1.3hr) at hour 12 but had minimal effects on the *Bmal1-dLuc* rhythm at hour 18 and 24, producing phase delays of less than 0.7hr. *Bmal1-dLuc* rhythms in differentiated adipocytes showed no clear signs of DHA- or palmitate-induced changes in amplitude. Acute palmitate treatment *in vivo* induced small phase shifts in locomotor activity rhythms, but its effects were not significant over all time points (Figure 16).

Since SFAs trigger NF-κB-mediated signaling that leads to the induction of pro-inflammatory cytokines (Ajuwon and Spurlock, 2005; Maloney et al., 2009; Wang et al., 2012), we next determined whether the effects of palmitate on these key mediators of fatty acid-induced inflammation are both time-dependent and coincident with the temporal variation in the phase-shifting responses of the clock mechanism to this SFA. Consistent with previous reports (Gladine et al., 2014; Novak et al., 2003), acute DHA treatment had no effect on NF-κB activation but modulated expression of the pro-inflammatory cytokine IL-6 in *Bmal1-dLuc* fibroblasts. In cultures treated with DHA at hour 6, 12, 18 or 24, levels of NF-κB phosphorylation (Figure 17A) were not significantly different from those in time-matched BSA controls. However, DHA administration at hour 12, 18 and 24 induced significant decreases ($p<0.05$) in IL-6 mRNA expression (≈40-75%) relative to BSA-treated fibroblasts (Figure 17B). In contrast to DHA, palmitate induced both NF-κB signaling and IL-6 mRNA expression in *Bmal1-dLuc* fibroblasts in a time-dependent manner. Relative to BSA-treated cultures, palmitate induced significant increases

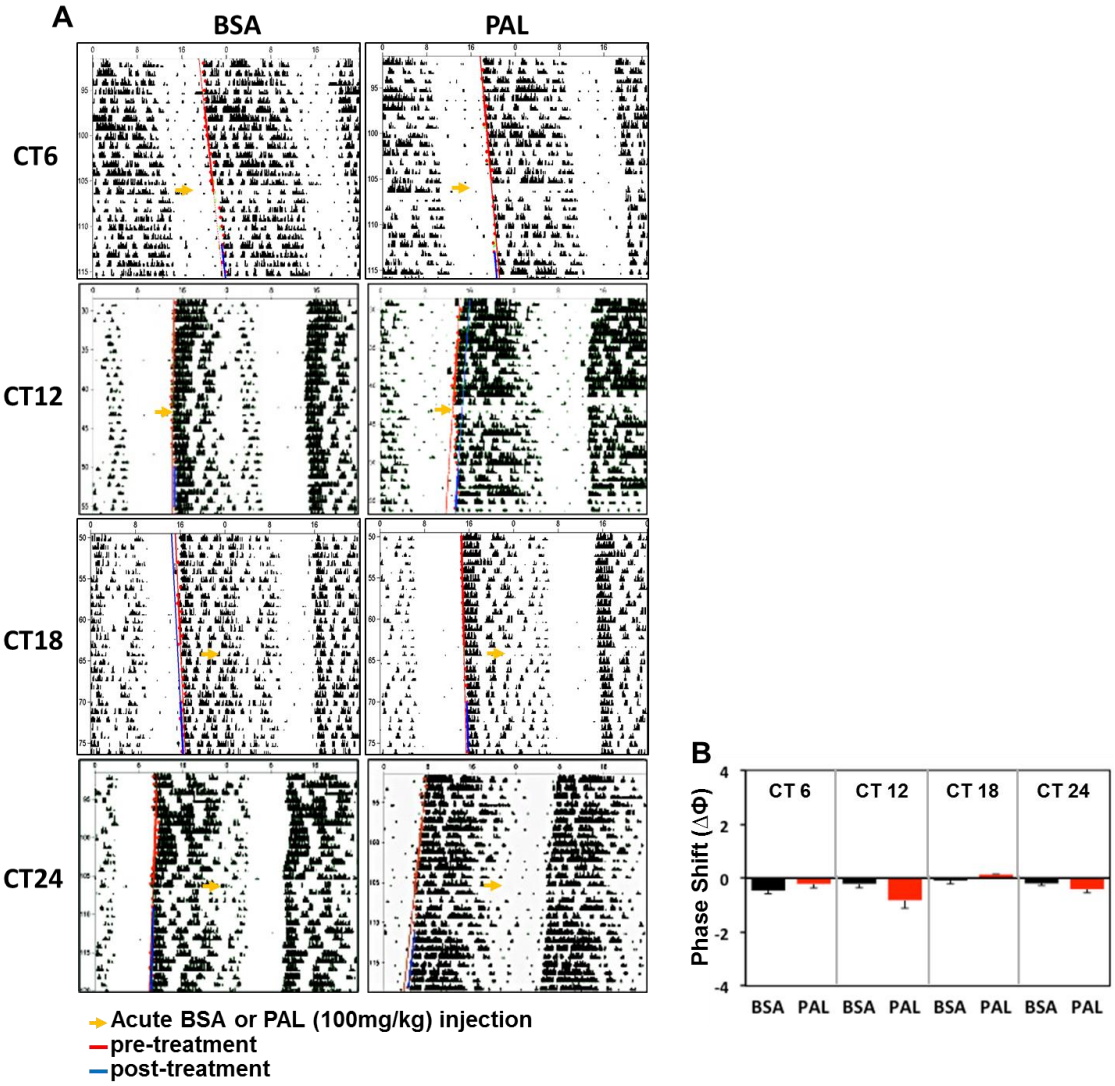

Figure 16: Phase shifting effect of acute palmitate (PAL) treatment on the free-running rhythm of locomotor activity *in vivo*. **(A)** Representative records of wheel-running activity in BSA- or PAL-treated mice under constant darkness. Actograms are plotted over a 48-hour period. At the arrow on the actograms, animals were received i.p. injection of BSA or palmitate (100mg/kg). **(B)** Time-dependent phase shifts (mean ± SEM) of locomotor activity rhythms in response to BSA or PAL treatment for at CT 6, 12, 18 and 24.

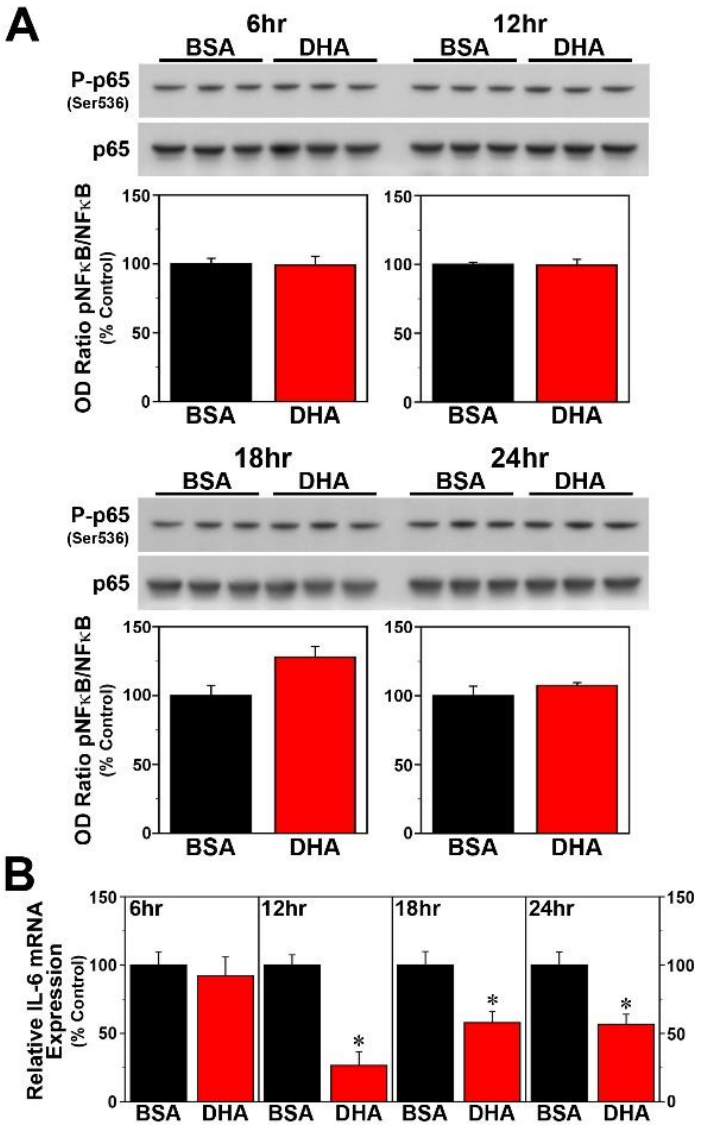

Figure 17: Time-dependent effects of acute DHA treatment on inflammatory signaling in *Bmal1-dLuc* fibroblasts. **(A)** Representative Western blots and densitometric analyses of NF-κB-mediated inflammatory signaling in fibroblast cultures treated for 4hr with BSA (n=3) or 250μM DHA (n=3) at hour 6, 12, 18 and 24. Bar graphs depict the ratios of P-p65/p65 immunoreactive signal that were adjusted in relation to control (BSA) values (% Control). **(B)** Real-time PCR determinations (mean ± SEM) of IL-6 mRNA expression in parallel groups of BSA- and DHA-treated fibroblast cultures. Plotted values correspond to the ratio of IL-6 mRNA signal normalized to β-actin mRNA levels in each sample and adjusted relative to the averages of time-matched BSA controls, which were set at 100%. Asterisks denote treatment times in which the relative expression of IL-6 mRNA in DHA-treated fibroblasts was significantly decreased ($p<0.05$) in comparison with that found in BSA controls.

($p<0.05$) in NF-κB phosphorylation at hour 12 and 18 but not at hour 6 and 24 (Figure 18A), coinciding with the temporal variation in the amplitude of phase-shifting responses to this SFA. Palmitate-induced IL-6 expression in *Bmal1-dLuc* fibroblasts provided further evidence of the close temporal coincidence between proinflammatory and phase-shifting responses to treatment; in palmitate-treated cultures, IL-6 mRNA expression was significantly increased ($p<0.05$) by 180-225% at hour 12 and 18 relative to BSA controls but this inductive effect was blunted (≈135%) at hour 6 and 24 (Figure 18B).

The effects of acute DHA treatment on NF-κB activation and IL-6 expression in differentiated adipocytes were similar to those observed in undifferentiated fibroblasts. Following administration at hour 6, 12, 18 and 24, DHA-treated adipocyte cultures and time-matched BSA controls exhibited no significant differences in levels of NF-κB phosphorylation (Figure 19A). However, IL-6 mRNA expression was significantly decreased ($p<0.05$) by ≈60-70% in response to DHA administration at hour 12, 18 and 24 relative to that observed in BSA-treated adipocytes (Figure 19B). At hour 6, IL-6 mRNA expression in DHA-treated cultures was decreased but not significantly different in comparison to time-matched controls. In differentiated adipocytes, palmitate treatment produced time-dependent increases in both NF-κB activation and IL-6 mRNA expression. Peak induction of NF-κB phosphorylation was observed in response to palmitate administration at hour 6 (Figure 20A), when phase-shifting responses to this SFA were maximal in differentiated adipocytes. Relative to time-matched BSA controls, palmitate

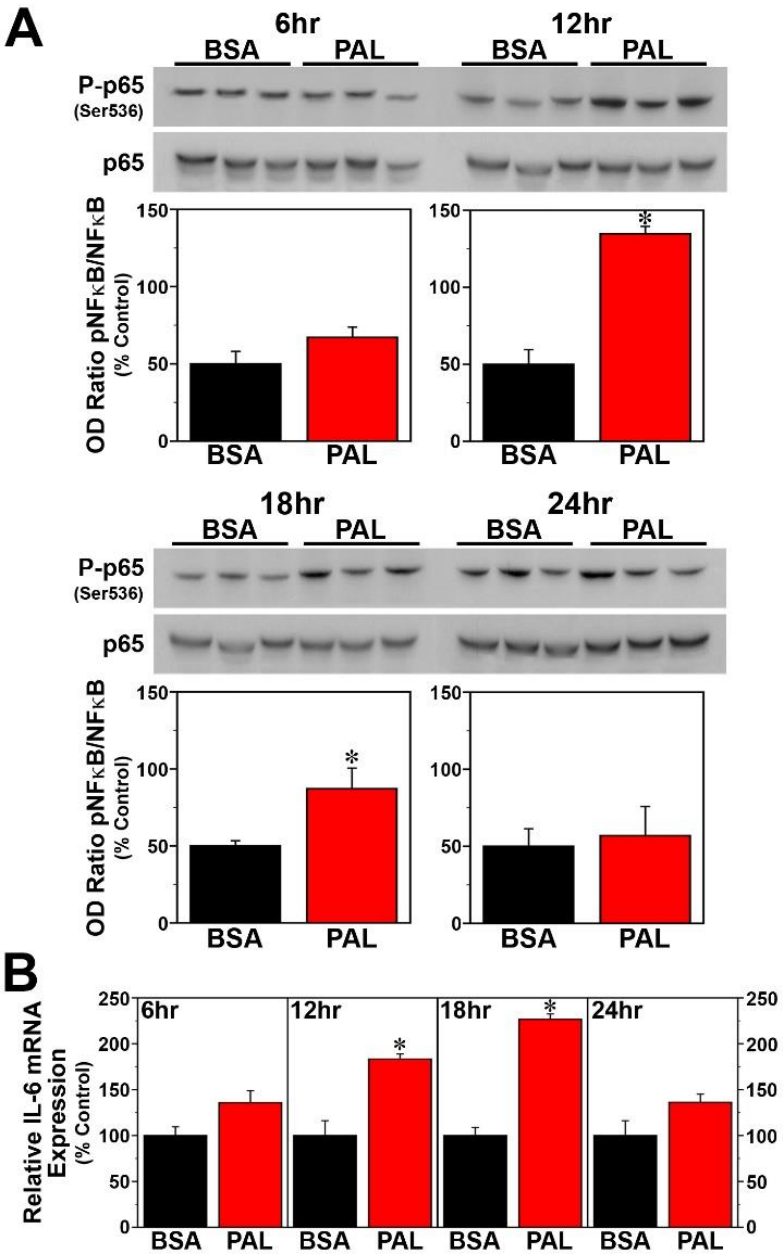

Figure 18: Time-dependent effects of acute palmitate (PAL) treatment on inflammatory signaling in *Bmal1-dLuc* fibroblasts. **(A)** Representative Western blot and densitometric quantification (mean ± SEM) of P-p65/p65 immunoreactive signal (% Control) in fibroblast cultures treated for 4hr with BSA (n=3) or 250μM PAL (n=3) at hour 6, 12, 18 and 24. **(B)** IL-6 mRNA expression (mean ± SEM) in parallel groups of BSA- and PAL-treated fibroblast cultures. Asterisks denote treatment times in which NF-κB phosphorylation or the relative expression of IL-6 mRNA in PAL-treated fibroblasts were significantly increased ($p<0.05$) compared to that found in control cultures.

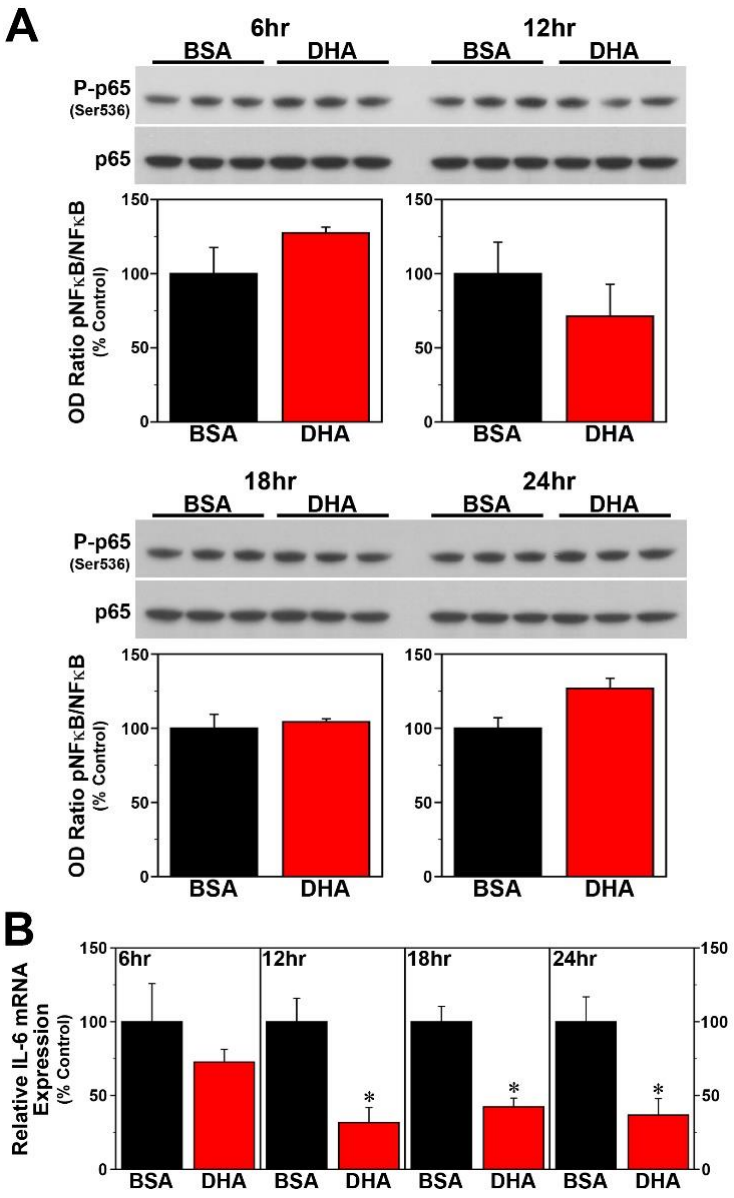

Figure 19: Time-dependent effects of acute DHA treatment on inflammatory signaling in differentiated *Bmal1-dLuc* adipocytes. **(A)** Representative Western blots and densitometric quantification (mean ± SEM) of P-p65/p65 immunoreactive signal (% Control) in differentiated adipocyte cultures treated for 4hr with BSA (n=3) or 250μM DHA (n=3) at hour 6, 12, 18 and 24. **(B)** Determinations (mean ± SEM) of IL-6 mRNA expression in parallel groups of BSA- and DHA-treated differentiated adipocyte cultures. Asterisks denote treatment times in which the relative expression of IL-6 mRNA in DHA-treated differentiated adipocytes was significantly decreased ($p<0.05$) in comparison with that found in BSA controls.

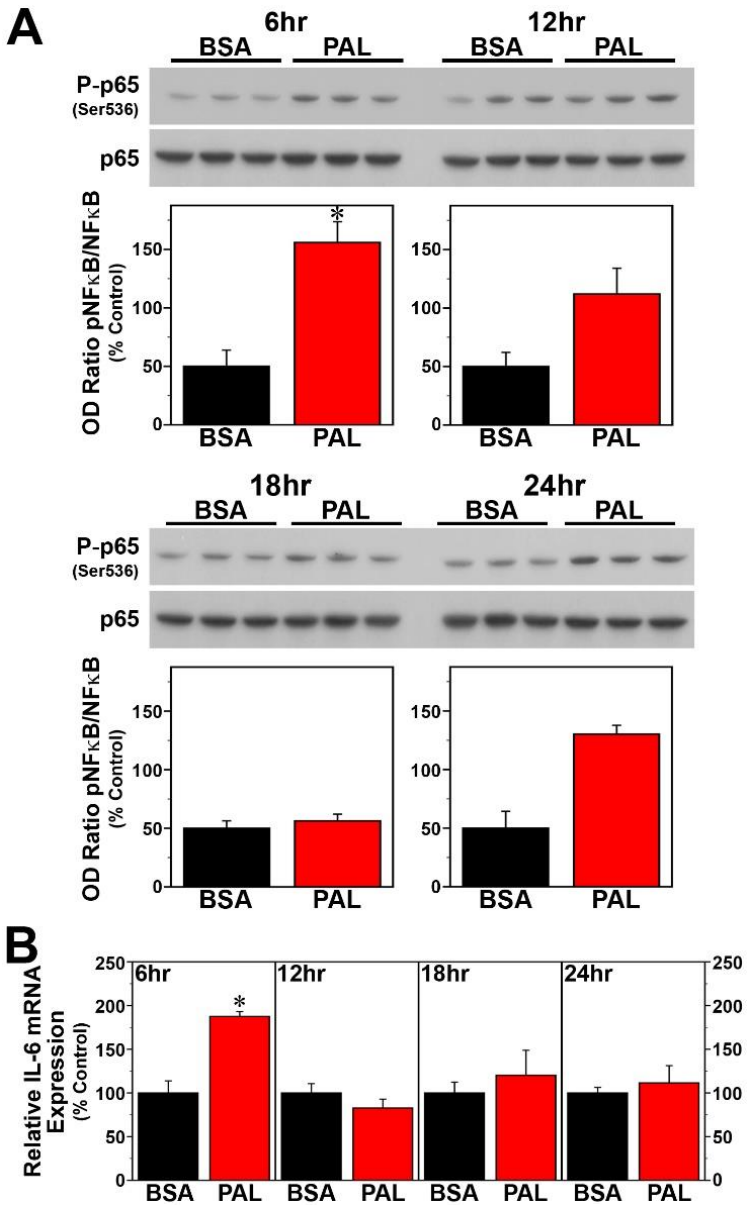

Figure 20: Time-dependent effects of acute palmitate (PAL) treatment on inflammatory signaling in differentiated *Bmal1-dLuc* adipocytes. **(A)** Representative Western blots and densitometric quantification (mean ± SEM) of P-p65/p65 immunoreactive signal (% Control) in differentiated adipocyte cultures treated for 4hr with BSA (n=3) or 250μM PAL (n=3) at hour 6, 12, 18 and 24. **(B)** IL-6 mRNA expression (mean ± SEM) in parallel groups of BSA- and PAL-treated differentiated adipocyte cultures. Asterisks denote treatment times in which NF-κB phosphorylation or the relative expression of IL-6 mRNA in PAL-treated differentiated adipocytes were significantly increased ($p<0.05$) compared to that found in control cultures.

induced significant increases ($p<0.05$) in NF-κB activation not only at hour 6 but also at hour 24. NF-κB phosphorylation in palmitate-treated adipocyte cultures was moderately elevated at hour 12 but unchanged at hour 18 in comparison with the levels observed in BSA controls. The effects of palmitate on IL-6 expression in differentiated adipocytes closely coincided with the timing of peak phase-shifting responses to this SFA. Palmitate had no significant effect on IL-6 mRNA levels following administration at hour 12, 18 and 24 (Figure 20B) but significantly increased ($p<0.05$) expression of this pro-inflammatory cytokine (≈188%) at hour 6, when treatment induced maximal phase advances of adipocyte *Bmal1-dLuc* rhythms.

***Effect of inhibiting inflammatory signaling on fatty acid-induced phase shifts of the circadian clock*:** To examine the role of inflammatory signaling in the mechanism by which palmitate modulates circadian clock function, we next determined whether the omega-3 fatty acid DHA and other inhibitors of inflammation abate peak inflammatory and phase-shifting responses to this SFA at hour 12 in *Bmal1-dLuc* fibroblasts. Because AMPK plays a role in regulating fatty acid oxidation and inhibiting NF-κB-mediated inflammatory responses (Salminen et al., 2011), the AMPK activator, AICAR, was utilized in parallel to inhibit palmitate-induced inflammatory signaling. Consistent with previous reports on AMPK regulation in response to palmitate and other SFAs (Lindholm et al., 2013; Sun et al., 2008), phospho-AMPK activity was significantly decreased ($p<0.05$) in *Bmal1-dLuc* fibroblasts treated with palmitate and was significantly increased

($p<0.05$) following exposure to AICAR alone (Figure 21). Palmitate administration at hour 12 induced a 35-40% increase in GFP-reported NF-κB activation in *Bmal1-dLuc* fibroblasts, but treatment with DHA (Figure 22A), AICAR (Figure 22B) or cardamonin (Figure 22C) significantly inhibited ($p<0.05$) the inductive effects of this SFA on NF-κB such that signaling activity was comparable to the basal levels found in BSA/vehicle controls. Treatment with DHA, AICAR or cardamonin similarly inhibited the phase-shifting responses of *Bmal1-dLuc* fibroblasts to palmitate. In DHA-treated cultures, palmitate-induced phase shifts of the *Bmal1-dLuc* rhythm were significantly decreased ($p<0.05$) relative to those observed in controls (Figure 23A). Administration of palmitate alone produced 2.5-hour phase advances in vehicle-pretreated controls whereas the amplitude of these shifts was reduced by 70% following pretreatment with DHA. Little or no phase-shifting effect was observed in response to DHA alone (+BSA at hour 12). Similar to the effects of DHA, AICAR significantly decreased ($p<0.05$) the phase-shifting responses of fibroblast *Bmal1-dLuc* rhythms to palmitate administration at hour 12 (Figure 23B). Once again, palmitate alone induced large phase advances of ≈2.8hr in vehicle-pretreated cultures. In comparison, the amplitude of these palmitate-induced phase shifts was abated by ≈80% in AICAR-treated fibroblasts. Phase-shifting effects of AICAR alone were minimal as only small advances of the *Bmal1-dLuc* rhythm (<0.5hr) were observed in cultures treated with BSA and AICAR at hour 12. Concurrent treatment with cardamonin, an anti-inflammatory chalconoid that inhibits LPS-stimulated NF-κB signaling (Chow et al., 2012), also had a significant effect ($p<0.05$) in repressing palmitate-induced phase shifts of fibroblast *Bmal1-dLuc* rhythms at hour 12. Palmitate

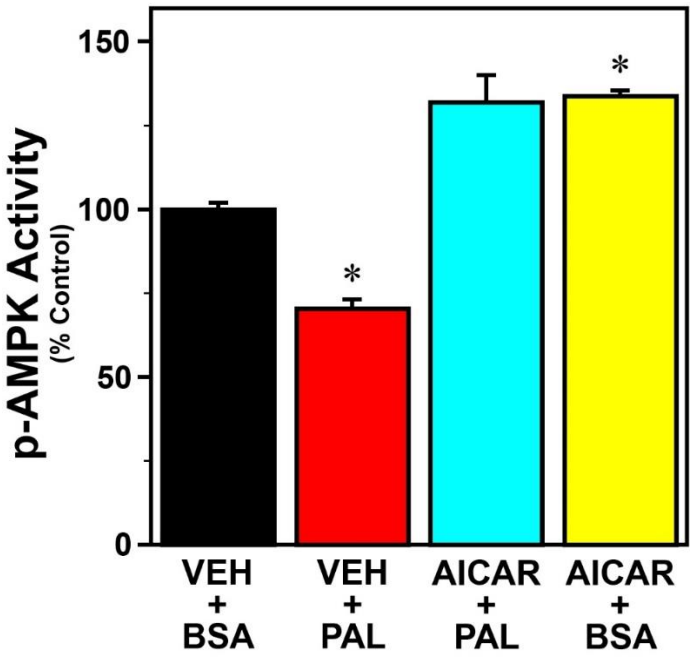

Figure 21: Effects of palmitate (PAL) and AICAR treatment on phospho-AMPK activity in *Bmal1-dLuc* fibroblasts. Using an ELISA assay for phospho-AMPKα (Invitrogen), p-AMPK activity was analyzed in cultures treated with AICAR (500uM) in conjunction with palmitate (250µM) administration. Effects of treatment with PAL (VEH+PAL) or AICAR (AICAR+BSA) alone on p-AMPK activity were also tested. Control cultures were treated with vehicle (DMSO) and BSA (VEH+BSA). Plotted values denote determinations of colorometric signal intensity (mean ± SEM) in each treatment group (n=4) that were normalized to the average signal for controls, which was arbitrarily set at 100%. Phospho-AMPK activity was significantly decreased ($p<0.05$) in *Bmal1-dLuc* fibroblasts treated with PAL (VEH+PAL) and was significantly increased ($p<0.05$) in cultures treated with AICAR alone (AICAR+BSA) relative to that found in controls (VEH+BSA).

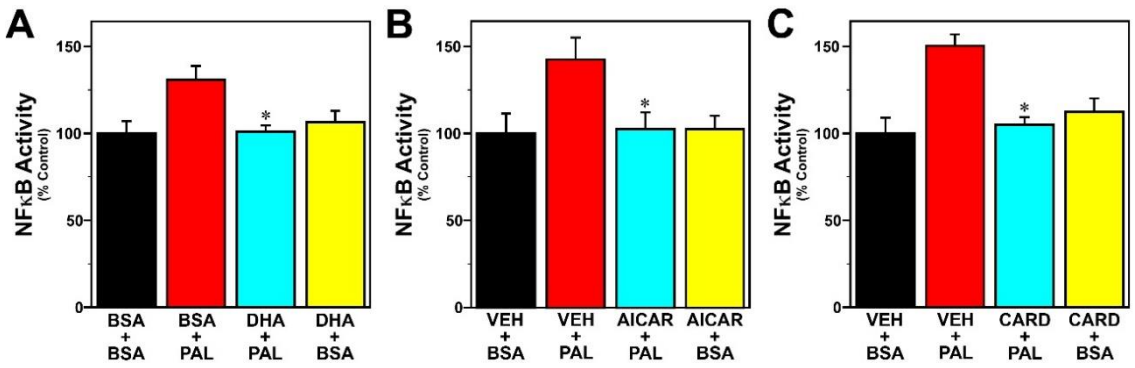

Figure 22: Effects of DHA **(A)**, AICAR **(B)** or cardamonin **(C)** treatment on NF-κB-regulated inflammatory responses of *Bmal1-dLuc* fibroblasts to palmitate (PAL; 250μM). Bar graphs depict real-time fluorometric analysis of cells transfected with an inducible NF-κB-responsive GFP construct that were treated with DHA (50uM), AICAR (500uM) or cardamonin (5μM) in advance of and/or during palmitate (250μM) administration for 4hr at hour 12. Effects of treatment with DHA, AICAR or cardamonin alone (DHA+BSA, AICAR+BSA, CARD+BSA) on NF-κB activity were also tested. Control cultures were treated with BSA or vehicle (DMSO) and BSA (VEH+BSA). Plotted values denote determinations of GRP signal intensity (mean ± SEM) in each treatment group (n=5) that were normalized to the average signal for controls, which was arbitrarily set at 100%. Asterisks indicate that palmitate-induced NF-κB activity was significantly decreased ($p<0.05$) in *Bmal1-dLuc* fibroblasts treated with DHA (DHA+PAL), AICAR (AICAR+PAL) or cardamonin (CARD+PAL) in comparison with those exposed to palmitate alone (BSA+PAL or VEH+PAL).

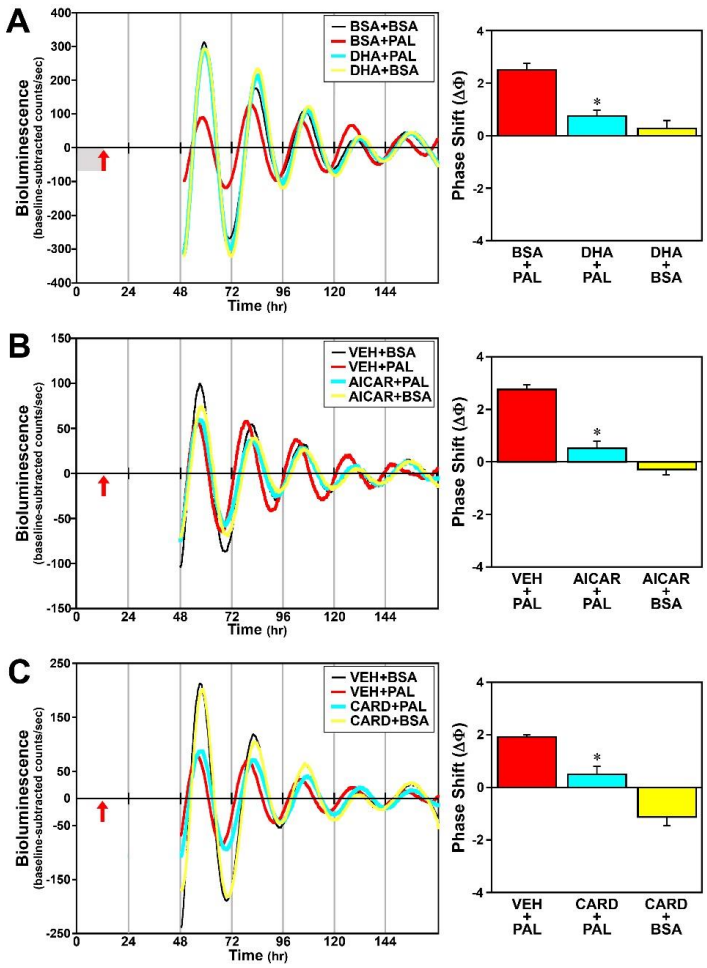

Figure 23: Effects of DHA **(A)**, AICAR **(B)** or cardamonin **(C, CARD)** treatment on the phase-shifting responses of *Bmal1-dLuc* fibroblasts to palmitate (PAL). Representative recordings of ensemble bioluminescence from individual fibroblast cultures treated with either vehicle (VEH; DMSO), DHA (50µM) for 12hr in advance or AICAR (500µM) or cardamonin (5µM) for 4hr in conjunction with PAL administration (250µM) for 4hr at hour 12. Phase-shifting effects of these anti-inflammatory treatments alone were also tested in BSA control cultures treated with DHA for 12hr, or with AICAR or cardamonin for 4hr. Specific experimental groups include: BSA+BSA or VEH+BSA (n=15), BSA+PAL or VEH+PAL (n=18), DHA+PAL (n=6), DHA+BSA (n=6), AICAR+PAL (n=10), AICAR+BSA (n=8), CARD+PAL (n=6) and CARD+BSA (n=6). Red arrows denote time of palmitate administration after 12hr pretreatment with DHA (adjacent shaded area in A) or during AICAR (B) or cardamonin treatment (C). Bar graphs depict the mean (±SEM) phase shifts (ΔΦ) in hours as a function of treatment group. Asterisks indicate that palmitate-induced phase shifts were significantly decreased ($p<0.05$) in *Bmal1-dLuc* fibroblasts treated with DHA (DHA+PAL), AICAR (AICAR+PAL) or cardamonin (CARD+PAL) in comparison with those observed in controls (VEH+PAL).

induced phase advances of ≈2 hours in vehicle-treated cultures but only advanced *Bmal1-dLuc* rhythms by 0.5hr or less in cultures concurrently treated with cardamonin (Figure 23C). Treatment with cardamonin alone (+BSA at hour 12) induced small phase delays (≈1hr).

Additional Results

Because acute palmitate induced time-dependent phase shifts of *Bmal1-dLuc* rhythm, we determined whether expression of core clock component BMAL1 protein is also altered by acute palmitate treatment in a time-dependent manner. Consistent with the time-dependent variation in its phase shifting effects on *Bmal1- dLuc* rhythms, palmitate treatment at hour 12 and 18, but not at hour 6 and 24, significantly increased ($p<0.05$) BMAL1 protein levels in fibroblast cultures (Figure 24).

Since AMPK is an energy-sensing enzyme that influences downstream inflammatory signaling pathway (Fullerton et al., 2013), we determined whether AMPK activity is regulated in a circadian manner. Wild-type fibroblast cultures exhibited circadian rhythms in phospho-AMPK levels with peak activity at hour 12 (after serum shock), but this rhythm was abolished in $Per1^{ldc}/Per2^{ldc}$ fibroblasts (Figure 25) indicating that AMPK activity is under the direct regulation of the fibroblast clock mechanism. Interestingly, the peak of AMPK phosphorylation coincided with the specific time when phase-shifting and proinflammatory effects of palmitate were maximal. Because palmitate decreases AMPK activity (Sun et al., 2008) and AMPK is directly involved in the regulation of core clock

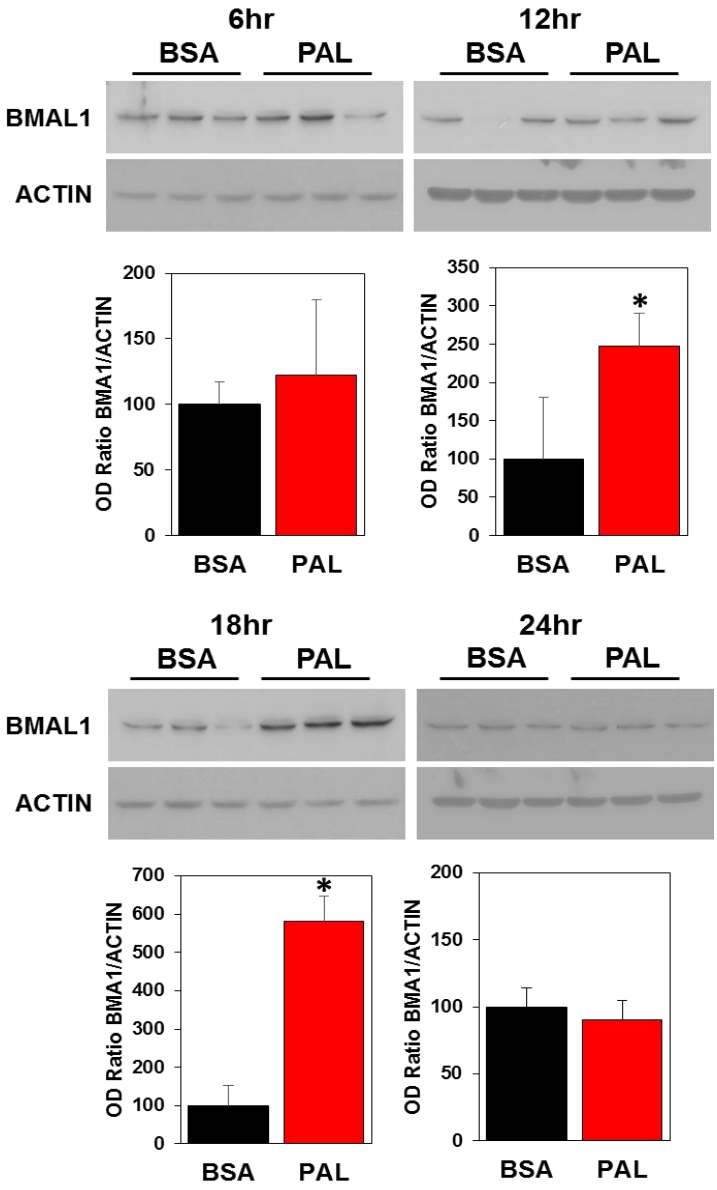

Figure 24: Time-dependent effects of acute palmitate (PAL) treatment on BMAL1 protein levels in *Bmal1-dLuc* fibroblasts. Representative Western blots and densitometric analyses of BMAL1 in fibroblast cultures treated for 4hr with BSA (n=3) or 250µM PAL (n=3) at hour 6, 12, 18 and 24. Bar graphs depict the ratios of BMAL1/ACTIN immunoreactive signal that were adjusted in relation to control (BSA) values (% Control). Asterisks denote treatment times in which BMAL1 protein levels in PAL-treated fibroblasts were significantly increased ($p<0.05$) compared to that found in control cultures.

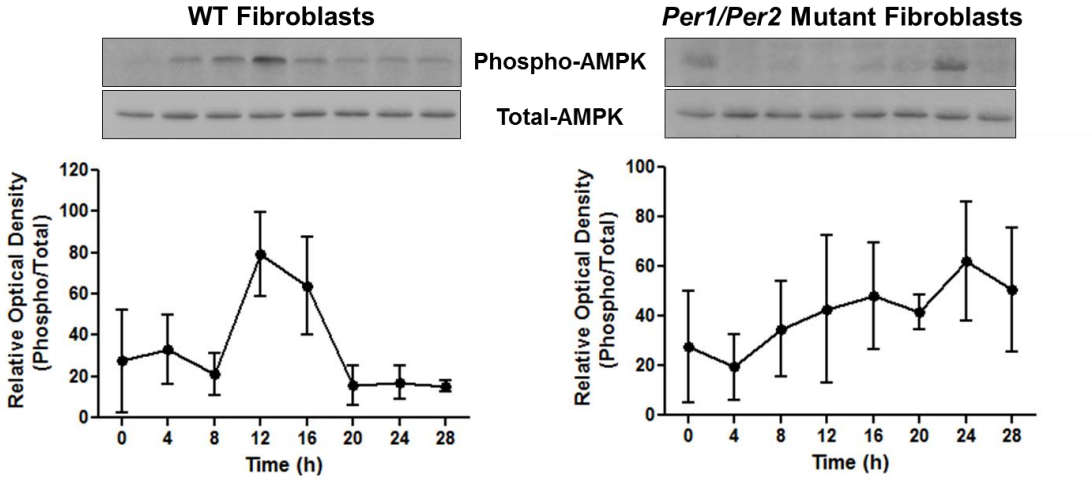

Figure 25: Circadian rhythm of phospho-AMPK protein (Molecular weight: 62 kDa) levels in wild-type and *Per1*^{ldc}/*Per2*^{ldc} fibroblasts. Representative Western blots and densitometric quantification (mean ± SEM) of p-AMPK immunoreactive signal (ratio of phospho-AMPK normalized to total AMPK) in wild-type (WT) (n=3) and *Per1*^{ldc}/*Per2*^{ldc} (n=3) fibroblasts collected at 4hr intervals for 28hr.

machinery (Lamia et al., 2009), we predict that palmitate treatment may suppress peak AMPK activity at hour 12, and thus yield maximal phase shifts. At hour 6, 18 and 24, palmitate may have a minor effect on AMPK activity because fibroblast AMPK activity is already low, thereby producing small phase shifts in response to this SFA. Taken together, these observations suggest that the rhythm in AMPK activity may play a role in the time-dependent phase shifts of the fibroblast clock in response to palmitate. This speculation may also apply to our AICAR data; this AMPK activator AICAR may attenuate palmitate-induced phase shifts by reversing the inhibitory effects of this SFA and restoring AMPK activity to peak levels that normally occur at hour 12. Circadian clocks in many peripheral tissues/cells of mammals coordinate daily rhythms in inflammatory responses, nutrient metabolism and secretion

Discussion

While circadian clocks throughout the body clearly play a role in regulating metabolic pathways and their homeostatic function, increasing evidence for diet-induced alterations in clock gene oscillations and behavioral rhythms suggests that nutrient metabolism may, in turn, feed back on the clock mechanism and modulate fundamental properties of circadian rhythms. HFD has been to alter circadian timekeeping function although its effects vary greatly among clocks in the brain and peripheral tissues. For example, HFD had no effect on core clock gene oscillations in the hypothalamus and only produced small increases in the free-running period of the SCN-regulated rhythm of locomotor activity (Kohsaka et al., 2007; Xu et al., 2014). Consistent with the effects of HFD on circadian

clocks in the brain, palmitate alters clock gene expression and the amplitude of their rhythmic profiles in a hypothalamic cell line and this effect is not accompanied by a corresponding induction of inflammatory cytokines such as IL-6 (Fick et al., 2011; Greco et al., 2014). In contrast, HFD-induced dysregulation of circadian timekeeping is more robust in peripheral clocks; in HFD-fed mice, clock gene oscillations are distinguished by severe damping in both liver and fat tissue (Kohsaka et al., 2007) and by circadian period increases of up to 9hr in adipose tissue and bone marrow-derived macrophages (Xu et al., 2014). Similar to the latter effect of HFD, prolonged treatment with palmitate induced large increases in the period of fibroblast *Bmal1-dLuc* rhythms, but small increases in the free-running period of the SCN-regulated rhythm in locomotor activity, suggesting that this pro-inflammatory SFA is a key mediator of HFD-induced modulation of peripheral circadian clocks.

Importantly, our findings indicate that the effects of HFD and its major constituent, palmitate, on circadian clock properties are not limited to changes in period and rhythm amplitude, but include differential phase shifts of clock gene oscillations in peripheral tissues. Previous studies have established that phase-shifting responses to HFD again differ among peripheral and central circadian clocks. In this regard, HFD treatment for 7 days has been shown to shift Per2 oscillations, producing large advances in the liver and small delays in the spleen but no effect on the phase of SCN rhythms (Pendergast et al., 2013). In the present study, 4-hour exposure of peripheral cell types to palmitate similarly induced marked phase shifts of clock gene oscillations that varied in amplitude depending on the time of treatment. It is noteworthy that the time-dependent nature of palmitate-

mediated phase shifts of the *Bmal1-dLuc* rhythm was cell-specific; the phase responses of undifferentiated fibroblasts to this pro-inflammatory SFA were maximal at hour 12 and negligible at hour 6 when palmitate-induced phase advances were at peak amplitude in differentiated adipocytes. Because the omega-3 fatty acid DHA had little or no phase-shifting effects on clock gene oscillations in both fibroblasts and differentiated adipocytes, these findings collectively suggest that SFAs may differentially reset the clock mechanism in some but not all cells within a given tissue, thereby altering the local coordination of circadian timekeeping among individual cellular clocks.

How HFD and palmitate modulate circadian timekeeping is unknown. The present evidence for the close coincidence between the time-dependent variation in inflammatory and phase-shifting responses to palmitate raises the possibility that mutual interactions between peripheral circadian clocks and inflammatory signaling pathways may play a key role in the underlying mechanism. In both *Bmal1-dLuc* fibroblasts and differentiated adipocytes, the maxima and minima for palmitate-induced inflammatory signaling were contemporaneous with the equivalent variation in the amplitude of the phase shifts induced by this SFA. Thus, the signaling cascades through which HFD and SFAs induce inflammation in peripheral tissues may govern the modulatory effects of palmitate on circadian timekeeping. The induction of inflammatory signaling by palmitate and other SFAs is mediated through activation of NF-κB, ultimately leading to the secretion of pro-inflammatory cytokines in various peripheral tissues (Ajuwon and Spurlock, 2005; Jove et al., 2006; Weigert et al., 2004). In addition, the nutrient sensor, AMP-activated protein kinase (AMPK), is involved in coupling fatty acid metabolism to inflammatory responses.

Palmitate and other SFAs decrease AMPK activity (Sun et al., 2008; Lindholm et al., 2013) and correspondingly their inductive effects on NF-κB activation and the expression of pro-inflammatory cytokines are repressed by AMPK activators (Green et al., 2011; Yang et al., 2010). The role of inflammatory signaling in SFA-mediated phase regulation of peripheral circadian clocks is directly supported by the present findings that the anti-inflammatory inhibitor of NF-κB signaling, cardamonin, and the AMPK activator, AICAR, suppress peak induction of both inflammatory and phase-shifting responses to palmitate at hour 12. Because DHA represses inflammation-induced NF-κB signaling and cytokine expression via activation of AMPK (Xue et al., 2012), the observed inhibition of palmitate-induced inflammation and phase shifts following pretreatment with this PUFA also underscores the importance of clock-gated inflammatory signaling in the mechanism by which palmitate alters circadian timekeeping. In conjunction with evidence for the circadian regulation of AMPK activity (Um et al., 2011) and inflammatory responses of the NF-κB pathway (Spengler et al., 2012) in different tissues, these observations indicate that mutual interactions between peripheral clocks and inflammatory signaling mediate the time-dependent variation in the extent to which SFA overload triggers inflammation and in turn, leads to the feedback modulation of circadian timekeeping.

The endoplasmic reticulum (ER) stress pathway may also be involved in the palmitate-mediated regulation of circadian clock properties. Recent evidence supports a link between the circadian clock and ER homeostasis involving mutual regulation of each other (Pluquet et al., 2014). Moreover, SFAs such as palmitate induce ER stress via multiple mechanisms (Ariyama et al., 2010; Baldwin et al., 2012; Milanski et al., 2009) whereas

the omega-3 fatty acid DHA attenuates ER stress (Kolar et al., 2011). Because ER stress has been linked to inflammation in metabolic disorders (Cao et al., 2016), further studies are needed to determine whether ER stress plays a role in palmitate-mediated modulation of circadian timekeeping in parallel to, or perhaps in concert with, the inductive effects of this SFA on inflammatory signaling.

The association between circadian clock dysfunction and metabolic disorders is largely based on studies that involve the complete disruption of rhythmicity using genetic or environmental approaches. However, the present data have novel implications for how differential and cell-specific modulation, rather than complete malfunction, of peripheral circadian clocks may be germane factors in the amplification of pro-inflammatory responses to HFD and SFA excess that are closely linked to systemic metabolic dysregulation. In both fibroblasts and differentiated adipocytes, palmitate-mediated inflammatory signaling is gated rhythmically and at times of peak induction, provides feedback that modulates the phase of the core clock mechanism. Because the phase-shifting responses to palmitate appear to differ among peripheral cell types, this feedback modulation is likely to disrupt the local coordination of circadian timekeeping among cell-autonomous clocks within a given tissue, thus further potentiating SFA-induced inflammation that contributes to systemic insulin resistance. The palmitate-induced inflammatory signals directly responsible for the feedback modulation of circadian phase remain to be determined, but future studies are warranted to examine the role of pro-inflammatory cytokines such as IL-6 in mediating the phase-shifting effects of this SFA. Finally, these interactions between peripheral circadian clocks and pathways linking fatty

acid metabolism to tissue inflammation suggest that chronotherapeutic strategies using DHA and/or metformin, an AMPK agonist exploited in the treatment of hyperglycemia in type II diabetes (Akbar, 2003), may be critical in the management of metabolic diseases associated with fatty acid overload.

CHAPTER V

ROLE OF PROINFLAMMATORY CYTOKINES IN FEEDBACK MODULATION OF CIRCADIAN TIME KEEPING MECHANISM BY SATURATED FATTY ACIDS

Introduction

Circadian clocks in the SCN and peripheral tissues mediate the generation and entrainment of tissue- or cell-specific rhythms in physiological processes such as inflammation and metabolism (Richards and Gumz, 2012). While light-dark cues are the primary signals for circadian entrainment in mammals, nutrient metabolism and food intake, especially high fat diet (HFD), can modulate the circadian timekeeping function of SCN and peripheral clocks. HFD has been shown to alter the period of the activity rhythm and clock gene rhythms in peripheral tissues (Kohsaka et al., 2007a; Xu et al., 2014). In addition, recent findings indicate that HFD and the saturated fatty acid (SFA) palmitate, which is a major component of HFD, disrupt the diurnal patterns of glucagon like peptide 1 and insulin secretion and damp clock gene rhythms in intestinal L-cells (Gil-Lozano et al., 2016). We have observed similar effects of HFD or palmitate on circadian clocks *in vivo* and *in vitro*; HFD and long-term treatment (48hr) with palmitate increased the period of activity rhythm and peripheral clock gene rhythms *in vitro* (Kim et al., 2016; Xu et al., 2014), and acute (4hr) treatment of palmitate induced time-dependent phase shifts of peripheral clock gene rhythms (Kim et al., 2016). In view of increasing evidence for the association between circadian dysregulation and metabolic phenotypes, this diet-mediated

modulation of circadian clock function may contribute to the mechanism by which HFD and SFA induce systemic metabolic disorders.

HFD and SFA are known to affect both the circadian system and metabolic homeostasis. However, it is unclear whether HFD- or SFA-induced circadian clock modulation and metabolic disorders are closely linked through a common mechanism. HFD and SFA initiate NF-kB- and JNK-mediated pro-inflammatory signaling cascades, leading to the release of pro-inflammatory cytokines such as IL-6 and TNFα (Fullerton et al., 2013). Therefore, it is possible that HFD-induced inflammatory signaling through pro-inflammatory cytokines is a common mediator of diet-induced metabolic and circadian dysregulation. This speculation is indirectly supported by our previous observation that in HFD-fed mice and palmitate treated fibroblasts, the resulting increases in inflammatory signaling, including the induction of NF-kB and JNK activity and IL-6 expression, are coincident with corresponding alterations of circadian clock properties (Kim et al., 2016; Xu et al., 2014). Direct, evidence is provided by our previous findings that DHA and other inhibitors of inflammatory signaling (AICAR, cardamonin) attenuated palmitate-induced proinflammatory responses and phase shifts of the fibroblast clock (Kim et al., 2016). Furthermore, HFD has been shown to disrupt circadian rhythms of PPARγ and AMPK (Barnea et al., 2009; Eckel-Mahan et al., 2013), which are directly involved in the regulation of metabolism and may also link the clock machinery to mediators of inflammation. Collectively, these data suggest that inflammatory signaling may play a key role in the mechanism by which HFD or SFA dysregulates circadian time keeping in peripheral clocks.

The immune system is clock-regulated, but also feeds back to modulate the timekeeping function of circadian clocks. The modulatory effects of the immune system on circadian timekeeping and the underlying clock mechanism have been observed in studies demonstrating that lipopolysaccharide (LPS) administration induces phase shifts of the activity rhythm in mice (Marpegan et al., 2009) and represses SCN expression of the clock genes, *Per2* and *Dbp* (Okada et al., 2008). Alterations in the functional properties of SCN and peripheral circadian clocks induced by LPS and other inflammatory stimuli such as HFD and SFA are presumably mediated through the release of pro-inflammatory cytokines. For example, intracerebroventricular injection of TNFα or IL-1β induces phase delays of circadian rhythm in locomotor activity at CT15 (Leone et al., 2012). Because our previous results demonstrate that the phase shifting responses to palmitate are associated with corresponding increases in IL-6 expression (Kim et al., 2016), our hypothesis is that the modulation of circadian phase (and period) in response to palmitate is derived from SFA-induced feedback on the clock mechanism via inflammatory signaling through pro-inflammatory cytokines. To test this hypothesis, we used two different approaches in this study: 1) we initially tested whether the pro-inflammatory cytokines, IL-6 and TNFα, mimic the phase shifting effects of palmitate at hour 12 when this SFA induces maximal phase shifts of the clock mechanism; and 2) conversely determined whether blockade of IL-6 or TNFα signaling using neutralizing antibodies abates palmitate-induced phase shifts in *Bmal1-dLuc* fibroblasts.

Material and Methods

***Cell culture*:** *Bmal1-dLuc* fibroblasts (Dr. Andrew Liu, University of Memphis, Memphis, TN; Ramanathan et al., 2012) were propagated on 60mm dishes in Dulbeco's Modified Eagle Medium (DMEM; HyClone) containing 10% Fetal Bovine Serum (FBS) and maintained at 37°C and 5% CO_2. Medium was replaced every 48hr and cultures were split 1:4 every 3 days.

***Fatty acid/drug preparation and treatment*:** Palmitate (Sigma) was dissolved in ethanol and then diluted (1:5.4 ratio) with 10% BSA (fatty acid-free and low endotoxin) diluted in 0.1M phosphate-buffered saline (PBS). Palmitate treatment in these studies was based on physiological concentrations that have been previously observed *in vivo* or used for *in vitro* studies (Ajuwon, and Spurlock, 2005; Han et al., 2010; Puri et al., 2009; Weldon et al., 2007). IL-6 recombinant protein (Invitrogen) was dissolved in 0.1M PBS, then diluted 1:100000 and 1:1000 in culture medium to achieve final concentration of 0.1ng/ml and 10ng/ml, respectively. TNFα recombinant proteins (Invitrogen) and TNFα neutralizing antibodies (R&D systems) were dissolved in 0.1M PBS, then diluted 1:1000 in culture medium to achieve final concentration of 20ng/ml and 0.4ug/ml, respectively. Vehicle controls for recombinant proteins and antibodies were treated with PBS and controls for fatty acid treatment were treated with BSA diluted in PBS with an equivalent ratio of ethanol.

***Phase shifting effects of recombinant IL-6 and TNFα on the circadian clock*:** IL-6 and TNFα recombinant proteins were used to test whether these proinflammatory cytokines mimic the phase shifting effect of palmitate. *Bmal1-dLuc* fibroblast cultures were exposed for 2hr to medium containing 15uM forskolin (hour 0) to facilitate circadian oscillation synchronization across cultures (Menger et al., 2007) and then treated for 4hr with IL-6 (final concentrations = 0.1ng/ml or 10ng/ml) at hour 12 when the phase shifting effects of palmitate are maximal (Kim et al., 2016). Parallel cultures were similarly treated with TNFα (final concentration = 20ng/ml) at 6hr intervals throughout the circadian cycle (i.e., hour 6, 12, 18 and 24). Vehicle controls for IL-6 and TNFα recombinant protein treatments consisted of cultures in which an equivalent amount of PBS was added to the medium. After treatment, all control and cytokine-treated cultures were placed in recording media for bioluminescence analysis of phase shifting effects on *Bmal1-dLuc* oscillations.

***Effect of neutralizing TNFα on palmitate-induced phase shifts of the circadian clock*:** Neutralizing antibody to TNFα were used to test whether inhibition of inflammatory signaling via these cytokines blocks or attenuates phase shifting effects of palmitate at hour 12 when this SFA induces maximal advances of the *Bmal1-dLuc* rhythm (Kim et al., 2016). Phase shifting responses were examined in *Bmal1-dLuc* fibroblasts treated with TNFα (0.4ug/ml) neutralizing antibody for 12hr in advance and during the 4hr exposure to palmitate (250μM). The vehicle control for TNFα neutralizing antibody consisted of cultures in which an equivalent amount of PBS was added to the medium.

***Real-time analysis of Bmal1-dLuc rhythms*:** Prior to bioluminescence analysis, growth medium including IL-6 and TNFα recombinant proteins, neutralizing antibodies, BSA and palmitate was removed. Cultures were rinsed, placed in DMEM recording medium containing 15uM forskolin, 25mM HEPES, 292µg/ml L-glutamine, 100units/ml penicillin, 100µg/ml streptomycin and 10uM luciferin (Promega) and then sealed airtight with sterile glass coverslips, and sterile silicon grease. The temporal patterns of *Bmal1-dLuc* bioluminescence were analyzed using an automated 32-channel luminometer (LumiCycle; Actimetrics) housed in a standard culture incubator at 32°C. Bioluminescence from individual cultures was continuously recorded for ~70sec at intervals of 10min. Rhythm parameters were determined from baseline-subtracted data using the damped sine fit and Levenberg–Marquardt algorithm. The amplitude of phase shifts in response to treatment with IL-6, TNFα recombinant proteins or palmitate was determined by measuring the time difference between the peaks of the *Bmal1-dLuc* rhythms during the third cycle in PBS, BSA or BSA/vehicle (PBS) control and experimental treatment groups.

***Statistical analysis*:** Time-dependent differences in phase-shifting effects of TNFα on the *Bmal1-dLuc* rhythm were first analyzed by one-way analysis of variance (ANOVA). Paired comparisons between peak phase-shifting responses and the corresponding minima were analyzed post hoc for statistical differences using the Student Newman-Keuls sequential range test. The p-value was set at 0.05 for these post-hoc analyses.

Results

***Effect of recombinant IL-6 and TNFα on the circadian clock*:** Based on our previous results showing that the saturated fatty acid, palmitate treatment at hour 12 induces maximal phase shifts of *Bmal1-dLuc* rhythms along with coincidental induction of NF-κB activation and IL-6 expression in fibroblasts *in vitro*, we first determined whether recombinant IL-6 mimics the phase shifting effects of palmitate at hour 12. Following to acute (4hr) treatment with 0.1ng/ml or 10ng/ml IL-6 at hour 12, the period of fibroblast *Bmal1-dLuc* rhythms was not significantly different from that observed in the PBS-treated control cultures. Phase shifting analysis revealed that the effects of IL-6 treatment were dose-dependent; 0.1ng/ml IL-6 induced small phase advances of about 0.5hr whereas 10ng/ml IL-6 induced large phase advances of *Bmal1-dLuc* rhythms (≈1.5hr) that were comparable in amplitude to the phase shifts in response to acute palmitate treatment alone (Figure 26). In addition to IL-6, we next tested whether another pro-inflammatory cytokine, TNFα, also induces phase shifts of fibroblast *Bmal1-dLuc* rhythms. In contrast to the direction of the shifts induced by IL-6 and palmitate, acute treatment with 20ng/ml TNFα induced phase delays of *Bmal1-dLuc* rhythms in a time-dependent manner (Figure 27). Peak phase-shifting responses to TNFα were observed at hour 12 when treatment induced large phase delays of ≈2.8hr (Figure 27B). TNFα treatment at hour 18 and 24 also induced phase delays albeit of reduced amplitude (≈0.5-0.9hr). At hour 6, TNFα had negligible effects on the *Bmal1-dLuc* rhythm.

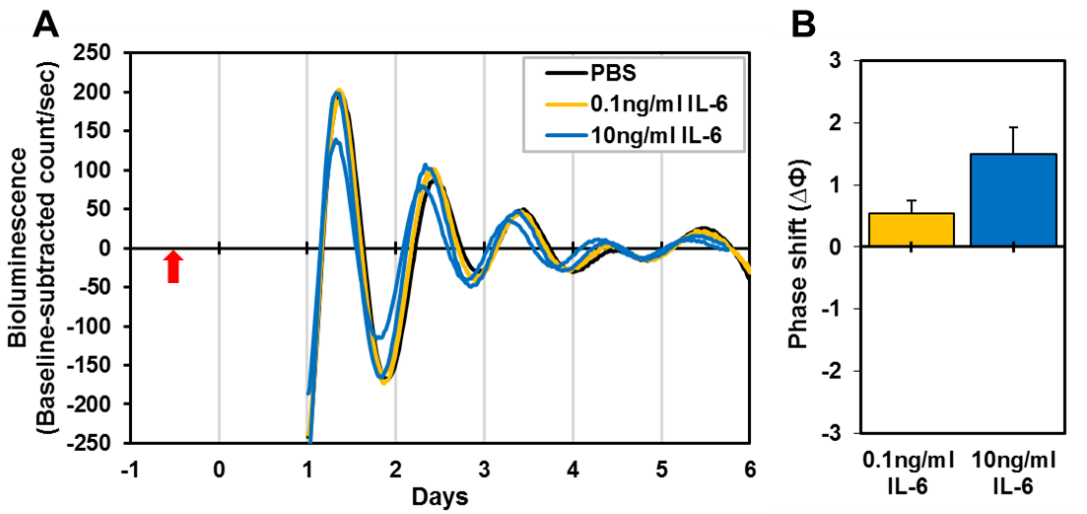

Figure 26: Phase-shifting effects of acute IL-6 cytokine treatment on ensemble *Bmal1-dLuc* rhythms in cultured fibroblasts. **(A)** Representative recordings of ensemble bioluminescence from individual cultures of *Bmal1-dLuc* fibroblasts treated for 4hr with PBS (control) (n=8), 0.1ng/ml IL-6 (n=8) or 10ng/ml IL-6 (n=8) at hour 12. Red arrow indicates time of PBS or IL-6 treatment. **(B)** Dose-dependent phase shifts (ΔΦ) of fibroblast *Bmal1-dLuc* rhythms (mean ± SEM) in response to IL-6 treatment for 4hr. Mean (+SEM) phase shifts in hours are plotted at hour 12.

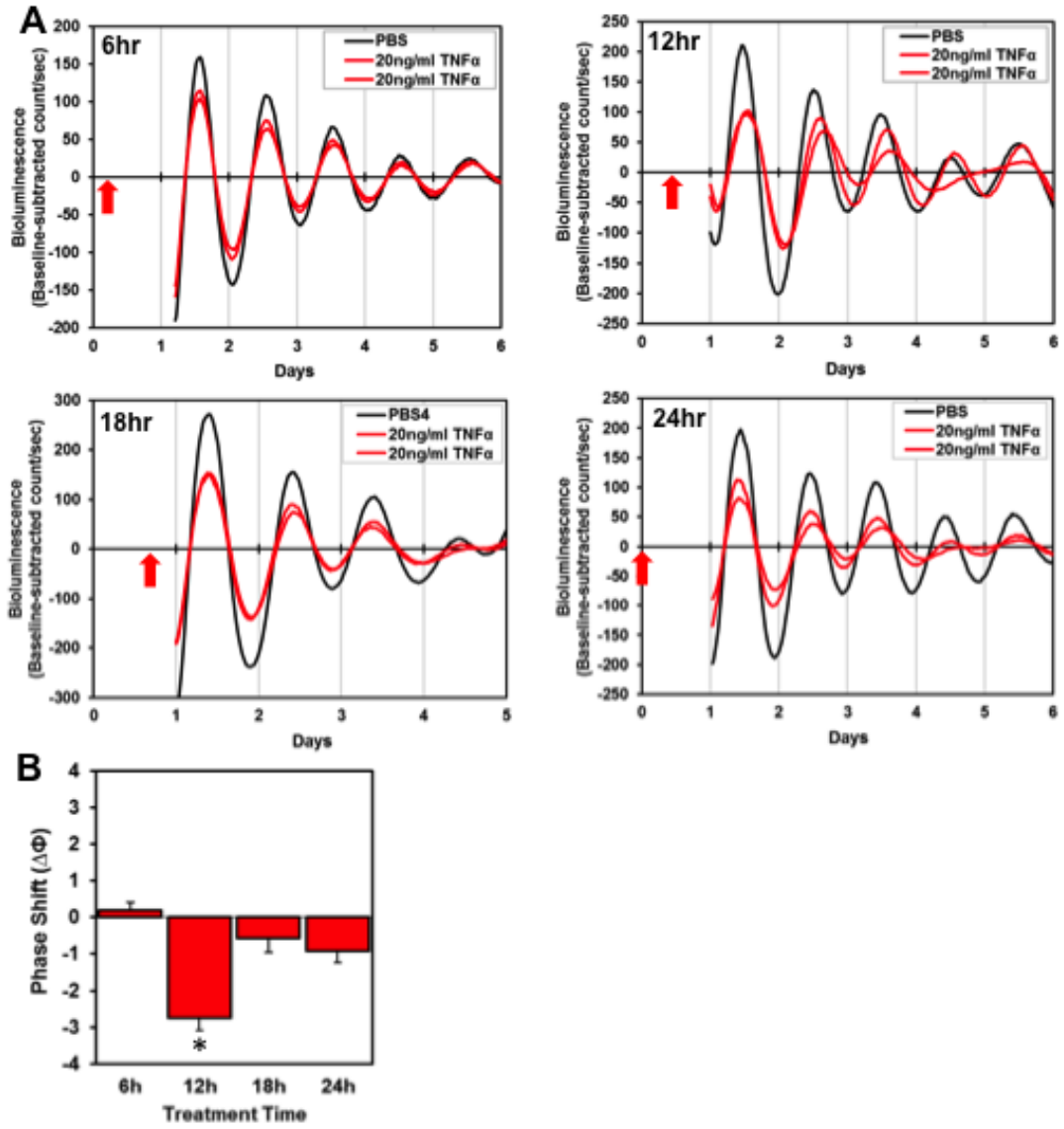

Figure 27: Phase-shifting effects of acute TNFα cytokine treatment on ensemble *Bmal1-dLuc* rhythms in cultured fibroblasts. **(A)** Representative recordings of ensemble bioluminescence from individual cultures of *Bmal1-dLuc* fibroblasts treated for 4hr with PBS (n=8) or 20ng/ml TNFα (n=8) at hour 6, 12, 18 and 24. Red arrows indicate time of PBS or TNFα treatment. **(B)** Time-dependent phase shifts of fibroblast *Bmal1-dLuc* rhythms in response to TNFα treatment for 4hr. Mean (+SEM) phase shifts (ΔΦ) in hours are plotted as a function of the timing of TNFα treatment (at hour 6, 12, 18 and 24). Phase delays of the fibroblast *Bmal1-dLuc* rhythm are indicated by negative values and advances are denoted by positive values. Asterisks indicate that peak phase-shifting responses to TNFα treatment at hour 12 were significantly greater ($p<0.05$) than those observed during the corresponding minima.

***Effect of neutralizing TNFα on palmitate-induced phase shifts of the circadian clock*:** In parallel with experiments using treatments with recombinant pro-inflammatory cytokines, we used the converse approach and determined whether neutralizing antibodies to TNFα that inhibit inflammatory signaling via this cytokine abate palmitate-induced phase shifts of fibroblast *Bmal1-dLuc* rhythms. Treatment with neutralizing antibody to TNFα inhibited the phase-shifting responses to acute palmitate treatment at hour 12. In fibroblast cultures treated with neutralizing antibody to TNFα, palmitate-induced phase shifts of the *Bmal1-dLuc* rhythm were significantly decreased ($p<0.05$) relative to those observed in controls (Figure 28). Acute palmitate alone induced about 1.8 hour phase advances in PBS-pretreated controls whereas the amplitude of these shifts was reduced approximately by 50% following pretreatment with TNFα neutralizing antibody. Antibody treatment alone had little or no phase-shifting effect on the *Bmal1-dLuc* rhythm.

Discussion

HFD and SFAs modulate the timekeeping function of peripheral circadian clocks in mammals. Long-term and short-term HFD treatment have been shown to alter circadian properties (i.e., decrease amplitude, increase period, phase shift) of clock gene rhythms in peripheral tissues (Kohsaka et al., 2007a; Pendergast et al., 2013; Xu et al., 2014). In addition, several investigations, including our recent study, have demonstrated that the proinflammatory SFA, palmitate, also modulate circadian clock properties in liver, fibroblasts and hypothalamic cells (Greco et al., 2014; Kim et al., 2016; Tong et al., 2015).

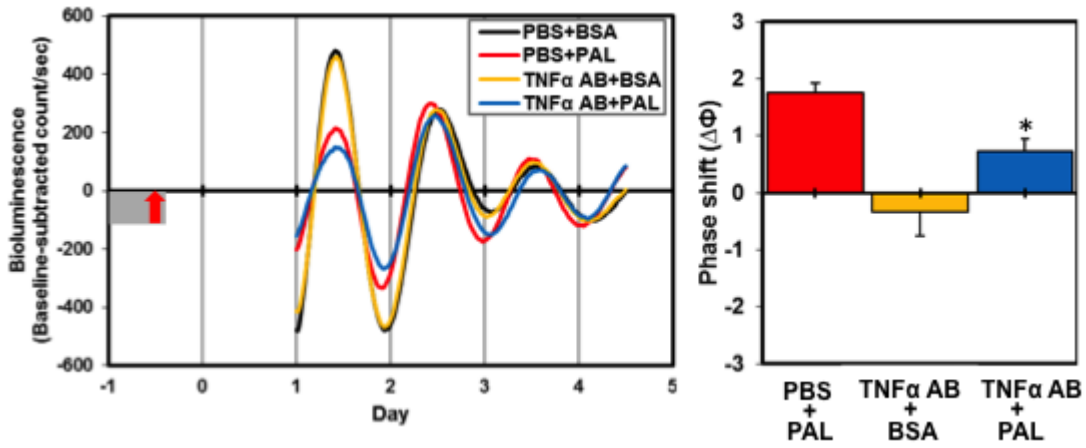

Figure 28: Effect of scavenging TNFα antibodies on palmitate-induced phase shifts of Bmal1-dLuc rhythms in cultured fibroblasts. Representative recordings of ensemble bioluminescence from individual *Bmal1-dLuc* fibroblast cultures treated with either PBS or TNFα antibody (0.4µg/ml) for 12hr in advance and during exposure to palmitate (250uM) for 4hr at hour 12. Phase-shifting effects of antibody treatment alone (with BSA) were also tested relative to control cultures. Red arrow denotes the time of palmitate administration after 12hr pretreatment with TNFα antibody (adjacent shaded area). Bar graphs depict the mean (±SEM) phase shifts (ΔΦ) in hours as a function of treatment group. The asterisk indicates that palmitate-induced phase shifts were significantly decreased ($p<0.05$) in *Bmal1-dLuc* fibroblasts treated with TNFα antibody in comparison with those observed in controls (PBS+PAL).

Although the specific mechanism is unknown, recent findings suggest that inflammatory signaling may play a role in HFD and SFA modulation of the circadian timekeeping function of peripheral clocks. LPS treatment alters clock gene rhythms in peripheral tissues (Paladino et al., 2014; Shimizu et al., 2017) and our previous results demonstrate that various inhibitors of inflammation attenuate palmitate-induced phase shifts of fibroblast clock gene rhythms (Kim et al., 2016). The present data unveil the potential roles of proinflammatory cytokines in the feedback modulation of peripheral circadian clocks by HFD- and SFA-mediated inflammation; we found that treatment with recombinant IL-6 mimics the phase-shifting effects of palmitate on fibroblast circadian clocks whereas administration of neutralizing antibodies that scavenge TNFα attenuate phase-shifting responses to this SFA. Implications for TNFα in the pathway by which inflammation modulates circadian timekeeping are reinforced by studies demonstrating that TNFα alters liver and fibroblast clock gene rhythms (Cavadini et al., 2007) and that LPS-induced phase shifts are abolished in TNFα receptor-deficient (1/p55, Tnfr1) mice (Paladino et al., 2014). Collectively, these observations suggest that TNFα, IL-6 and other proinflammatory cytokines may be key elements in the signaling cascade by which HFD- and SFA-induced inflammation feeds back and modulates circadian properties of peripheral clocks.

How proinflammatory cytokines like TNFα and IL-6 modulate the timekeeping function of peripheral circadian clocks is unknown. Recent evidence suggests that key mediators of inflammation may directly interact with core clock components and regulate clock gene expression. NF-κB is a major transcription factor that governs downstream

cytokine expression as well as the regulation of core clock components. The regulatory NF-κB subunit, RelB, has been shown to physically interact with BMAL1 and repress CLOCK/BMAL1-mediated circadian transcription in fibroblasts (Bellet et al., 2012). Furthermore, NF-κB also directly interacts with CLOCK protein (Spengler et al., 2012). Based on these observations, it is possible that elevated NF-κB levels may lead to increased binding with BMAL1 or CLOCK, perhaps modulating the clock mechanism by sequestering available binding partners and inhibiting the formation of BMAL1-CLOCK heterodimers during TNFα- and IL-6-induced inflammation in the current study. The role of cytokines in mediating communication between the immune system and circadian clocks is supported by studies demonstrating that cytokines themselves directly interact and regulate molecular components of the clock mechanism in peripheral tissues. For example, TNFα treatment suppresses *Per1-3* expression by direct inhibition of CLOCK/BMAL1-driven E-Box transactivation in fibroblasts (Cavadini et al., 2007). In addition, IL-6 induces *Per1* gene expression in hepatoma cells (Motzkus et al., 2002). These findings have further implications for the role of proinflammatory cytokines in HFD- and SFA-mediated inflammatory feedback and its modulatory effects on the timekeeping function of peripheral clocks.

Although our current results demonstrate that the proinflammatory cytokines IL-6 and TNFα induced phase shifts of fibroblast *Bmal1-dLuc* rhythms in a time dependent manner, it is unclear why the directionality of TNFα-induced shifts was different from those induced by IL-6 and palmitate treatment. It is noteworthy that another study using the same concentration of palmitate treatment, but longer treatment duration, yielded

concentrations of approximately 0.6ng/ml IL-6 and 0.05ng/ml TNFα in peripheral cell culture medium (Ajuwon and Spurlock, 2005). Since the TNFα concentration in our experiments (20ng/ml) was much higher than physiological concentrations observed in palmitate-treated cultures, it is possible that the observed effect is pharmacological, producing an increase in circadian period rather than the expected phase advance like palmitate. This possibility is indirectly supported by our published findings that long-term palmitate treatment increased the period of *Bmal1-dLuc* rhythm, but acute treatment with this SFA induced mainly phase advances of the fibroblast clock (Kim et al., 2016). Similarly, chronic HFD treatment induced robust increases in period of peripheral clock gene rhythms (Xu et al., 2014), whereas short-term consumption of HFD advanced the phase of liver circadian rhythms (Pendergast et al., 2013; Xu et al., 2014). Thus, future studies are warranted to examine the dose-dependent effects of TNFα on inflammatory signaling and circadian timekeeping in peripheral clocks. In addition, it will be important to further determine whether blockade of proinflammatory cytokines, or NF-κB and other key proinflammatory signals, provides resistance against HFD-induced circadian dysregulation and in turn its exacerbating effect on metabolic disorders.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

Circadian clocks in many peripheral tissues/cells of mammals coordinate daily rhythms in inflammatory responses, nutrient metabolism and secretion of hormones such as insulin and leptin that mediate the regulation of immune and metabolic homeostasis in response to immune challenge or overnutrition. However, increasing evidence strongly indicates that clock regulation of inflammatory and metabolic processes in peripheral tissues is not strictly unidirectional because inflammatory signals and nutrient metabolism feed back on peripheral clocks and modulate fundamental circadian properties. Thus, this dissertation focuses on mutual interactions between circadian clocks and key mediators of inflammation, along with their role in in linking diet-associated circadian dysregulation and metabolic phenotypes.

Role of inflammatory signaling in circadian disruption- or desynchronization-induced metabolic disorders

HFD-induced obesity is associated with low-grade inflammation, which contributes to insulin resistance. Increasing evidence indicates that macrophages mediate diet-induced insulin resistance by stimulating proinflammatory signaling pathways in adipose tissue. Macrophage infiltration into adipose tissue is considered as the first step of chronic inflammation associated with HFD-mediated obesity. In conjunction with HFD-induced infiltration, adipose tissue macrophage polarization is shifted from an alternative anti-

inflammatory M2 to a proinflammatory M1 status. Proinflammatory M1 activation results in increased macrophage production of proinflammatory cytokines such as IL-6 and TNFα (Fujisaka et al., 2009), leading to the induction of TLR4-mediated proinflammatory responses in adipose tissue. HFD-induced stimulation of TLR4, which is a pattern recognition receptor for innate immune responses, triggers downstream inflammatory signaling pathways via NF-κB and JNK activation, again resulting in proinflammatory cytokine expression in adipose tissue. Furthermore, AMPK is also involved in diet-induced insulin resistance. In the presence of high cellular energy source such as HFD, the activity of this energy sensing enzyme is suppressed. Since AMPK inhibits NF-κB activation, the HFD-induced decrease in AMPK activity contributes to the pathogenesis of insulin resistance through the disinhibition of inflammation in diet-induced obesity (Salminen et al., 2011). Collectively, the elevated adipose tissue inflammation caused by macrophage proinflammatory activation in response to HFD mediates the development of metabolic disorders by interfering with insulin signaling and action.

The studies described in this dissertation indicate that proinflammatory macrophage activation and the corresponding induction of inflammatory signaling are key processes in the mechanism by which circadian disruption or desynchronization exacerbate HFD-mediated metabolic disorders (see below; Figure 29). Circadian desynchronization in response to chronic shifts of the LD cycle was observed to further amplify HFD-induced macrophage infiltration and M1 polarization in adipose tissue. Consistent with these observations, Xu et al. (2014) demonstrated that myeloid cell-specific disruption of the

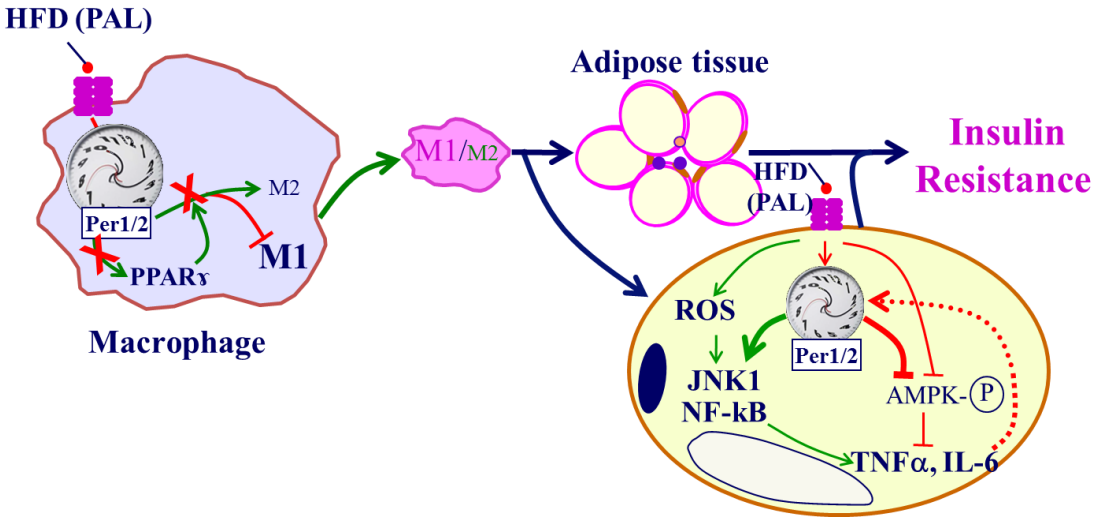

Figure 29: Model of mechanism by which circadian disruption or desynchronization exacerbates HFD- or palmitate-mediated metabolic disorders. Circadian disruption or desynchronization decreases PPARγ levels, thus amplifying diet-induced macrophage infiltration and M1 polarization in adipose tissue. Infiltrated M1 macrophages further potentiate HFD- or palmitate-mediated induction of NF-κB and JNK activity and decreased AMPK activity, resulting in TNFα and IL-6 expression. In turn, increased inflammation and fat deposition promotes systemic insulin resistance.

clock genes, *Per1* and *Per2*, promotes adipose tissue macrophage infiltration and polarization to the proinflammatory M1 phenotype. PPARγ, which is required to maintain anti-inflammatory M2 macrophage activation (Odegaard et al., 2007b), may play a key role in mediating this clock disruption-associated shift in macrophage inflammatory status because the enhanced proinflammatory activation of *Per1/2*-disrupted macrophages was accompanied by decreased PPARγ levels and was reversed, at least in part, by PPARγ overexpression. Because the PPARγ promoter contains multiple E-boxes (Herzig et al., 2003) and PPARγ is under circadian control in white adipose tissue (Yang et al., 2006), it is possible that circadian desynchronization in the present study may similarly amplify HFD-induced M1 macrophage activation by inhibiting PPARγ activity. Coupled with its amplification of M1 macrophage activation, circadian desynchronization potentiates inflammatory signaling in macrophages as exposure to chronic shifts in the LD cycle elevates LPS-stimulated IL-6, IL-1β and TNFα expression in macrophages from HFD-fed mice. Moreover, *Per1/2* disruption similarly increases LPS-stimulated JNK1 and NF-κB p65 phosphorylation and IL-1β and TNFα expression in adipose tissue macrophages (Xu et al., 2014). Further implications for inflammatory processes in circadian desynchronization/disruption-induced metabolic dysregulation are manifested by the findings that exposure to shifted LD cycles and myeloid cell-specific disruption of *Per1/2* potentiated HFD-induced inflammation and concomitantly exacerbated insulin resistance and glucose intolerance. Altogether, these observations provide strong evidence that further amplification of macrophage proinflammatory is a key process in the physiological

cascade by which circadian desynchronization and disruption exacerbate diet-induced metabolic dysfunction.

Role of HFD- and SFA-induced inflammation in the feedback modulation of circadian clock function

Recent studies suggest that key mediators of inflammation have a strong influence on circadian clock properties. In this regard, data presented in this dissertation provide some insight into how HFD- and SFA-induced inflammation feed back on circadian clocks and modulate their timekeeping function (Figure 30). HFD induced a substantial increase in the period of macrophage clock gene rhythms. Consistent with our observations, Shi et al. reported that HFD feeding similarly lengthened the period of the *Per2* rhythm in cultured adipose tissue (Shi et al., 2013b). This alteration of circadian clock properties may be caused by the activation of inflammatory signaling pathways because HFD-induced circadian dysregulation was associated with increased levels of LPS-stimulated JNK1 and NF-κB p65 phosphorylation, as well as increased IL-6 and TNFα expression in macrophages. The proinflammatory fatty acid palmitate, which is the most prevalent SFA in HFD, recapitulated the modulatory effects of HFD on fundamental properties of circadian clocks, whereas the anti-inflammatory fatty acid DHA had negligible effects on circadian period and phase. The opposing effects of proinflammatory versus anti-inflammatory fatty acids further suggest that inflammatory signaling pathways may mediate HFD- and SFA-induced circadian dysregulation in peripheral tissues.

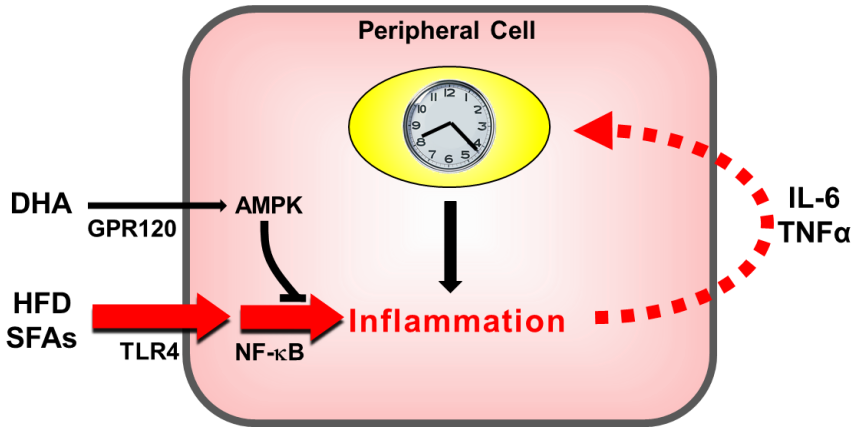

Figure 30: Model for the role of HFD- and SFA-induced inflammation in altering fundamental properties of peripheral circadian clocks. DHA blocks NF-κB-mediated inflammatory signaling pathway by activating AMPK. On the other hand, HFD and SFAs decrease AMPK activity and increase NF-κB activity, thus stimulating the production of proinflammatory cytokines (i.e. IL-6 and TNFα) in peripheral tissues. Increased IL-6 and TNFα levels feed back on circadian clock machinery and modulate its time keeping function.

Our study using acute palmitate treatment provides a better understanding how key components of inflammatory signaling pathways mediate HFD- and SFA-induced modulation of peripheral circadian clocks. Acute palmitate treatment induced time-dependent phase shifts of clock gene rhythms, and the timing of the maximal phase shifts coincided with peak induction of NF-κB phosphorylation and IL-6 expression in fibroblasts and adipocytes. Because pharmacological inhibition of NF-κB repressed palmitate-induced phase shifts in fibroblasts, NF-κB activation may be a key signal in the cascade underlying HFD- and SFA-mediated circadian dysregulation. Similar to our observations, the phase shifting effect of LPS on circadian rhythms of locomotor activity is suppressed by NF-κB inhibition (Marpegan et al., 2005). As transmembrane receptors responding to fatty acids (Oh and Walenta, 2014), the pattern recognition receptor TLR4 and GPR120 may be upstream elements of the mechanism by which palmitate-induced inflammatory signaling alters circadian properties of peripheral clocks. Because TLR4 is activated commonly by SFAs and its agonist LPS, leading to the induction of inflammatory signaling pathways via NF-κB (Wong et al., 2009), TLR4 may provide a critical mediator of palmitate-induced alterations of circadian clock function. Alternatively, it is possible that palmitate may decrease AMPK activity, leading to disinhibition of NF-κB, and in turn, to the alteration of circadian rhythms because AMPK activation was found to suppress palmitate-induced NF-κB activity and phase shifts. The inhibitory effect of DHA on palmitate-induced NF-κB activity and phase shifts further supports this possibility because DHA increases AMPK activity in macrophages via G-protein coupled receptor GPR120 (Xue et al., 2012).

Current evidence indicating that IL-6 treatment mimics the phase shifting effects of palmitate and scavenging TNFα using a neutralizing antibody attenuates palmitate-induced phase shifts, it is speculated that proinflammatory cytokines themselves may directly feed back on the clock mechanism during HFD- and SFA-induced inflammation. In conjunction with other studies demonstrating that TNFα phase shifts the circadian rhythms of locomotor activity *in vivo* and PER2 levels in cultured SCN astrocytes *in vitro* (Leone et al. 2012), our observation provides primary evidence that HFD- and SFA-induced inflammatory signaling via NF-κB and proinflammatory cytokines plays a critical role in the feedback modulation of circadian timekeeping in peripheral clocks.

Role of mutual interactions between inflammatory signaling and circadian clocks in diet-induced metabolic disorders

All data presented in this dissertation point to key mediators of inflammation and inflammatory signaling as the central hub linking diet-mediated circadian dysregulation and metabolic dysfunction (Figure 31). HFD and SFAs activate the NF-κB-mediated inflammatory signaling pathway directly or indirectly through inhibition of AMPK activity in peripheral tissues. The transcription factor NF-κB promotes proinflammatory responses by inducing the production of cytokines such as TNFα and IL-6, thus exacerbating diet-related insulin resistance and glucose intolerance. In parallel, NF-κB-mediated inflammatory responses modulated circadian clock properties through proinflammatory cytokines. Disrupted or desynchronized circadian rhythms amplified HFD- and SFA-induced macrophage M1 polarization and infiltration into adipose tissue,

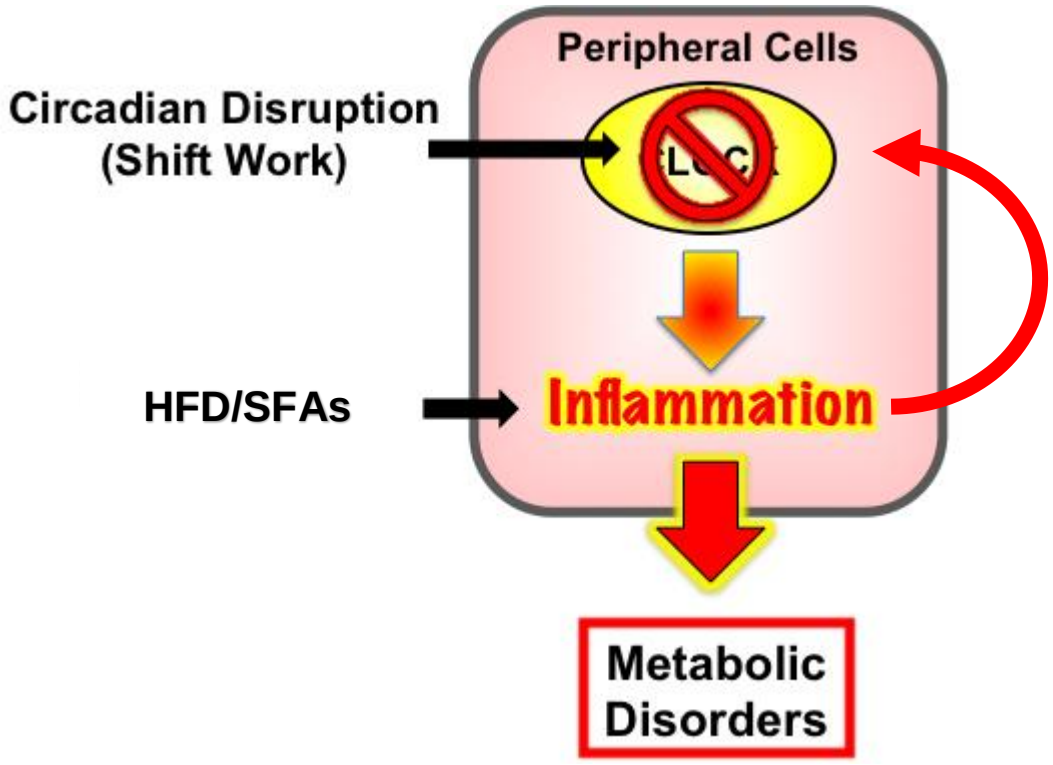

Figure 31: Model for the role of mutual interactions between inflammatory signaling and circadian clocks in diet-induced metabolic disorders. HFD- and SFAs-induced inflammation feed back on the clock mechanism and modulate fundamental properties of circadian rhythms in peripheral tissues. In turn, this circadian dysregulation further amplifies HFD- and SFAs-mediated inflammation, and exacerbates diet-induced systemic insulin resistance and glucose intolerance.

perhaps by repressing macrophage PPARγ expression, and then exacerbated NF-κB-mediated proinflammatory responses in adipose tissue. In turn, this dysregulation of circadian clock function exacerbates diet-induced insulin resistance. This amplification of HFD- and SFA-induced immune responses may exacerbate systemic insulin resistance and glucose intolerance, thus implicating circadian dysregulation in the pathogenesis of diet-induced metabolic disorders. Taken together, our study suggests that mutual interactions between inflammatory signaling pathways and circadian clocks may govern the effects of HFD and SFAs on metabolism.

Remaining Questions and Concluding Remarks

The studies in this dissertation have yielded novel evidence for the role of key mediators of inflammation in diet-induced circadian modulation and pathogenesis of metabolic disorders. We have shown that circadian desynchronization in response to shifted LD cycles was accompanied by amplification of diet-induced inflammatory responses and increased severity of insulin resistance and glucose intolerance. However, these observations are correlative in nature so it remains unclear whether circadian desynchronization-associated exacerbation of proinflammatory responses was directly responsible for the elevations in insulin resistance and glucose intolerance. To address this issue, it will be necessary to test the effects of various inflammatory inhibitors on metabolic phenotypes in mice exposed to chronic shifts of the LD cycle. The AMPK activator, AICAR, metformin which has been used to treat diabetes in humans, and the PUFA DHA are optimal for this analysis due to their well-established anti-inflammatory

effects. If amplified proinflammatory signaling directly mediates the effects of circadian dysregulation in increasing the severity of insulin resistance and glucose intolerance, then treatment with AICAR, metformin, DHA or other inhibitors of inflammatory responses should "rescue" metabolic function in animals on shifted LD cycles. Because PPARγ is a key upstream mediator of anti-inflammatory macrophage activation (Odegaard et al., 2007b) and myeloid cell-specific *Per1*/*Per2* disruption decreased macrophage PPARγ levels in conjunction with increased proinflammatory M1 activation and systemic insulin resistance (Xu et al., 2014), future studies are also warranted to examine the role of PPARγ in the signaling cascade by which circadian dysregulation exacerbates diet-induced macrophage infiltration and M1 activation, inflammatory signaling and metabolic phenotypes. If macrophage levels of PPARγ are suppressed in response to chronic shifts of the LD cycle similar to those observed in *Per1/2*-disrupted macrophages, then treatment with a PPARγ activator such as rosiglitazone should reverse the circadian dysregulation-associated increases in proinflammatory macrophage activation and cytokine production, thus ameliorating diet-induced metabolic alterations.

In Chapter II, IV and V, our experiments have yielded some insight into the mechanism by which HFD and SFAs modulate peripheral circadian clocks. Although the results of these studies directly implicate AMPK, NF-κB and cytokines in the HFD- and palmitate-induced modulation of peripheral circadian clocks, there are other critical components of inflammatory signaling pathways, such as TLR4, that also deserve some consideration. TLR4 is a transmembrane receptor that recognizes HFD and SFAs and initiates intracellular proinflammatory signaling cascades in peripheral tissues. Because

of its role in gating diet-induced inflammation as an upstream element of NF-κB, it would be interesting to test whether TLR4 inhibitors block the effects of HFD and palmitate on circadian clock properties (i.e., period or phase). Furthermore, G-protein coupled receptor GPR120 would be another interesting upstream target in future studies because it mediates the anti-inflammatory effect of DHA in macrophages via AMPK activation (Xue et al., 2012). Like a DHA treatment in our study, GPR120 activators (i.e., Grifolic acid and GSK 137647) would be expected to attenuate HFD- and palmitate-induced circadian dysfunctions. Similarly, the effect of PPARγ activators and inhibitors are other drugs to be tested in future studies because it regulates lipid metabolism and inflammatory responses in response to HFD and fatty acids. Based on the fact that PPARγ is a target of many anti-diabetic drugs due to its anti-inflammatory effects (Martin, 2010), a PPARγ inhibitor would be expected to mimic the modulatory effect of HFD and SFAs on circadian clocks, while a PPARγ activator would be expected to ameliorate HFD- and SFA-induced circadian dysregulation. Since PPARγ is under circadian control in adipose tissue (Yang et al., 2006), PPARγ inhibitors and activators would be expected to have time-dependent effects of circadian rhythms.

The research described here has established the feedback modulation of circadian clocks by HFD- and palmitate-induced inflammation, but one intriguing question that remains unanswered is how/why are these nutrient-related modulatory effects more robust on peripheral clocks than on the SCN clock? In our *in vivo* study, it is possible that HFD and palmitate had greater effects on peripheral clock oscillations than on SCN clock-regulated rhythms because HFD and palmitate are processed and metabolized by

peripheral tissues first. To investigate this possibility, it will be necessary to test the effects of palmitate microinjections into SCN on circadian period or phase of the activity rhythm. Since HFD- and SFA-induced macrophage inflammation is the key signaling pathway in feedback modulation of circadian clocks, it will need to be determined whether peripheral treatment of palmitate would have a greater effect on inflammatory responses in adipose tissue macrophages than on microglia (the resident macrophages in brain). Given the circadian control of immune responses, it would be also interesting to test whether peripheral or central treatment of palmitate alters the fundamental properties of circadian rhythms in microglia.

In summary, the research described here suggests that inflammatory mediators may play a key role in the underlying mechanism by which HFD and SFAs modulate fundamental properties of circadian clocks, and in turn this circadian dysregulation exacerbates diet-induced systemic insulin resistance and glucose intolerance. Our findings provide possible therapeutic targets for metabolic disorders during circadian disruption, or desynchronization (i.e., shift work), and serves as a foundation for further studies to examine interactions between metabolism, the circadian clock, and immune systems.

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