이학박사 학위논문

생체시계가 대사기능에 미치는 영향에 관한 연구

Studies on circadian clock regulation on metabolic functions

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노 한 상

Studies on circadian clock regulation on metabolic functions

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School of Biological Sciences
at
Seoul National University
by
Hansang Row

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2015년 12월

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ABSTRACT

Studies on circadian clock regulation on metabolic functions

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In mammals, the suprachiamatic nucleus (SCN) of the anterior hypothalamus acts as master circadian rhythm generator, which regulates the molecular organizations of clock oscillations residing in peripheral organs. Molecular circadian clock organization have evolved to adapt day and night environmental changes and to regulate daily physiological occurrences. And one of the important roles of circadian clock is to control daily metabolism in molecular and in systemic levels.

5'-AMP activated protein kinase, AMPK, is known as the key cellular energy sensor, which interacts with circadian clock to maintain cellular energetic status. AMPK is involved with various metabolic functions, such as glycolysis, gluconeogenesis and lipid metabolism. Therefore, AMPK has been a key target for developing therapeutic agents

to treat metabolic disorders. Metformin is known as the first-in class therapeutic choice for treatment of type 2 diabetes. It has been used since early 1950s, and growing mechanistic studies have identified AMPK as key modulating factor by metformin to decrease hyperglycemia and increase insulin sensitivity. Here, following the previous results of metformin's effects on circadian oscillation, I have investigated effects metformin-derived novel chemical compound, H271, on circadian clock. Compared to metformin, I was able to find HL271 was much more potent on AMPK phosphorylation. Under ~1000x lower dosage concentration, HL271 displayed similar molecular effects as compared to metformin. HL271, also modulated molecular circadian clock by increasing degradation rates of PER2 and CYR1 and shortening of Per2 expression dose-dependently. Interestingly, HL271 did not appear to lower blood glucose level as compared to metformin, and the difference of physiological outcome demonstrated that HL271 may act differently in systemic level.

Omega-3 fatty acids (FA) are known as essential fatty acids, which mammals require for normal development. Although omega-3 FA is known to elicit health benefits such as improved inflammatory system and, retinal- and neural development, there are paucity of studies regarding the effects of omega-3 FA on circadian clock.

By utilizing a unique omega-3 FA animal model, *fat-1* transgenic

mouse, which converts omega-6 FA to omega-3 FA endogenously, I have investigated effects of omega-3 FA on circadian oscillation. By cross-breeding *fat-1* TG mouse with Per2::luc KI mouse and created *fat-1*/Per2::luc mouse. *Fat*-1/Per2:luc mouse displayed enhanced Per2 expression in the peripheral tissues, including liver, kidney and submandibular gland. In *Fat-1* TG mouse, clock mRNA levels were elevated in liver and kidney, however, clock gene expression in the hypothalamus only displayed shifting of period. Molecular analysis revealed that in *Fat-1* TG mouse, the core clock gene, BMAL1 was highly sumoylated and ubiquitinated eliciting enhanced proteosomal-mediated degradation and protein turnover. In accordance with the alteration of clock oscillation in the SCN, *Fat-1* TG mouse displayed elongated period during free-running activities in constant darkness (DD) condition. Therefore in the present study, I investigated effects of omega-3 FA on circadian clock system and provided evidence for role of omega-3 FA on molecular circadian clock oscillation, and illustrated possible benefits of omega-3 FA influencing pronounced circadian oscillation which may yield longer life.

***Key words*:** Circadian rhythm, metformin, HL271, Omega-3, Fat-1

***Student number*:** 2010-20313

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BACKGROUND AND PURPOSE

BACKGROUND

1. Mammalian circadian clock

1-1. Circadian rhythm

We live on a planet that is governed by the diurnal cycle, which is caused by rotation of the Earth consisting about 24-hours. This led to the evolution of internal biological clocks virtually in all living organisms to anticipate the changes in the environment (Lowery and Takahashi, 2004). The term “circadian” comes from the Latin *circa*, “around”, and *diem* or *dies,* “day”, meaning literally “approximately one day”. Daily events ranging from sleep/wake cycle in humans to photosynthesis in plants represents the evolutionarily conserved adaptation to the environment that can be traced back to early life forms. The principal factor for synchronization between the environment and the internal rhythm is light. Light is a strong zeitgeber, which is a synchronizing agent capable of resetting a pacemaker or synchronizing a self-sustaining oscillation. For example, under conditions of steady environment, the period of the self-sustaining oscillation conforms to that of the zeitgeber. Another features of rhythmic synchronization from environment is through temperature

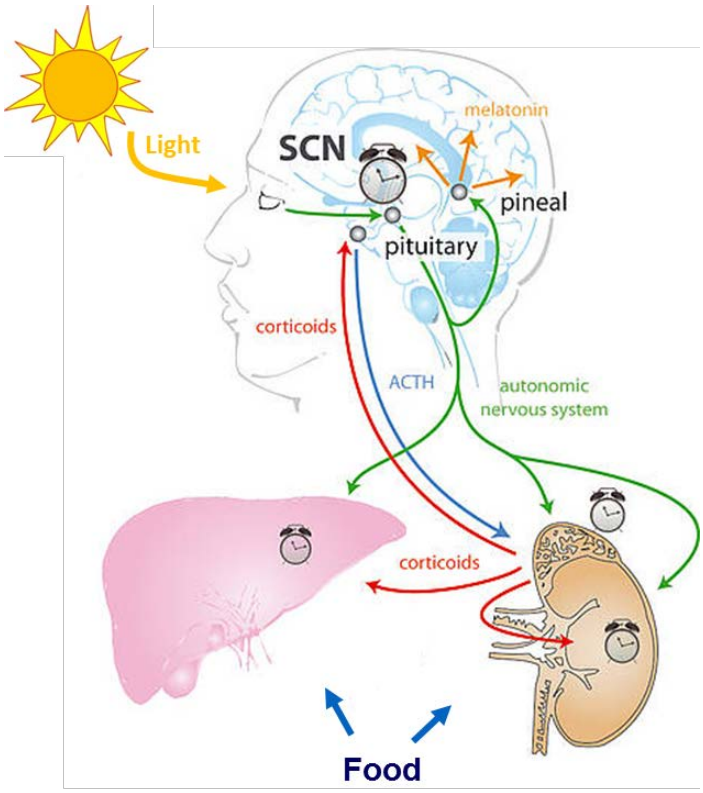
compensation. Most of the biological systems in living organisms relies on the temperature, as the temporal oscillation of circadian rhythm is almost the same over a broad range of physiological temperature (Viaterna et al., 2001).

1-2. Hierarchical organization of circadian clock system

In mammals, light is mediated by special photo-receptors in our eyes in cells that are known as intrinsically photo-sensitive receptor ganglion cells (ipRGC). Photoresponse in the ipRGC integrate light information through retino-hypothalamic tract (RHT) and reaches suprachiasmatic nucleus (SCN) located in anterior hypothalamus and situated directly above the optic chiasm. SCN has been identified as the dominant circadian pacemaker responsible for regulating circadian rhythm. It is composed of over ~20,000 neurons, each of which is thought to contain a cell-autonomous circadian oscillator generating dynamic spatial and temporal coordination upon synchronization (Yamaguchi et al., 2003). Rhythmic oscillation have been observed in cells and tissues throughout the body in mammals, and these rhythms persist in *in vitro* culture, demonstrating that non-SCN cells also contain endogenous circadian oscillators (Balsalobre et al., 1998, Yamazaki et al., 2000). Therefore, for biological clocks to be effective and functional in an

organized circadian system, this requires SCN control of peripheral oscillators (Figure 1).

Figure 1. Hierarchical organization of circadian clock system. The master pacemaker encoding the mammalian clock resides within the SCN. Clock genes are expressed in other regions of the brain and in most of the peripheral tissues. Peripheral clocks are synchronized through humoral, nutrient, and autonomic wiring and that the cell-autonomous function of the clock is important in pathways involved in fuel storage and consumption. A hierarchical model indicates that all peripheral clocks are subordinate to the SCN.


Light
SCN
melatonin
pineal
pituitary
corticoids
ACTH
autonomic
nervous system
corticoids
Food
Activity
Body temp

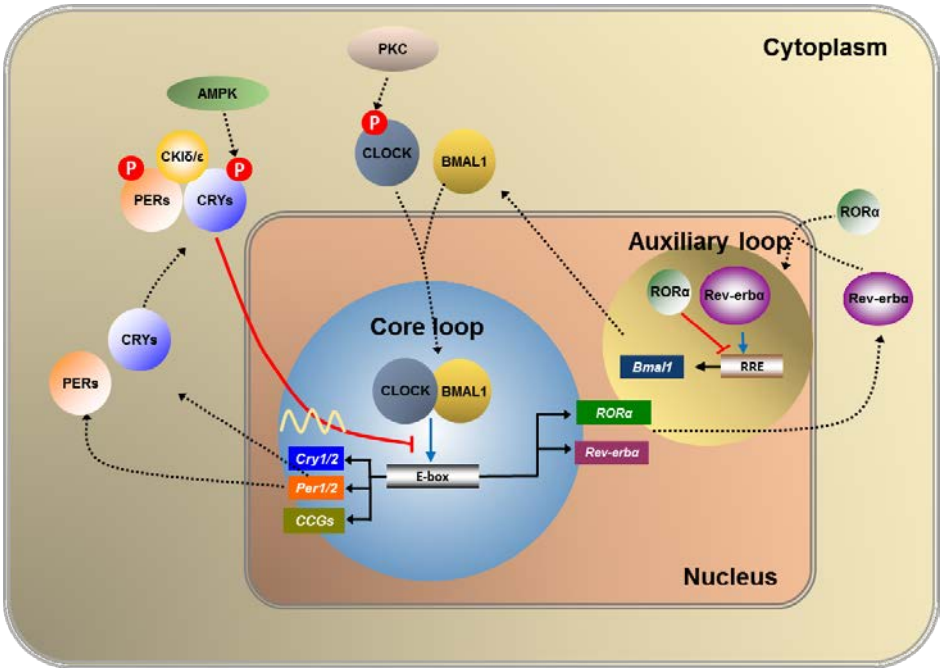
1-3. Coupling of central and peripheral clocks

The SCN controls peripheral clock oscillation through both sympathetic and parasympathetic pathways (Kalsbeek et al., 2010). Sympathetic innervation from the SCN to the liver results in daily rhythms of plasma glucose by direct influence of the rhythms of hepatic gluconeogenesis (Kalsbeek et al., 2004). Autonomic pathways from the SCN influence oscillators in the adrenal gland and liver, which sympathetic innervation modulates the sensitivity of the adrenal gland to adrenocorticotropic hormone (ACTH) and influencing glucocorticoid (GC) release (Buijs et al., 1999, Kalsbeek et al., 2010). GC is considered as a strong humoral entraining signals, since level of GC oscillates in the circadian manner and reaches almost all parts of the body. Independent of the SCN, local signaling pathways can also affect peripheral oscillators, such as body temperature, circulating GC and feeding-related cues (Albrecht, 2010). Body temperature can reset the circadian rhythm of liver, kidney, lung and fibroblasts (Abraham et al., 2010, Buhr et al., 2010). And also, food-related signals are able to produce circadian patterns and the change of feeding/fasting schedule can uncouple the phase relationship between the SCN and peripheral clocks, suggesting that changes in metabolic signaling can influence systemic regulation of circadian clock (Albrecht, 2010).

1-4. Molecular mechanism of the mammalian circadian clock

The cell-autonomous driven circadian rhythm arise from an autoregulatory negative-feedback transcriptional and translational network (Lowery and Takahashi, 2004). Two interlocking feedback loops generate molecular mechanism of the circadian clock in mammals, consisting of core loop involving the activators, Circadian Locomoter Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like protein 1 (BMAL1) and their target gene Period (Per) 1-3, Cryptochrome (Cry) 1-2, Rev-erbα and RAR-related orphan receptor α (RORα) whose gene products form a negative-feedback repressor complex. In addition to core transcriptional feedback loop, other feedback loop involving REV-ERBα and RORα that represses Bmal1 transcriptional leading an antiphase oscillation in Bmal1 gene expression stabilizing the circadian oscillation (Gallega and Virshup, 2007). Therefore, the ubiquitous, cell-autonomic clocks in central and peripheral oscillators carry similar mechanisms although they function in different cellular contexts (Figure 2).

Figure 2. Molecular bases of circadian system. The clock mechanism consists of two main parts: (1) a transcriptional-translational feedback loop (TTL) consisting of a positive and a negative limb and (2) oscillating post-translational modification of gene products in the TTL, which regulate degradation and nuclear localization of these proteins. The two transcription factors, CLOCK and BMAL1, bind to E-box on the promoter of clock-controlled genes, including Pers, Crys, Rev-erbα and Rorα. Translated PER and CRY proteins are post-translationally modified, accumulate in the cytoplasm and, then. Translocate to the nucleus to repress CLOCK:BMAL1 transcription activity. On the other hand, Rev-erbα and Rorα regulates Bmal1 expressing through the binding of RRE in Bmal1 promoter.


Cytoplasm
PKC
AMPK
CKIδ/ε
P
PERs
CRYs
CLOCK
BMAL1
RORα
Auxiliary loop
Rev-erbα
Core loop
Bmal1
RRE
E-box
Cry1/2
Per1/2
CCGs
Nucleus

2. Circadian clock and metabolism

2-1. Congruent relations between circadian rhythm and metabolism

The circadian system orchestrates the temporal organization of many aspects of physiological events, including metabolism. Circadian regulation of metabolic homeostasis is intimately linked and disruption of circadian rhythms can contribute to many metabolic diseases (Green et al., 2008). The importance of circadian clock function in metabolic regulation in the periphery has been observed in mice genetic models. Bmal1 knock-out (KO) mice, *Bmal1*$^{-/-}$, exhibit complete loss of rhythmicity causing abolished oscillations in plasma glucose and triglyceride (Rudic et al., 2004). In addition, tissue-specific deletion of *Bmal1* in liver or pancreas display altered blood glucose level resulting hypoglycemia during the fasting phase of the feeding cycle and eventually leads to diabetes (Marcheava et al., 2010, Sadacca et al., (2005). *Cry1*$^{-/-}$*Cry2*$^{-/-}$ KO mice display salt-sensitive hypertension due to elevated plasma aldosterone and also hyperglycemia eventually leads to diabetes mellitus. (Doi et al., 2010). Mutations in *Per2* results in altered lipid metabolism and lower body weight and appears to show increased urinary sodium excretion (Grimaldi et al., 2010).

2-2. Clock control of cellular metabolic process

The timing of metabolism can be influenced by the systemic cues of circadian system arising from the SCN or through local clocks. Temporal regulation of various metabolic pathways are regulated by circadian clock system. This regulation is achieved by controlling the expression of enzymes controlling the expression of rate-limiting steps of a metabolic pathway and by integrating proteins such as nutrient sensor with the clock machinery (Sahar and Sassone-Corsi, 2012). Recent development of genome-wide and microarray studies, it has been estimated that ~22-30% of the transcriptome has been shown to be under circadian regulation (Panda et al., 2002; Storch et al., 2002). Among the rhythmic genes identified, many carry a role in metabolic processes, including lipid metabolism, glycolysis and gluconeogenesis, oxidative phosphorylation and detoxification pathways (Green et al., 2008). Mostly, feedback system represented the communication between circadian clock and metabolism including AMP/ATP ratio sensors, glucose and fatty acid sensors and redox sensors.

2-3. Interplay between regulators of circadian clock and metabolism

The communication between circadian clock and metabolism, sensors affecting both systems include redox sensors, AMP/ATP ratio sensors, glucose and fatty acid sensors. Circadian clock controls the expression of NAD^+ salvage pathway by controlling the expression of nicotinamide phosphoribosytransferase (NAMPT), a key rate-limiting enzyme in the NAD^+ biosynthesis (Nakahata et al., 2009). And inhibition of NAMPT promotes oscillation of clock gene *Per2* by releasing CLOCK:BMAL1 from suppression of nutrient sensor, Sirtuin 1 (SIRT1). In turn, the circadian transcription factor CLOCK binds to upregulate Nampt, thus completing a feedback look involving NAMPT/NAD^+ (Nahakata et a., 2009; Ramsey et al., 2009).

Glucose homeostasis involves GC and its receptors. Application of high glucose concentration to fibroblast cell culture leads to transcriptional repression of *Per1, Per2,* and *Bmal1* genes leading synchronization of fibroblast clocks (Hirota et al., 2002). A recent study has demonstrated that Cry family interacts with GR and modulates glucose signaling by reducing GR activation potential for the expression of the phosphoenolpyruvate caboxykinase 1 (Pck1) gene which encodes rate-limiting enzyme in gluconeogenesis, PEPCK.

One measure of cellular metabolic status is the ratio between AMP and ATP. A major sensor for the AMP/ATP ration is adenosine monophosphate-dependent protein kinase (AMPK), which is activated

when AMP/ATP ratio is high during low cellular energy status (Carling et al., 2011). The activity of AMPK is involved in various circadian clock mechanisms. Once activated, AMPK directly phosphorylates CRY1, leading to destabilization and degradation by ubiquitin ligase, F-box leucine-rich-repeat protein 3 (FBXL3) (Lamia et al., 2009). In addition, AMPK phosphorylates of casein kinase 1ε (CK1ε) modulating PER2 protein stability leading accelerated degradation (Um et al., 2007). Activation of AMPK also leads to increase of NAD^+ level possibly modulating circadian gene expression indirectly through SIRT1 activation (Frescas et al., 2005).

2-4. Circadian disturbance and metabolic diseases

Many aspects of metabolic physiology are known to occur at specific times each day, suggesting circadian disruption may lead to augmentation and progression of metabolic syndrome. Circadian disruption can be caused by rotating shift-work, frequent transmeridian flights and constant stress leading to de-synchronization of the various body clocks (Albrecht, 2012). Many clinical and epidemiological provides an evidence that confounding factors of circadian disruption is associated with obesity, diabetes, cardiovascular complications and neurological disorders (Laposky et al., 2007). Shift-work and sleep deprivation leads

to dampen rhythms in growth hormone and melatonin, reduce insulin sensitivity, and elevates circulating cholesterol (Spiegel et al., 2009). And these changes eventually leads to weight gain, obesity and development of metabolic syndromes. Chronic shift-work can also favor the development of mood disorders due to a misalignment of rhythms in body temperature, melatonin and sleep (Hasler et al., 2010). This association has been demonstrated in *Clock* gene mutated mouse model. These animal displayed metabolic problems and obesity and behavior comparable to bipolar disorder patients, such as hyperactivity and mania-like behavior (Roybal et al., 2007). Jet lag is commonly caused by frequent transmeridian flights. Constant experience of jet lag induces de-synchronization of circadian rhythm between SCN and the peripheral clocks inducing symptoms like, sleep disturbance, daytime sleepiness, decrease cognitive skills, indigestion, nausea and performance impairment (Cho et al., 2000). These symptoms are associated with accelerated cardiomyopathy and premature mortality and also neuroendocrine control of appetite and glucose control (Figure 3) (Spiegel et al., 2004. Taheri et al., 2004).

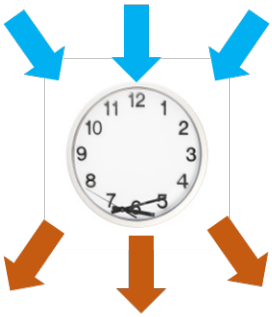
Figure 3. Circadian clock and diseases. Many aspects of metabolic physiology are known to occur at specific time of the day. Circadian disruption can be arise by continuous irregular lifestyle, constant jetlag and shiftwork leading sleep disorder, mood disorder and metabolic syndrome. This suggests a potential link between disruption of synchrony between periods of rest/activity with feeding/fasting and energy storage/utilization may be tied to dysregulation of not only metabolism and also physiological homeostasis.

❖ **Daily lifestyle**

Irregular lifestyle

Constant jet-lag

Shift work



❖ **Diseases**

Sleep disorder

Mood disorder

Metabolic syndrome

3. Fatty acids

3-1. Types of fatty acids

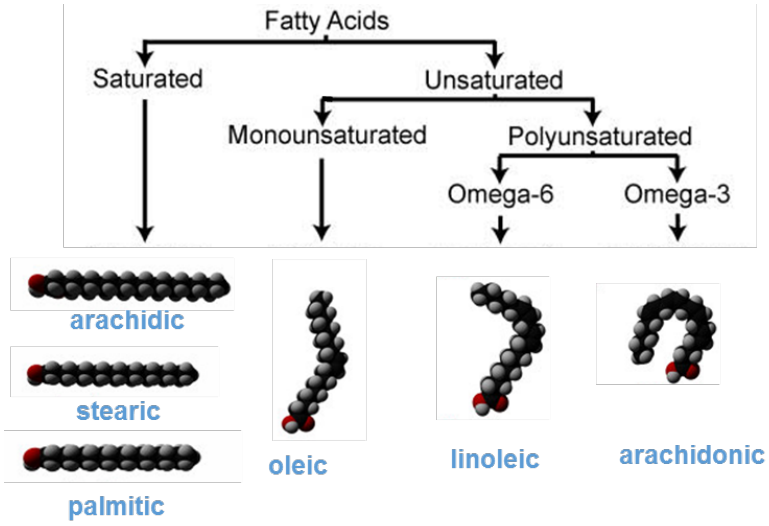
Fatty acids (FAs) are long-chain hydrocarbon molecules containing a carboxylic acid. FAs are usually derived from triglycerides or phospholipids and when they are not attached to other molecules, they are known as “free fatty acids”. Depending on the types of carbon-carbon bond, FAs can be classified as either saturated or unsaturated. FAs that contain no carbon-carbon double bonds are termed as saturated fatty acids (SFAs) and fatty acids carrying carbon-carbon double bonds are known as unsaturated and fatty (UFAs) acids with multiple sites of unsaturation are termed polyunsaturated fatty acids (PUFAs). The number of carbon atoms followed by the number of sites of carbon-carbon double bond designates the numeric FAs. FAs carry important functions in our body serving as building blocks of cellular membrane, source of fuel and act as a second messenger to maintain physiological homeostasis. In class of PUFAs, essential fatty acids are required by the human body development but cannot be made from other substrates, and therefore must be obtained from diet. Mammals including humans lack the ability to introduce double bonds in fatty acids beyond carbon 9 and 10 from the carboxylic acid, hence Omega-6 linolenic acid (LA) and the omega-3 α-linolenic acid (ALA) are essential for humans in the diet. In

addition longer-chain omega-3 FAs LA and ALA converts into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are, which conversion is limited in humans. The main physiological requirement for omega-6 and omega-3 FAs is associated with arachidonic acid. Presence of arachidonic acid play a vital role in cell signaling by acting as a major precursor of prostaglandins, leukotrienes, and anandamides. (Sanders and Emery, 2003 *from wiki*). Dietary source of omega-3 and omega-6 can be found in fish, flaxseed, hemp seed, soya oil, canola oil, sunflower seed and walnuts. In turn, fish is the main source of the omega-3 EPA and DHA which has been shown to provide many health benefits (Figure 4).

3-2. Functions of fatty acids

Fat has traditionally been regarded as important source fuel, nutrients and source of essential fatty acids. FAs have been increasingly recognized as major biological regulators and varying intake of dietary FAs leads to changes in cell membrane structure and functions. In addition, FAs have differential effects on the production of cytokines, chemotaxis, and other factors relating to the immunological response (Deckelbaum et al., 2006). Some important biological functions of FAs includes, modulating pathways of blood coagulation, influence sterol metabolism, signal transduction, enzyme activities, cell proliferation and

Figure 4. Types of fatty acids. Fatty acids are made up of carbon and hydrogen molecules. There are three types of fatty acids: saturated, monounsaturated and polyunsaturated. The basic difference between each of these are the number of carbon atoms or without two hydrogen atoms bonded to them. In a saturated fatty acids, each carbon atom has bonded with two hydrogen atoms. In a monounsaturated fatty acids, on pair of carbon atoms forms a double bond with each other that replaces the bon each would have with one hydrogen atom. A polyunsaturated fatty acid has two or more carbon pairs that have bonded together rather than with a hydrogen atoms.


Fatty Acids
Saturated
Unsaturated
Monounsaturated
Polyunsaturated
Omega-6
Omega-3
arachidic
stearic
palmitic
oleic
linoleic
arachidonic

receptor expression (Clandinin et al., 1991). Therefore, many of the effects of FAs in both cell biology and human health and disease relate to their abilities to regulate gene expression and subsequent downstream events.

3-3. Benefits of omega-3 fatty acids

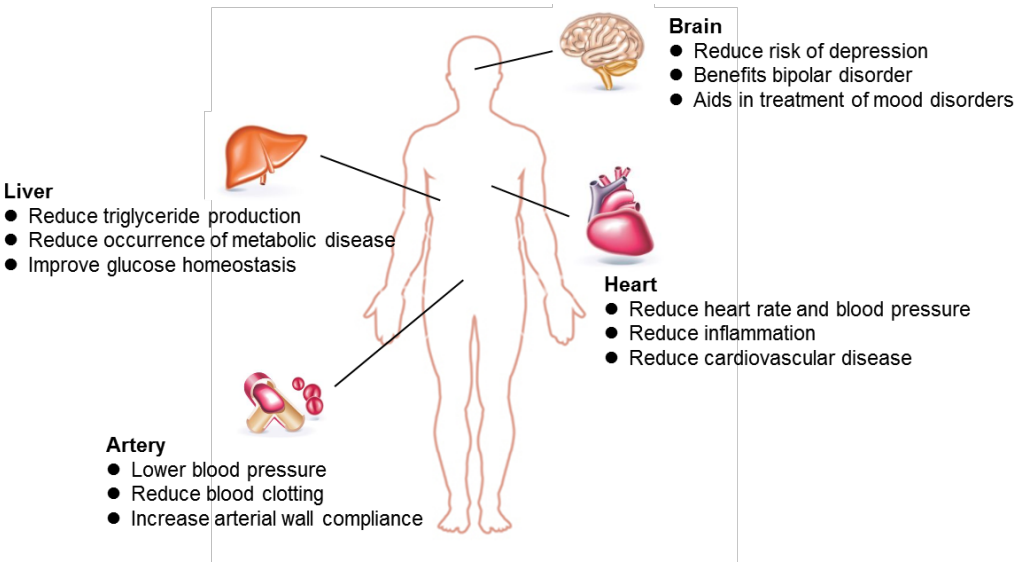
Increasing clinical and epidemiological studies demonstrates that increased omega-3 FAs consumption ameliorates or decrease the risk of a variety of diseases. Omega-3 FAs have shown definite roles in cognitive development and learning, visual function, the immune-inflammatory response, pregnancy, cardiovascular disease and cancer (Seo et al., 2005). Distinct capacities of omega-3 FAs appears to modulate both cellular metabolic functions and gene expression. Their longer chain length, high number of double bonds and the presence of the first double bond gives omega-3 FAs distinct and unique properties compare to common omega-6 and omega-9. Overall, the positive effects of omega-3 FAs on health relates to inhibition of eicosanoid pathways, leading alteration of inflammatory response, modulation of molecules or enzymes associated with various signaling pathways involving normal and pathological cell function and direct effects of gene expression (Seo et al., 2005).

Because of the abilities of omega-3 to modulate metabolic pathways and suppress the gene expression related to lipid metabolism, it is being considered as therapeutic agents in dyslipidemia, various metabolic syndromes, type 2 diabetes and steatohepatitis (Deckelbaum et al., 2006). Omega-3 FAs, in particular EPA and DHA, have been shown to have similar regulatory effects as glitazones, a member of the thiazolidinedione drugs, on a large number of related pathways. Similar to glitazones, omega-3 FAs act as potent agonist for peroxisome proliferator-activated receptor γ (PPARγ) and have been used for treatment of a variety of lipid-related disorders. In addition, omega-3 FAs suppress large number of inflammatory proteins and in enhancing gene expression and activity of a large number of transcription factors that would help to ameliorate adverse pathways related to obesity, metabolic syndromes and type 2 diabetes. Dietary consumption of omega-3 FAs have shown to reduce risk of cardiovascular diseases by diminishing key contributors (Figure 5) (Li et al., 2004).

3-4. Fat-1 transgenic mice: a novel animal model for omega-3 research

Dietary supplementation has been principal method to modify tissue nutrient composition in animal studies. However, feeding animals with different types of diets that consist of many components can bring in

Figure 5. Benefits of omega-3 fatty acids. Not only omega-3 is required for proper body development and functions, but also they deliver large health benefits. Omega-3 can lower elevated triglyceride levels and decrease risk of heart disease. And also provides beneficial effect on bipolar disorder and reduce mania-like behavior and depression. In addition, several lines of evidence provides omega-3 can reduce the incidence of sudden cardiac arrest, and reduce the total mortality and sudden death in patients with coronary heart disease.


Brain
Reduce risk of depression
Benefits bipolar disorder
Aids in treatment of mood disorders
Liver
Reduce triglyceride production
Reduce occurrence of metabolic disease
Improve glucose homeostasis
Heart
Reduce heart rate and blood pressure
Reduce inflammation
Reduce cardiovascular disease
Artery
Lower blood pressure
Reduce blood clotting
Increase arterial wall compliance

many confounding factors between experimental groups. Although the total energy can be examined with precise measurement of dietary intake, it is difficult to make all the dietary supplements to be identical between the diets. For example, currently there are no pure omega-3 and omega-6 FAs available for animal diet. Generally, diet consisting of increased amounts of fish oil, plant seeds and vegetable oils have been used to provide omega-3 and omega-6 FAs. However, theses FAs are derived from different natural sources and may contain other unwanted bioactive compounds that may affect functions of omega-3 and omega-6. Therefore, inevitable differences between the diets act as confounding factors in nutritional studies and may contribute to inconsistent or conflicting results.

To overcome this experimental dilemma, groups of scientists have generated a transgenic (TG) mouse capable of converting omega-6 to omega-3 FAs. Mammals cannot convert omega-6 to omega-3 FAs because they lack the enzyme responsible for this conversion. However, by inserting *fat-1* gene from *Caenohabditis elegans*, which encodes an omega-3 fatty acid desaturase that can introduce double bond into omega-6 FAs, by microinjection into fertilized eggs led to creation of novel transgenic mouse lines (Kang et al., 2004). As result, *fat-1* TG mouse were capable of producing omega-3 FAs from omega-6 FAs, resulting in enrichment of omega-3 FAs in virtually all organs and tissues without the need of dietary omega-3 supplementation (Kang et al., 2004). Therefore,

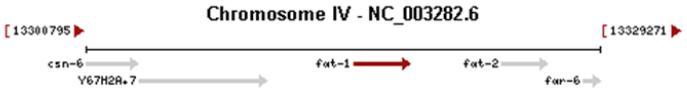
the use of the *fat-1* TG mice allowed scientists to overcome complications of conventional diet supplementation and concede with well-controlled studies (Figure 6).

3-5. Interaction between fatty acids and circadian clock

Fatty acid (FA) metabolism is influenced by daily fluctuations in activity and nutritional studies where during periods of intermittent fasting leads to increased FAs oxidation rates. The whole body FA metabolism can be compromised by manipulation of dietary nutrients quantities and qualities as well as timing of the meals throughout the 24 h period (Bray and Young, 2011). Observation of the time-dependent oscillation of FA metabolism have led to external environmental influences principally drive daily rhythms of FA metabolism by associated oscillations in circulating nutrients and metabolically relevant neural and hormonal inputs. Recent studies have demonstrated that mice fed with high fat diet (HFD) developed disrupted behavior circadian rhythms (Kohsaka et al., 2007). Mice subjected to HFD led to altered rhythms in core clock gene transcript expression and in clock target genes involved in metabolic homeostasis by delaying circadian expression of adiponectin signaling in the liver (Barnea et al., 2009). In addition, female mice fed with HFD for 6 wks did not show increased diurnal food intake, but did developed obesity, hyperlipidemia and hyperglycemia (Yanagihara et al.,2006). Overall,

feeding mice with high-fat diet could lead to disruptions in normal circadian patterns of behavior and lipid and glucose homeostasis.

Figure 6. Generation of *fat-1* TG mice. The *fat-1* gene of *C. elegans* encodes an *n-3* fatty-acid desaturase enzyme that converts omega-6 to omega-3 and which is absent in most animals, including mammals. Modified *Fat-1* gene encoding this protein by optimization of codon usage for mammalian cells and coupled to chicken beta-actin promoter. The expression vector is microinjected into fertilized eggs to produce transgenic mouse line.



n-3 desturase located in chromosome IV

Fat-1 gene from *C.elegans*

Chicken β-actin promoter + *fat-1* gene
microinjection to fertilized eggs

Fat-1 transgenic mice

PURPOSE

With rising population of patients holding metabolic diseases, such as obesity and diabetes mellitus, it is becoming an epidemic issue worldwide. Since, circadian clock and metabolic system is tightly integrated systematically, the manipulation of circadian clock oscillation has been a new way of approach to treat metabolic diseases. In this regards, the present study aims to elucidate circadian clock regulation of metabolism as following:

1. To investigate effects of metformin, the first-line of drug for type 2 diabetes treatment, and metformin-derived novel chemical compound, HL271 on molecular oscillation of circadian clock and clock-controlled metabolic regulator.
2. To investigate the effects of omega-3 fatty acids on circadian oscillation by utilizing novel transgenic animal model, *fat-1* TG mouse.

These investigations will be addressed in detail in the following chapters.

CHAPTER 1.

Effects of metformin and metformin-derived novel chemical compound, HL271 on circadian rhythm

ABSTRACT

Self-sustaining circadian clock regulates daily physiological and metabolic events. Cell-autonomous clocks regulate metabolism by interacting with various factors responsible for metabolic homeostasis. For example, 5'-AMP activated protein kinase, AMPK, is known as the key cellular energy sensor, which interacts with circadian clock to maintain cellular energetic status. AMPK is involved with various metabolic functions, such as glycolysis, gluconeogenesis and lipid metabolism. Therefore, AMPK has been a key target for developing therapeutic agents to treat metabolic disorders. Metformin is known as the first-in class therapeutic choice for treatment of type 2 diabetes. It has been used since early 1950s, and growing mechanistic studies have identified AMPK as key modulating factor by metformin to decrease hyperglycemia and increase insulin sensitivity. Here, following the previous results of metformin's effects on circadian oscillation, I have investigated effects metformin-derived novel chemical compound, H271, on circadian clock. Compared to metformin, I was able to find HL271 was much more potent on AMPK phosphorylation. Under ~1000x lower dosage concentration, HL271 displayed similar molecular effects as compare to metformin. HL271, also modulated molecular circadian clock by increasing degradation rates of PER2 and CYR1 and shortening of Per2 expression

dose-dependently. Interestingly, HL271 did not appeared to lower blood glucose level as compared to metformin, and the difference of physiological outcome demonstrated that HL271 may act differently in systemic level.

Key words: circadian rhythm, AMPK, metformin, Hl271

INTRODUCTION

Cell-autonomous endogenous circadian clocks drive daily physiological and behavioral rhythms with a period of about 24 h (Reppert and Weaver, 2002). The SCN is the master circadian clock that synchronizes intrinsic clocks located throughout the peripheral organs of mammals (Froy, 2010). The timing of circadian clocks in peripheral organs can be reset by external cues, such as food intake and activity (Schibler, 2005), indicating that metabolic cues can provide important feedback that organizes peripheral clock timing.

Molecular circadian clocks in mammals are driven by transcriptional-translational feedback loops, in which heterodimers of the proteins circadian locomoter output cycle kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1) initiate the transcription of clock-controlled genes such as periods (Per1, Per2, and Per3), cryptochromes (Cry1 and Cry2), Rev-erbα, and RORα. The competitive binding of Rev-erbα and RORα to a ROR responsive element (RORE) transcriptionally fine-tunes the circadian clock oscillation of Bmal1 (Yagita et al., 2001; Mohawk et al., 2012).

5’-Adenosine monophosphate (AMP)-activated kinase (AMPK) is a primary sensor of cellular energy and nutrient status in eukaryotic cells (Hardie, 2014). This enzyme generally senses changes in the AMP:ATP

ratio and is activated under conditions of low cellular energy. The kinase activity at the Thr172 phosphorylation site of the AMPK-α1 catalytic subunit phosphorylates downstream factors, switching the cell from a catabolic to an anabolic state and generating ATP (Hardie, 2014). AMPK activity is also involved in the molecular machinery of the cell's circadian clock. Activation of AMPK was shown to regulate the direct phosphorylation of CRY1 at the Ser71 residue and partly at Ser280, leading to degradation by F-box and leucine-rich repeat protein 3 (FBXL3) (Lamia et al., 2009; Jordan et al., 2013). In addition, activated AMPK phosphorylates casein kinase I ε (CKIε) to promote the proteosomal-dependent degradation of PER2 protein (Um et al., 2007). Thus, AMPK has been known to be a key link between circadian rhythm and cellular energy metabolism.

Metformin, one of the most widely prescribed drugs to treat patients with type 2 diabetes, was shown to phosphorylate AMPK in primary hepatocytes, lowering serum glucose concentration partly by reducing net hepatic glucose production (Zhou et al., 2001). Pharmacologically, metformin was found to inhibit the electron transport chain in mitochondria, specifically inhibiting complex I and increasing the AMP:ATP ratio, thus further activating AMPK (Fortez et al., 2014; Viollet et al., 2012). Metformin was shown to have tissue-specific effects on metabolic processes of circadian clocks (Barnea et al., 2012), but its

detailed physiological and molecular effects on these processes are still not well-understood. Although AMPK is activated by a wide range of physiological concentrations of metformin (He et al., 2015), the associations between the effects of metformin on the circadian clock and AMPK activation and its glucose-lowering effects remain unclear.

Based on the biguanide structure of metformin, Hanall Biopharmaceuticals recently developed HL271 as a next-generation anti-hyperglycemic agent (Figure 7). In present study, I investigated the effects of HL271 with those of metformin on the circadian clock and the metabolic system. From the investigation, I demonstrate that HL271 affected AMPK phosphorylation, circadian clock oscillations and clock expression, and degradation similar to metformin, but much potently. I also provide that the net systemic effects of these two agents on glucose lowering and lipid metabolism differed markedly, suggesting that the biguanide-derived compound HL271 acts via alternative mechanisms.

Materials and methods

Cell culture and transfection

NIH3T3, HepG2, and wild-type mouse embryonic fibroblast (MEF) cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) at 37°C in a humidified atmosphere containing 5% CO2. One day after seeding, cells were transfected with LipofectAMIN Plus (Invitrogen), lysed, and subjected to western blot or mRNA analysis.

Western blot analysis

Cells were harvested in 4X sodium dodecyl sulfate (SDS) sample buffer (200 mM Tris [pH 6.8], 8% SDS, 40% glycerol, 400 mM β-mercaptoethanol [Sigma], 1 mM NaF, 1 mM Na3VO4, 1 mM PMSF, and 1× protease inhibitor cocktail [Roche]). Whole-cell extracts were resolved on 8% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). The membranes were incubated with anti-AMPKα, anti-phospho-AMPK (Thr172) (Cell Signaling Technology), anti-Flag M2 (Sigma), and anti-V5 (Invitrogen) antibodies.

The immune complexes were visualized with an ECL detection kit (Pierce) (Lee et al., 2007).

Quantitative real-time PCR analysis

qPCR analysis was performed as described (Chung et al., 2014). Total RNAs were extracted from cells by using the guanidine thiocyanate method (TRIZOL; Life Technologies Inc.). A 2 μg aliquot of RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega). The cDNA was PCR amplified by Chromo4 DNA Engine (BioRad), and expression was quantified by SYBR green I real-time analysis. The levels of expression of all genes were normalized to that of gluceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used for real-time PCR were as follows: *mG6PD*, 5'-CAGCGGCAACTAAACTCAGA-3'(forward) and 5'-TTCCCTCAGGATC CACAC-3' (reverse);*mPck1*,5'-GTGCTGGAGTGGATGTTCGG-3' (forw ard) *and* 5'-CTGCTGATTCTCTGTTTCAGG-3'(reverse); *hG6Pase*, 5'-AGAATCATCGTGGATGTAGACTCT-3' (forward) and 5'-GCTTG GTG GTGATTGCTCTGCTA TG-3' (reverse); *hPck1*, 5'-CGGTCAATA AACCATGTTCTGA-3' (forward) and 5'-ACG CGGACTTTGATT CTGTATT-3' (reverse); and *GAPDH*, 5'-CATGGCCTTC CGTGTTCCTA-3' (forward) and 5'-AGTTCTTCCACCACTTGCGTCC-3' (reverse)

(Fortez et al., 2010).

Recording of real-time luminescence

Wild-type MEFs were plated at 4 × 106 cells per dish in 35 mm dishes the day before monitoring luminescence. The cells were transduced with adenoviral Per2 promoter construct, as described (Cheon et al., 2013). Two hours before recording, the cells were treated with 1 μM dexamethasone (Sigma); at zero time, the medium was replaced with 2 mL of cultured medium (DMEM, supplemented with 10% FBS, 1% PS, and 0.1 mM luciferin [Promega], and containing DMSO [control], metformin, HL271, or HL271IA, an inactive compound). Bioluminescence was measured with a dish-type luminometer (AB-2500; ATTO Co.) at 36°C in a humidified atmosphere containing 5% CO2.

Animal diet and housing

Mice were housed individually in a sound-proof isolated room in a conventional animal facility at a constant temperature (22–23°C) and humidity (50 ± 10%). Illumination for the LD cycle was provided by 32 W cool white fluorescence bulbs, with a measured light intensity of 200–250 lux at the bottom of each cage. Lights were turned on at 08:00 and off at 20:00 (L:D = 12 h:12 h). To generate diet-induced obese mice, wild-type

C57/BL/6J mice were fed ad libitum with a high-fat diet (HFD, with 60% kcal from fat; D12492, Research Diets) for 5 weeks. As a control group, mice were fed a normal diet (ND, with 12% kcal from fat; Purina 5001, Lab Diet). All animal experiments were performed according to the guidelines of the Laboratory Animal Centers at Seoul National University and DGIST.

Drug administration

Diet-induced obese mice were orally administered with HL271 (10 mg/kg or 30 mg/kg), diluted in drinking water, every morning at 09:30 for 4–5 weeks. Negative control mice were administered drinking water alone, and positive control mice were administered metformin (400 mg/kg) in drinking water.

Body composition and metabolic parameter analysis

The body weight and fat mass composition of mice were measured using a quantitative magnetic resonance whole-body composition analyzer (EchoMRI, Echo Medical Systems). Mice were starved for 12 h, and blood samples were drawn. Plasma insulin concentrations were measured using Ultra-Sensitive Mouse Insulin ELISA (Crystal Chem, Inc.), and plasma glucose concentrations were

measured by the Accu-Check® Performance kit (Roche). Total cholesterol, HDL, VLDL, and triglyceride concentrations were measured using the CR-3000 apparatus (CallegrariTM), according to the manufacturer's protocols.

Statistical analysis

All statistical analyses were performed using GraphPad PRISM software (GraphPad Prism Software Inc.). Between-group comparisons were assessed by one-way ANOVA, followed by post-hoc Tukey's test. All values are expressed as the mean ± SEM, with statistical significance defined as $P < 0.05$.

RESULTS

HL271 potently phosphorylates AMPK in a dose- and time-dependent manner

To determine whether HL271 phosphorylates AMPK like metformin, I assess AMPK phosphorylation level utilizing NIH 3T3, mouse firbroblast cells. HL271 dose-dependently phosphorylated AMPK under ~1000x lower dosage as compared to metformin (Figure 8A and B). By contrast, HL271IA, an inactive form of HL271 used as a negative control, did not alter the phosphorylation of AMPK at several concentrations, indicating the specificity of HL271 on AMPK phosphorylation (Figure 8C). A time-course experiment showed that HL271, at a much lower concentration (10 μM), induced AMPK phosphorylation 4 h faster than metformin (Figure 9). Based on these dose- and time-dependent effects, 10 μM HL271 and 2 mM metformin were used as positive controls in subsequent experiments. (Figure 10) Therefore, comparing between HL271 and metformin, I provide an evidence that HL271 under potent dosage, phosphorylated AMPK dose- and time- dependently.

HL271 shortens the period and dampens the amplitude of mPer2-dsLuc expression

To determine whether HL271 and metformin regulate clock gene expression in similar manner I have investigated the effects of HL271 on mPer2 promoter-driven destabilized luciferase activity in real-time were analyzed. Because metformin was shown to affect mPer2 oscillation (Um et al., 2007), MEFs stably expressing a mPer2-dsLuc construct were treated with metformin, HL271, or HL271IA. Consistent with previous findings (Um et al., 2007), metformin shortened the period and dampened the amplitude of mPer2-Luc expression in a dose-dependent manner (Figure 11A). HL271 showed similar activities, but at much lower concentrations than metformin (Figure 11B). By contrast, inactive HL271IA did not alter the period or amplitude of mPer2-dsLuc oscillation (Figure 11B-D).

HL271 induces PER2 and CRY1 protein degradation

In previous studies, metformin was shown to degrade PER2 and CRY1 proteins, from the result of AMPK phosphorylation (Um et al., 2007; Lamia et al., 2009). To determine whether the effects of HL271 of the period and amplitude of mPer2 expression were due to an increased rate of molecular clock turnover, I have measured the degradation levels of PER2 and CRY1 proteins in cells treated with HL27. NIH3T3 transfected with V5-tagged PER2 (PER2-V5) or FLAG-tagged CRY1 (FLAG-CRY1)

were treated with metformin, HL271, or HL271IA in the presence of cycloheximide, an inhibitor of protein synthesis. I was able to find PER2 and CRY1 protein degradation were more rapid in HL271 than in metformin treated cells (Figure 12A and B), whereas the inactive compound HL271IA had no effect on PER2 or CRY1 degradation. To further determine whether the degradation of these proteins is proteasome-dependent, I have repeated the experiment in the presence of the proteasome inhibitor MG132, together with cycloheximide. Neither metformin nor HL271 inhibited the degradation of PER2 and CRY1 proteins (Figure 13 A and B). Taken together, these results indicate that HL271, like metformin, induced the degradation of PER2 and CRY1, probably affecting the amplitude and/or period of cellular oscillation (Figure 11).

HL271 does not reduce body weight, blood glucose, or insulin level in diet-induced obese mice

Since metformin is used clinically as an anti-hyperglycemic and anti-diabetic agent, I have created diet-induced obese mice, an animal model of human type 3 diabetes, and investigated the effects of HL271 on food intake and body weight. HFD-fed mice were orally administered metformin (400 mg/kg) or HL271 (10 and 30 mg/kg) dissolved in drinking

water for 4 weeks, and their daily food intake and total body weight were measured. A control group fed a normal diet (ND) displayed higher food intake than the HFD-fed group, but there were no differences in daily food intake between mice administered metformin (400 mg/kg) and HL271 (10 and 30 mg/kg) (Figure 14A). Although metformin treatment resulted in a significant reduction in body weight gain, I found out that body weight gain in HL271-treated mice was the same as that of mice fed a ND and HFD-fed mice (Figure 14B).

The major physiological effects of metformin is to lower blood glucose level in diabetic patients. Therefore, to assess the anti-hyperglycemic effects of HL271, I have measured blood-glucose and insulin levels after a 12 h fast at 0, 2, and 4 weeks. Mice-treated with metformin showed significant reductions in blood-glucose and insulin levels compared with mice fed a ND (Figure 14C and D). Surprisingly, mice fed both 10 and 30 mg/kg HL271 showed no reductions in blood-glucose and insulin levels (Figure 14 C and D).

Different effects of metformin and HL271 on gluconeogenic gene expression in HepG2 cells and in wild-type mouse liver

To further investigate the differences in glucose-lowering effects between metformin and HL271, I have investigated the expression of

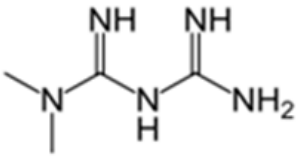
G6pase and Pck1 mRNAs were assayed in HepG2 cells, human liver hepatocellular carcinoma cell-line. Although treatment with 2 mM metformin decreased the levels of G6pase and Pck1 mRNAs, 10 μM HL271 had no effect (Figure 15A and B). The effects of HL271 on circadian expression of G6pase and Pck1 *in vivo,* I have interperitoneally injected metformin (500 mg/kg) or HL271 (100 mg/kg) into wild-type mice and liver tissues were collected every 6 h for 24 h to analyze G6pase and Pck1 expression. Liver G6pase did not display circadian oscillations in the three groups. By contrast, liver Pck1 expression peaked at CT2400, just before lights-on, and was minimal between CT 0600 and CT 1200, showing mild circadian oscillation (Figure 15C and D). Compared with control mice, the levels of G6pase and Pck1 were significantly reduced in mice injected with metformin (500 mg/kg), but not HL271 (100 mg/kg). Taken together, I was able to find that metformin reduced the blood-glucose level partly by reducing the expression of the key gluconeogenic genes, G6pase and Pck1, in liver. By contrast, HL271 did not repress G6pase and Pck1 expression in the liver, with no net changes in blood-glucose levels.

HL271 does not alter levels of lipids and metabolites in diet-induced obese mice

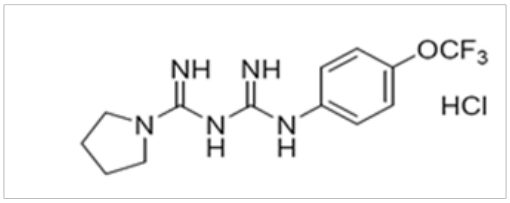
Since metformin is also involved in lipid metabolism, the effects of

metformin and HL271 on fat mass and cholesterol and triglyceride levels were compared in diet-induced obese mice. Mice treated with metformin displayed decrease of total fat mass, however fat mass levels were not significantly changed in HL271 treated mice (Figure 16). In addition, following the metformin (400 mg/kg) treatment significantly reduced total fat mass, including epidermal fat and white adipose tissues, but not brown adipose tissue (Figure 17). Metformin also reduced LDL, but not HDL, levels , as well as total cholesterol and triglycerides, in liver and muscles. Neither concentration of HL271 (10 and 30 mg/kg) had any effects on total fat mass, organ-specific fat mass, and cholesterol levels, including HDL and LDL, or on liver- and muscle-specific triglyceride levels (Figure 17). Therefore, although HL271 has molecular effects similar to metformin on AMPK phosphorylation and circadian clock gene modifications, the net systemic effects of HL271 on glucose lowering and lipid metabolism differed substantially from those of metformin.

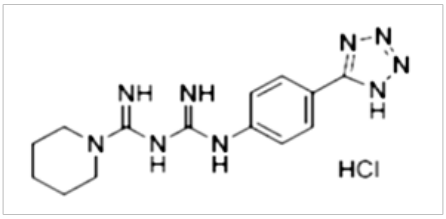
Figure 7. Chemical structure of metformin, HL271 and HL271IA. Chemical structures and molecular weight of metformin, HL271 and HL271IA. Based on the biguanide structure of metformin, which molecular weight consist of 129.164 g/mol, HL271 contain pyrolidine and benzyl trifluoromethyl group weighting 375.35 g/mol. HL271IA contains pyrolidine and benzyl urea groups consisting 349.82 g/mol.



Metformin (mw. 129.16 g/mol)



HL271 (mw. 375.35 g/mol)



HL271IA (mw. 349.82 g/mol)

Figure 8. Dose-dependent AMPK phosphorylation by metformin and HL271. (A) Detection of AMPKα1 Thr172 phosphorylation in NIH3T3 cells after treatment for 4 h with 0.125, 0.25, 0.5, 1, 2, and 4 mM metformin, and (B and C) 0.31, 0.62, 1.25, 2.5, 5, and 10 μM HL271 and HL271IA. Phosphorylated AMPK is normalized by the endogenous AMPK and displayed in bar graph. Vales are the mean ± standard error of the mean (SEM) of three or four independent experiments performed in triplicates (*, *P<0.05*).

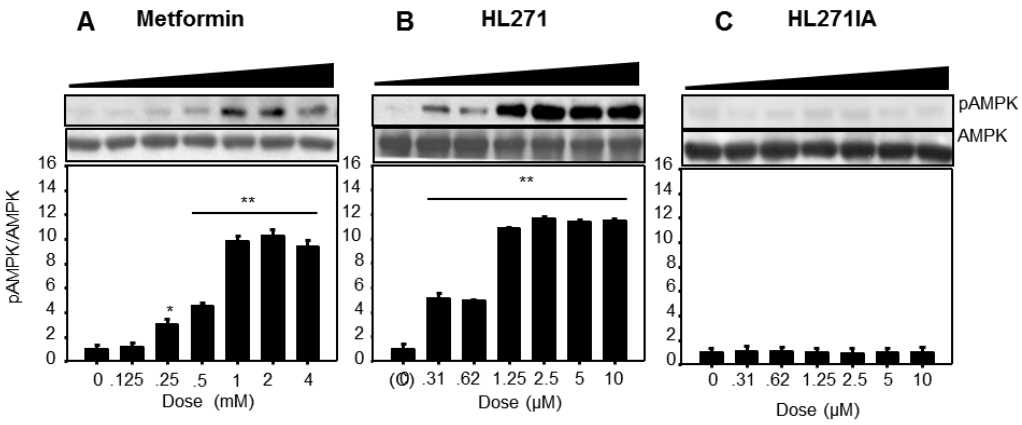
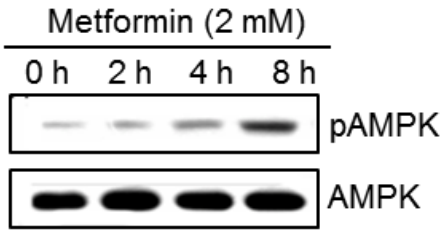

A
Metformin
B
HL271
C
HL271IA
pAMPK
AMPK
pAMPK/AMPK
16
14
12
10
8
6
4
2
0
**
*
0 .125 .25 .5 1 2 4
Dose (mM)
(0) .31 .62 1.25 2.5 5 10
Dose (μM)
0 .31 .62 1.25 2.5 5 10
Dose (μM)

Figure 9. Time-dependent AMPK phosphorylation by metformin and HL271. Time-dependent measurement of AMPKα1 Thr 172 phosphorylation level after treatment with metformin (2 mM) or HL271 (10 uM) in NIH3T3 cells. 0, 2, 4 and 8 hours after metformin and HL271 treatment, cells were harvested proceeded to western blotting. Phosphorylated AMPK level were analyzed by normalization between endogenous AMPK and phosphorylated AMPK.


Metformin (2 mM)
0 h
2 h
4 h
8 h
pAMPK
AMPK

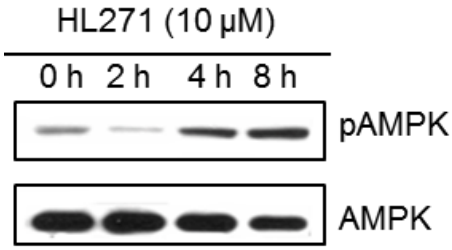

HL271 (10 μM)
0 h
2 h
4 h
8 h
pAMPK
AMPK

Figure 10. AMPK phosphorylation by HL271 under ~500x lower concentration. Comparison and quantification of AMPKα1 Thr172 phosphorylation level normalized relative to non-phosphorylated AMPK level after treatment for 4 h with metformin (2 mM), HL271 (10 μM), or HL271IA (10 μM). Each number represents the mean ± SEM values of three independent experiments, with each experiment performed in triplicate and compared by one-way ANOVA, followed by Tukey's test. *P < 0.05 and **P < 0.01 vs. control treatment.

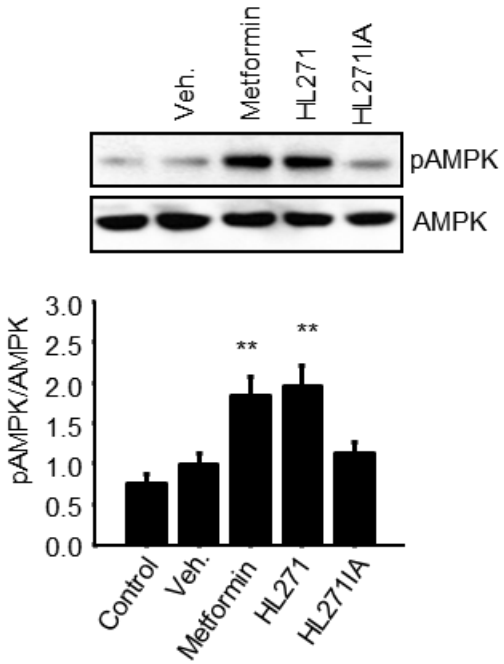

Veh.
Metformin
HL271
HL271A
pAMPK
AMPK
pAMPK/AMPK
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
Control
Veh.
Metformin
HL271
HL271A

Figure 11. Dose-dependent effects of metformin and HL271 on Per2 expression. Real-time luminescence recording of WT MEFs transduced with an adenoviral Per2 promoter-driven luciferase construct. Treatments with (A) 1, 2, and 4 mM metformin, (B) 2.5, 5, and 10 μM HL271, and (C) 2.5, 5, and 10 μM HL271IA. (D) Luminescence recordings of cells treated with DMSO (negative control), metformin (2 mM), HL271 (10 μM), and HL271IA (10 μM) after dexamethasone (1 μM) administration. Left, actual luminescence recordings. Middle, rhythms derived from luminescence recordings after subtracting baseline. Right, Period calculation for each dose. Each number represents the mean ± SEM of three independent experiments, with each bioluminescent recording performed in triplicate. *P < 0.05. (One-way ANOVA, followed by Tukey's test.)

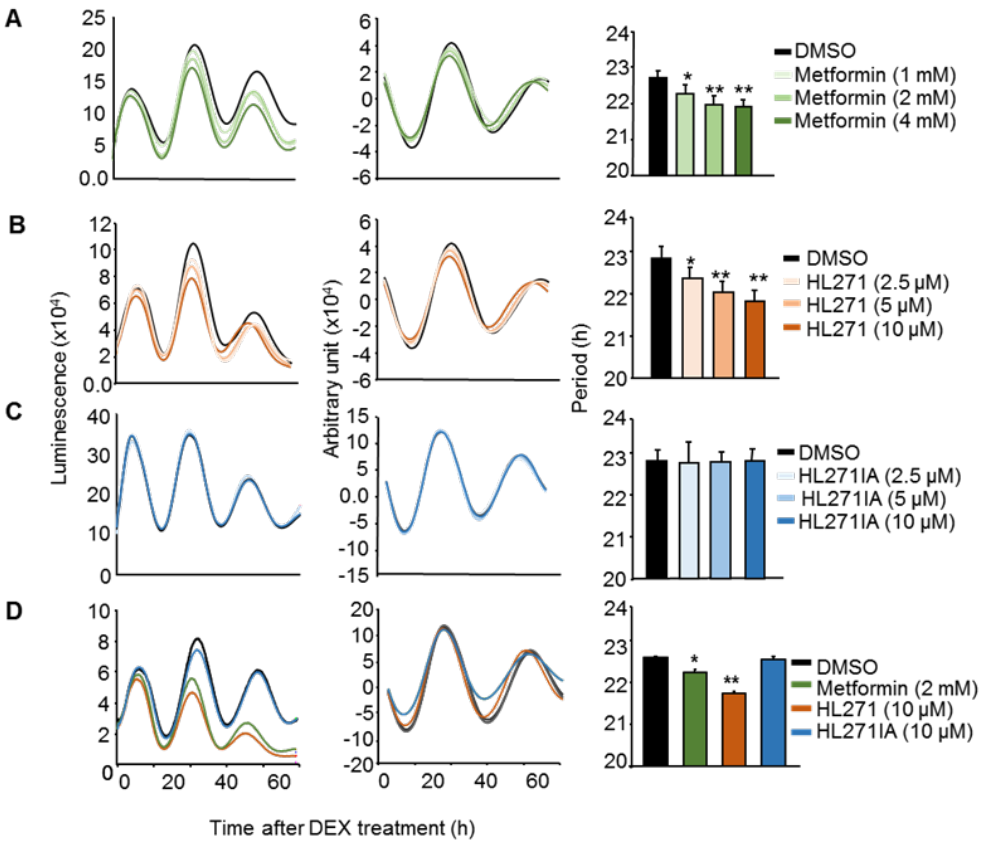

A
B
C
D
Luminescence (x10⁴)
Arbitrary unit (x10⁴)
Period (h)
Time after DEX treatment (h)
DMSO
Metformin (1 mM)
Metformin (2 mM)
Metformin (4 mM)
HL271 (2.5 μM)
HL271 (5 μM)
HL271 (10 μM)
HL271IA (2.5 μM)
HL271IA (5 μM)
HL271IA (10 μM)

Figure 12. PER2 and CRY1 protein stability after metformin and HL271 treatment in presence of CHX. (A and B) Measurement of V5-tagged PER2 and FLAG-tagged CRY1 degradation after treatment with cycloheximide (30 μg/mL) plus DMSO (control), metformin (2 mM), HL271 (10 μM), or HL271IA (10 μM). Transfection efficiency were normalized by GFP. Each number represents the mean ± SEM of three independent experiments, with each experiment performed in triplicate. Comparisons by one-way ANOVA, followed by Tukey's test. *P < 0.05 and **P < 0.01 vs. control treatment.

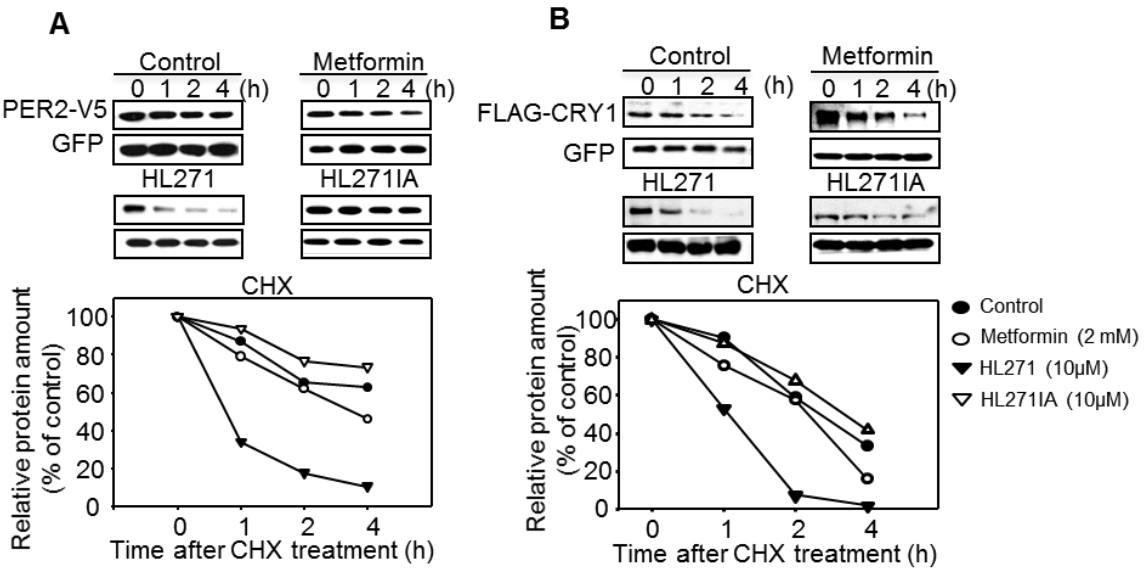

A
Control
Metformin
0 1 2 4 (h)
0 1 2 4 (h)
PER2-V5
GFP
HL271
HL271IA
CHX
Relative protein amount (% of control)
100
80
60
40
20
0
0 1 2 4
Time after CHX treatment (h)
B
Control
Metformin
0 1 2 4 (h)
0 1 2 4 (h)
FLAG-CRY1
GFP
HL271
HL271IA
CHX
Relative protein amount (% of control)
100
80
60
40
20
0
0 1 2 4
Time after CHX treatment (h)
Control
Metformin (2 mM)
HL271 (10μM)
HL271IA (10μM)

Figure 13. PER2 and CRY1 protein stability after metformin and HL271 treatment in presence of CHX and MG132. Stability of V5-tagged PER2 and FLAG-tagged CRY1 protein treated with CHX (30 μg/mL) plus MG132 (50 μM) in the presence of DMSO, metformin, HL271, or HL271IA. Each number represents the mean ± SEM of three independent experiments, with each experiment performed in triplicate. Comparisons by one-way ANOVA, followed by Tukey's test.(*P < 0.05)

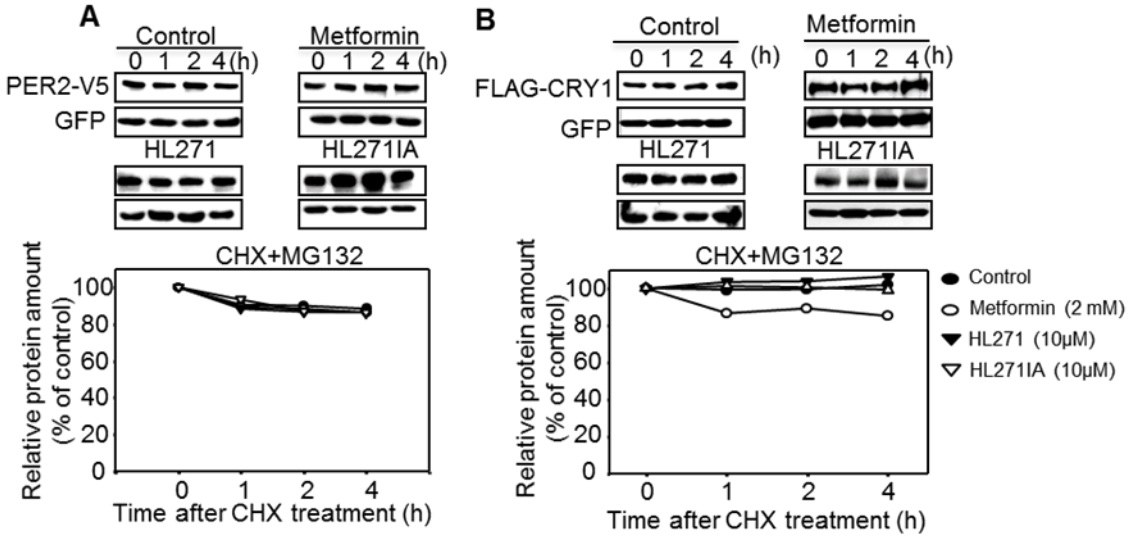

A
Control
Metformin
0 1 2 4 (h)
0 1 2 4 (h)
PER2-V5
GFP
HL271
HL271IA
CHX+MG132
Relative protein amount (% of control)
100
80
60
40
20
0
0 1 2 4
Time after CHX treatment (h)
B
Control
Metformin
0 1 2 4 (h)
0 1 2 4 (h)
FLAG-CRY1
GFP
HL271
HL271IA
CHX+MG132
Relative protein amount (% of control)
100
80
60
40
20
0
0 1 2 4
Time after CHX treatment (h)
Control
Metformin (2 mM)
HL271 (10μM)
HL271IA (10μM)

Figure 14. Changes of daily food intake, body weight, glucose and insulin level in mice treated with either metformin or HL271. (A) Daily food intake over 4 weeks in mice fed a normal diet (ND) or high-fat diet (HFD). Diet-induced obese mice were orally administered drinking water, metformin (400 mg/kg), or HL271 (10 and 30 mg/kg) at 09:30 every day for 4 weeks. (B) Measurements of whole body weights. Blood was collected from the tail veins of mice after a 12 h fast at baseline and after 2 and 4 weeks. Blood samples were collected at the first day of drug injection (0 weeks), in the middle (2 weeks), and on the last (4 weeks) day following drug administration. Glucose and insulin concentrations were measured. The mean ± SEM for each group of ten mice is shown. Results compared by one-way ANOVA, followed by Tukey's test. *P < 0.05 and **P < 0.01 vs. HFD control.

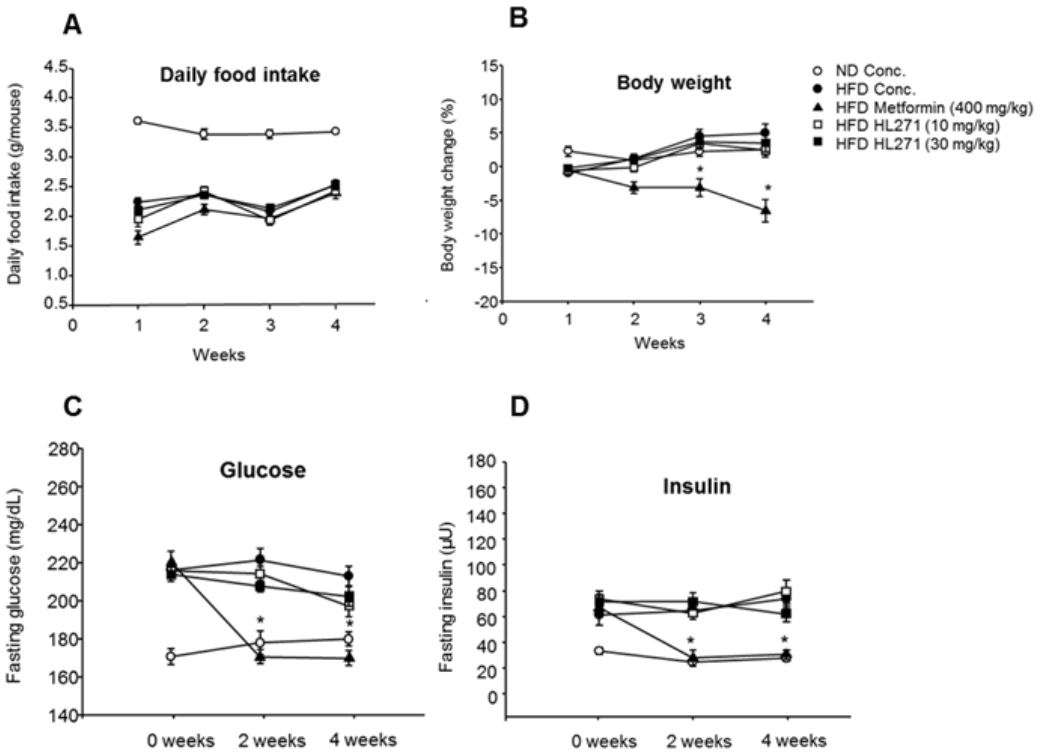

A
Daily food intake
Daily food intake (g/mouse)
Weeks
B
Body weight
Body weight change (%)
Weeks
ND Conc.
HFD Conc.
HFD Metformin (400 mg/kg)
HFD HL271 (10 mg/kg)
HFD HL271 (30 mg/kg)
C
Glucose
Fasting glucose (mg/dL)
0 weeks
2 weeks
4 weeks
D
Insulin
Fasting insulin (μU)
0 weeks
2 weeks
4 weeks

Figure 15. Effects of metformin and HL271 on liver *G6pase* and *Pck1* mRNA expression. (A and B) HepG2 cells were treated with DMSO (control), metformin (2 mM), or HL271 (10 μM). After 4 h, the cells were collected, and mRNA was extracted and analyzed by real-time RT-PCR for expression of (A) hG6pase and (B) hPck1 mRNA levels. Values shown are the mean ± SEM of two independent experiments performed in triplicate. (C and D) Eight-week-old wild-type mice were intraperitoneally administered metformin (500 mg/kg) or HL271 (100 mg/kg) dissolved in saline. A liver sample was obtained every 6 h for 24 h, and mRNA was extracted and the levels of (C) G6pase and (D) Pck1 mRNAs were analyzed by real-time PCR. The level of each cDNA was normalized relative to that of GAPDH. Results were compared by one-way ANOVA, followed by Tukey's test. *P < 0.05 and **P < 0.01 vs. control.

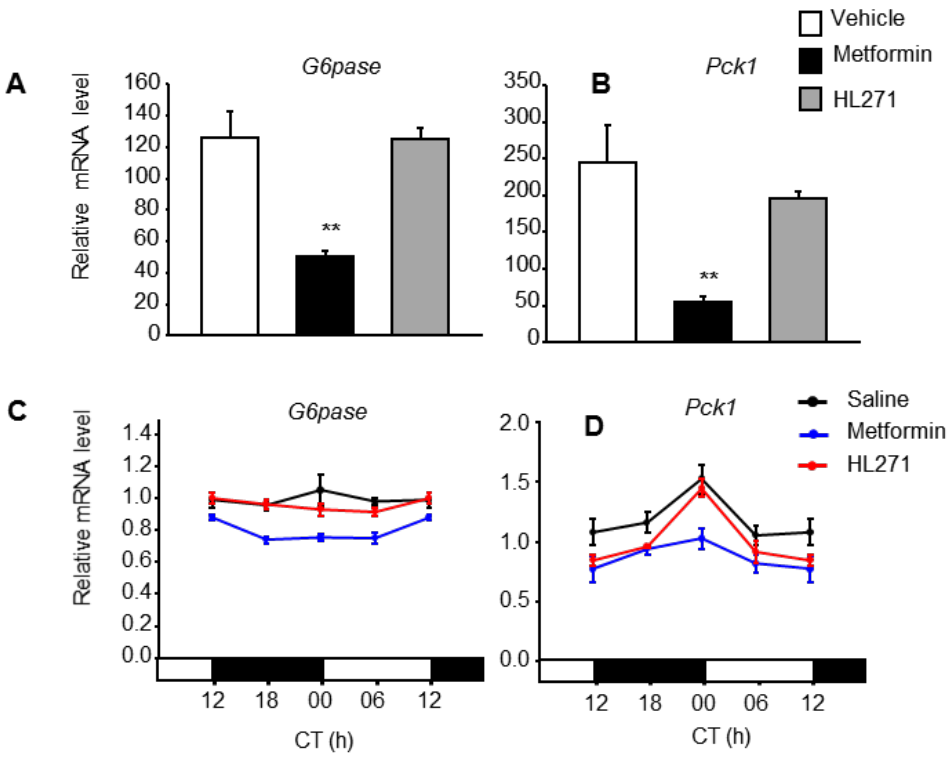

A
Relative mRNA level
G6pase
160
140
120
100
80
60
40
20
0
**
B
Pck1
350
300
250
200
150
100
50
0
**
Vehicle
Metformin
HL271
C
Relative mRNA level
G6pase
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
12
18
00
06
12
CT (h)
D
Pck1
2.0
1.5
1.0
0.5
0.0
12
18
00
06
12
CT (h)
Saline
Metformin
HL271

Figure 16. Total body fat mass changes. Total body fat was measured before treatment (0 weeks) and after 2 and 4 weeks of drug administration. Metformin (400 mg/kg) and HL271 (10 and 30 mg/kg) were administered orally dissolved in drinking water. The mean ± SEM for each group of ten mice is shown. Results compared by one-way ANOVA, followed by Tukey's test. *P < 0.05 and **P < 0.01 vs. HFD control.

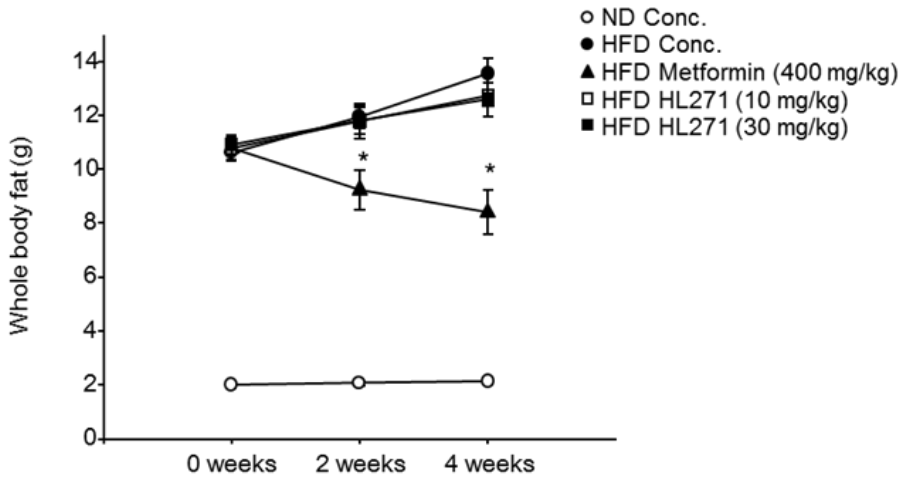
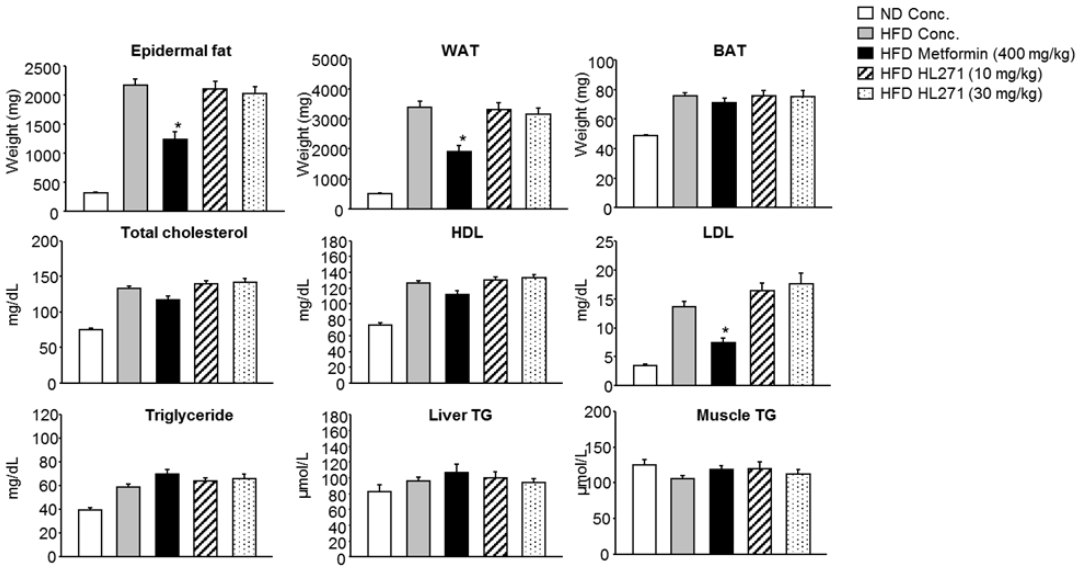

ND Conc.
HFD Conc.
HFD Metformin (400 mg/kg)
HFD HL271 (10 mg/kg)
HFD HL271 (30 mg/kg)
Whole body fat (g)
0
2
4
6
8
10
12
14
*
*
0 weeks
2 weeks
4 weeks

Figure 17. Changes of lipid metabolites between metformin or HL271 treated mice. Epidermal fat, white adipose tissue (WAT), and brown adipose tissue (BAT) were removed and weighed to the closest 0.01 mg. Blood samples were collected, and total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides were measured. Liver and gastrocnemius muscle were collected from each mouse, and triglyceride levels were measured. Each number represents the mean ± SEM for each group of ten mice. Results compared by one-way ANOVA, followed by Tukey's test. *P < 0.05 vs. HFD control.


ND Conc.
HFD Conc.
HFD Metformin (400 mg/kg)
HFD HL271 (10 mg/kg)
HFD HL271 (30 mg/kg)
Epidermal fat
Weight (mg)
WAT
Weight (mg)
BAT
Weight (mg)
Total cholesterol
mg/dL
HDL
mg/dL
LDL
mg/dL
Triglyceride
mg/dL
Liver TG
μmol/L
Muscle TG
μmol/L

DISCUSSION

In Chapter 1, I have compared the effects of metformin and a novel metformin-derived compound, HL271, on molecular circadian clock functions and glucose and lipid metabolism. HL271 was more potent than metformin in phosphorylating AMPK and at modifying circadian clock oscillators. However, HL271 differed from metformin in metabolic regulation, including body weight, blood glucose, and insulin levels and lipid metabolite content in HFD-induced obese mice. Thus, from the results, I demonstrated that although molecular effects of metformin and HL271 displayed similar effects, the net physiological effects of metformin and HL271 differ and possibly due to its systemic effects.

Circadian clock timing is tightly integrated, with daily metabolic cycles including glucose homeostasis. AMPK phosphorylation of CRY1 provided direct evidence for the molecular connection between circadian clock timing and glucose homeostasis (Lamia et al., 2009). In addition, CRY1 can modulate gluconeogenesis by inhibiting glucagon mediated cAMP-PKA signaling (Zhang et al., 2010) and by interfering with glucocorticoid receptors (Lamia et al., 2011). The faster CRY1 degradation rate displayed by HL271 than by metformin may be due to the increased phosphorylation of AMPK by HL271. Therefore, the system-wide difference between HL271 and metformin may be affected by the

stability of cryptochrome, which is important for maintaining circadian clock oscillation and glucose homeostasis.

The glucose-lowering and insulin-sensitizing effects of metformin were shown to involve the AMPK signaling pathway (Pernicova and Korbonits, 2014; Fortez et al., 2014). Net glucose lowering may be achieved by either an AMPK-dependent or AMPK-independent pathway (Quinn et al., 2013). In the former, metformin can lower the blood-glucose level by initiating the phosphorylation of AMPK, increasing the activity of insulin receptor substrate-2 (IRS-2), and thereby inducing the translocation of glucose transporters (Gunton et al., 2003). Metformin activation of AMPK can also inhibit fatty acid synthesis and lipogenesis by suppressing acetyl-CoA carboxylase (ACC) activity (Zhou et al., 2001; Fullerton et al., 2013). Even in the absence of AMPK, however, metformin can lower blood-glucose concentrations by rapidly inhibiting glucagon-stimulated, cAMP-PKA-CREB-mediated gluconeogenesis (Fortez et al., 2010). In addition, this AMPK-independent action of metformin causes the depletion of cAMP, affecting the expression of gluconeogenic genes such as G6pase and Pck1, by repressing transcription induced by cAMP-responsive element binding protein (CREB-1) (Miller et al., 2013).

Altering the physiological concentrations of metformin can alter its effects (He et al., 2015). For example, at supra-pharmacologic concentrations (~5 mM), metformin inhibited mitochondrial respiratory

chain complex 1 in hepatocytes, leading to an increase in the AMP/ATP ratio and altering AMPK activation (El-Mir et al., 2000). A metformin concentration ≥2 mM was also required to suppress gluconeogenesis and to increase the AMP/ATP ratio (Fortez et al., 2010). However, at therapeutic concentrations in hepatocytes (40~70 μM), metformin was able to suppress gluconeogenesis by suppressing the expression of cAMP-stimulated gluconeogenic genes, reducing glucose production without affecting mitochondrial respiratory chain 1 (Cao et al., 2014). In addition, oral administration of a therapeutic concentration of metformin (50 mg/kg), either acutely or chronically, inhibited mitochondrial glycerophosphate dehydrogenase (mGDP), increasing cytosolic NADH and inhibiting lactate and glycerol functions, resulting in reduced glucose production (Madiraju et al., 2014). Taken together, the differences between metformin and HL271 may be caused by concentration-dependent AMPK action and/or a direct or indirect effect of AMPK on multiple target tissues systematically influencing glucose and lipid metabolism.

Abundant clinical evidence indicates that the secondary effect of metformin can reduce the rates of various cancers, suggesting that metformin has anticancer activity (Giovannucci et al., 2010; Evans et al., 2005). The antitumor effects of metformin are mediated by reducing circulating insulin and insulin growth factor-1 (IGF-1) levels, inhibiting

downstream tyrosine kinase signaling including through the PI3K-Akt-mTORC1 pathway (Shackelford et al., 2013; Inoki et al., 2003). Metformin was also shown to regulate the expression of c-MYC, insulin receptor substrate 1 (IRS-1), and hypoxia-inducible factor 1α (HIF-1α) (Blandino et al., 2012). Therefore, along with on-going clinical trials, studies on the mechanisms underlying the secondary effects of metformin on tumor formation and development are necessary for the repositioning of this drug (Quinn et al., 2014).

In conclusion, this study compared the effects of HL271 and metformin on circadian clock regulation, glucose homeostasis and lipid metabolism. Although HL271 and metformin phosphorylated AMPK and influenced circadian clock oscillations in a similar manner, the two had different effects on glucose lowering and lipid contents.

CHAPTER 2.

Systemic role of omega-3 fatty acids on circadian oscillation

ABSTRACT

Omega-3 fatty acids (FA) are known as essential fatty acids, which mammals require for normal development. Although omega-3 FA is known to elicit health benefits such as improved inflammatory system, retinal- and neural development, there are paucity of studies regarding the effects of omega-3 FA on circadian clock. In this chapter, I have utilized a unique omega-3 FA animal model, *fat-1* transgenic (TG) mice, which converts omega-6 FA to omega-3 FA endogenously, to investigate effects of omega-3 FA on circadian oscillation. I have cross-mated *fat-1* TG mice with Per2:: luc KI mice and created *fat-1*/Per2::luc mice. First, I have investigated *In vivo* imaging analysis and *fat*-1/Per2:luc mice displayed enhanced Per2 expression in the peripheral tissues, including liver, kidney and submandibular gland. In f*at-1* TG mice, clock mRNA levels were elevated in liver and kidney, however, clock gene expression in the hypothalamus only displayed shifting of period. Molecular analysis revealed that in f*at-1* TG mice, the core clock gene, BMAL1 was highly sumoylated and ubiquitinated eliciting enhanced proteosomal-mediated degradation and protein turnover. In accordance with the alteration of clock oscillation in the SCN, f*at-1* TG mice displayed elongated period during free-running activities in constant darkness (DD) condition. Therefore, in present study I demonstrate that ubiquitous

actions of omega-3 FA elicit systematic influence on circadian clock oscillation.

Key words: Circadian rhythm, fatty acids, omega-3, *fat-1*

INTRODUCTION

Daily metabolic process is governed by circadian clock system. Self-sustainable circadian clock is synchronized by the neuronal and hormonal signals from suprachaismatic nucleus (SCN) residing in the hypothalamic nucleus (Asher and Schibler et al., 2011; Eckel-Mahan and Sassione-Corsi, 2013). However, the environmental factors such as daily food intake, exercise, and behavioral activity can also synchronize peripheral clocks away from signaling factors from SCN. The molecular circadian clock is composed of two transcriptional- and translational-feedback loops (Brown et al., 2012; Saini et al., 2011). The core loop consists of circadian locomoter output cycles kaput (*CLOCK)* and brain and muscle Arnt-like-1 *(BMAL1)* forming a heterodimer and initiating transcription of Periods *(Per1-3)* and Cryptochromes *(Cry1 and Cry2)* in the nucleus (Crane and Young, 2014). Following post-translational modifications, PERs and CRYs binds to each other and translocate back into the nucleus to repress CLOCK::BMAL1 heterodimer binding to its own promoter, eliciting a negative-feedback loop. To fine tune this feedback mechanism, nuclear receptor Rev-erbα and Rorα binds to their specific responsive element (RORE) in Bmal1 promoter to either repress or induce transcriptional process, respectively (Robles and Mann, 2013).

High-fat diet (HFD) rich in saturated fatty acids (SFA), are known to disrupt circadian clock system by altering core clock gene expression and clock target genes in metabolic homeostasis (Kohsaka et al., 2007). Palmitic acid is most abundant form of SFA found in major body component and excessive palmitic acid leads to increase risk of developing cardiovascular disease, obesity and metabolic syndrome (Tahara et al., 2013). In circadian clock system, palmitic acid is known to destabilize circadian clock by either increasing expression of *Bmal1* and *Clock* or decreasing *Per2* and *Rev-erbα* mRNA transcripts in hypothalamic neuronal cells (Fick et al., 2011). Unlike SFA, polyunsaturated fatty acids (PUFA) containing more than one double bond in their carbon-carbon bond, are known to have beneficial effects on health. PUFA, especially omega-3 FA, are known as an essential fatty acids, because mammals, including humans, cannot synthesize them and can only obtain from diet. And in class of omega-3 FA, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) plays an important role in normal development, signal transduction and also is an important source of fuel (Hainault et al., 1993; Clarke SD., 2000). Despite the beneficial effects of omega-3 FA on normal metabolism and development, there are pittance amount of studies of its effects on circadian clock system.

In previous studies, investigating the effects of omega-3 FA on circadian clock consisted of external treatment of DHA or EPA. One study

have utilized the immortalized hypothalamic neuronal cell model, mHypoE-37, and investigated effects of omega-3 FA on transcriptional expression of circadian clock genes (Greco et al., 2014). And others have utilized dietary supplementation method to deliver DHA or EPA to investigate effects of omega-3 FA on circadian clock *in vivo* (Furutani et al., 2015). However, application of DHA or EPA abundant fish oil or diet consisting of highly concentrated omega-3 FA, may hold experimental consequences. For instance, PUFA is highly unstable and prone to oxidation which dietary supplementation of omega-3 fatty acids to animals may provide many variables potentially leading to confounding effects. In addition, equivalent delivery of omega-3 FA supplementation would be difficult for each animals, since individual variance in feeding and digestion cannot be controlled.

To overcome this experimental dilemma, Kang and colleagues have recently engineered a transgenic (TG) mice that can endogenously produce omega-3 fatty acid by inserting modified *fat-1* gene from *Caenorhabditis elegans*, which encodes n-3 desaturase enzyme responsible for conversion of omega-6 FA to omega-3 FA (Kang et al., 2004). *Fat-1* TG mice displayed high level of omega-3 FA in most of the tissues and reduction of omega-6 FA when fed with normal diet in absence of dietary omega-3 FA, while displaying normal development and healthy phenotype (Kang et al., 2001). This led to novel murine model

to study effects of omega-3 FA on various disease and cancer models. Recent researches demonstrated that in *fat-1* TG mice prostaglandin E_3 (PGE3) level was increased resulting reduced melanoma formation (Xia et al., 2006), prevented age-related glucose metabolic syndromes (Romanatto et al., 2014), and also observed enhanced dendritic spine density and neurogenesis leading improved spatial learning tasks (He et al., 2009). Although *fat-1* TG mice has been utilized as murine model to investigate influence of omega-3 FA in various studies, there are scarce amount of research involving its effects on circadian rhythm.

In the present study, I have investigated the underlying mechanism of omega-3 fatty acids on circadian clock oscillation by utilizing *fat-1* TG mice which can endogenously convert omega-6 FA to omega-3 FA.

Materials and methods

Animals and locomoter activity

Fat-1 transgenic mice were gifted kindly provided by Dr. Lim Kyu (Chungnam National University). Fat-1 TG mice mixed background of C57/BL6J were cross-mated with Homozygous Per2::luc KI mice to generate fat-1/Per2::luc KI mice. All mice were raised in a sound-proof isolated room with a constant temperature (22-23℃) and humidity (50 ± 10%). Lights were on at 08:00 AM and off at 20:00 PM (L:D=12 h:12 h) light intensity of 100-150 lux at cage level and provided with standard diet. Mice were housed with a wood-chip bedding (North Eastern Products Corporation, Warrenburg, NY). All mice experiments were carried out by the guidelines of the Laboratory Animal Center at Seoul National University and Daegu Gyeongbuk Institute of Science and Technology.

In vivo imaging and analysis

C57/BL6J based male fat-1/Per2::luc KI and Per2::luc KI mice were dilapidated dorsal and ventral side to monitor organ bioluminescence. Mice were intraperitoneally [i.p] injected with 15 mg/kg D-luciferin potassium salt (Promega) dissolved in phosphate-buffer saline (PBS) under isofluorane anesthesia (Mylan Inc.) with mixture of oxygen.

After 8 to 10 minutes after D-luciferin injection mice were subjected to luminescence image acquisition by an in vivo imaging system (IVIS Kinetics; Caliper Life Sciences). All bioluminescence data were automatically calculated by Living Image 4.4 software (Caliper Life Sciences). Briefly, for each organ photon counts were measured same shape and size of the region of interest (ROI) at indicated times.

Cell Culture

Wild type (WT), Per2::luc KI, fat-1 and fat-1/Per2::luc KI mouse embryonic fibroblasts (MEFs) were generated as previously described (Bea et al., 2001; Bunger et al., 2000; Todaro and Green. 1963a; Yoo et al., 2004). Primary cell line were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

Recording of real-time luminescence

Per2::luc KI and fat-1/Per2::luc KI MEFs were treated with1 μM dexamethasone 2 hr before recording. Before the start of recording, media were changed to normal culture media with 100 μM D-luciferin (Promega). Bioluminescence were measured for 1 min for each dish at

10 min interval by real-time luminescence monitoring device (Kronos-Dio; ATTO) at 35°C in a humidified chamber containing 5% CO2. Data were normalized by the average of the initial minimum value.

Immunoblotting and immunoprecipitation

Tissues and cells were harvested as previously described (Lee et al., 2008). Briefly, cells and tissues were harvested in radioimmunoprecipiration assay (RIPA) buffer containing protease inhibitor cocktail (Roche). Equal amounts of total protein were incubated with 2 μg of anti-BMAL1 (Kwon et al., 2006) antibodies for 1.5 h at 4°C and then A/G-Sepharose bead slurry was added. For immnoblot analysis, target protein were detected by anti-BMAL1 generated in rabbit (Kwon et al., 2006), anti-SUMO2/3 (Abcam), anti-SUMO1 (Santa Cruz) antibodies. The immune complex were detected by ECL solution kit (Pierce).

Quantitative real-time PCR

Total RNA from cells and tissues were extracted by TRIzol reagent (Invitrogen) according to manufacturer's protocol. RNA was reverse-transcribed reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega). Single stranded cDNA was PCR amplified by Chromo4 DNA Engine (BioRad), and expression was

quantified by SYBR green I analysis. The levels of expression of all genes were normalized to that of gluceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

All statistical analyses were performed using GraphPad PRISM software (GraphPad Prism Software Inc.). Between-group comparisons were assessed by one-way ANOVA, followed by post-hoc Tukey's test. All values are expressed as the mean ± SEM, with statistical significance defined as $P < 0.05$.

Results

Per2 expression is elevated in kidney, liver, and sub mandibular gland of fat-1 TG mice

To investigate and visualize clock gene expression in fat-1 TG mice in vivo, I have cross-mated with Per2::luc KI mice, which endogenous mPer2 gene is fused with luciferase at the 3' end, posing PER2::LUC fusion protein (Yoo et al., 2004). Minutes after luciferin injection I have imaged bioluminescence of Per2::luc, as a positive control, and fat-1/Per2::luc mice in either dorsal or ventral position. In dorsal position we were able to visualize Per2 expression from kidney where at ZT 12 and ZT 18 fat-1/Per2::luc mice displayed significant increase of luminescence signal as compared to Per2::luc mice (Fig. 18 A up and B left). In ventral position, luminescence signals from liver and sub mandibular gland also showed significant induced expression of Per2 at ZT 12 and ZT 18 in fat-1/Per2::luc mice (Fig. 18 A down and B middle and right). These results demonstrates that clock gene expression is generally elevated in peripheral tissues of fat-1 TG mice throughout 24 h period.

Hypothalamic clock gene and neuropeptide expression in fat-1.

Since, only a certain peripheral organs can be visualized by in vivo

luminescence system, I investigated the clock gene and clock-controlled hypothalamic neuropeptide expression in hypothalamic region of fat-1 TG mice. First, I have monitored Per2 expression from SCN tissues of either Per2::luc or fat-1/Per2::luc mice (Fig. 19). Compared to elevated Per2 expression in the peripheral tissues in fat-1 mice, the amplitude of Per2 expression in the SCN were not elevated, however, oscillating period were significantly shifted close to ~26 h. The peak expression period of Bmal1 and Per1 mRNA transcripts were also shifted in fat-1 hypothalamic tissue, however amplitude of clock gene transcript levels were not elevated (Fig 20). Since fatty acids are known to influence hypothalamic neuropeptide regulating energy homeostasis, we have examined the level of orexigenic neuropeptides, Agouti-related protein (Agrp) and neuropeptide Y (NyP), and anorexigeneic neuropeptides, pro-opiomelanocortin (POMC) and the cocain-and amphetamine-regulated transcript peptide (CART). In fat-1 TG mice, Agrp expression at CT 06 were significantly elevated, however, Nyp, Pomc, and Cart expression at CT 06 and 18 were not changed as compared to wildtype (WT) mice (Fig. 21)

Clock gene expression in fat-1 liver and kidney

To further investigate the elevated clock gene expression in

peripheral tissues in fat-1 TG mice, I have monitored Per2 expression in the livers of Per2::luc and fat-1/Per2::luc TG mice. In accordance with the in vivo imaging results (Fig. 18), the amplitude of Per2 expression in the fat-1 TG mice liver tissue were significantly increased (Fig. 22). Following the results, clock gene transcripts from liver and kidney displayed elevated amplitude of expression throughout 24 h period (Fig. 23). In both liver and kidney, compared to Bmal1, Clock and Per1 expression, Rev-erbα expression were not significantly elevated (Fig. 23). Interestingly, compared to Per2 expression in the SCN, period shifting were not present in liver and kidney.

BMAL1 sumoylation and proteaosomal degradation is increased in fat-1 TG

To understand the enhanced clock gene expression in fat-1 TG mice, I have investigated the turnover process of core clock component, BMAL1. Since oscillating levels of BMAL1 controls the transcription and regulation of clock controlled genes, including Per2, I have questioned whether BMAL1 modifications were changed in fat-1 TG mice. First, I have investigate the stability of BMAL1, I have utilized WT and fat-1 mouse embryonic fibroblast (MEF) cells and treated with cycloheximide (CHX), protein synthesis inhibitor. BMAL1 protein in fat-1 MEF rapidly

diminished, with an estimated half-life of ~2 h, whereas BMAL1 protein from WT MEF displayed estimated half-life of ~3 h (Fig. 24). To delineate the role of proteasome-dependent degradation of BMAL1, I have further treated WT and fat-1 MEF with MG132, 26S specific proteasome inhibitor in presence of CHX. In accordance with previous results, MG132 treatment strongly stabilized BMAL1 protein in both WT and fat-1 MEFs, indicating that increased sumoylation of BMAL1 in fat-1 TG mice demonstrates the induced level of proteaosomal-dependent degradation pathway leading to its enhanced turnover as compared to WT.

Modification of BMAL1 by SUMO and uniquitin in fat-1 MEFs

To elucidate the turnover of BMAL1 in fat-1 TG mice, I further investigated sequential modifications in the circadian regulation of clock gene transcription and analyzed BMAL1 modification by SUMO and ubiquitin during the period of maximal and minimal transcription activation in cellular compartments. First, I treated Per2::luc and fat-1/Per2::luc MEFs with dexamethasone (DEX) for cellular synchronization and monitored luciferase activities. Fat-1/Per2::luc MEF oscillation displayed increased amplitude of Per2 expression, however, length of period were not changed between fat-1/Per2::luc and Per2::luc MEF (Fig. 25A). And the proceeding maximal and minimal transcriptional expression state at 6

h and 18 h, respectively after DEX treatment. Following the luciferase activity, I measured the amounts of modified BMAL1 by SUMO and ubiquitin in cytoplasm and nucleus (Fig 25B). The levels of both the sumoylated and ubiquitinated proteins were markedly higher in the transcriptionally active phase than in the inactive phase which were more prominent in the nucleus than in the cytoplasm. In addition, modified BMAL1 in fat-1 MEF displayed markedly higher sumoylation and ubiquitination in both cellular compartments as compared to WT MEF (Fig 25B). Thus, this demonstrates that the sequential modification of BMAL1 by SUMO and ubiquitin in fat-1 TG mice is highly elevated as compared to WT mice. `

Locomoter activity of wild type and fat-1 TG mice under 12:12 light-dark (LD) cycle following constant darkness (DD) condition

Lastly, to find out the behavioral outcome of fat-1 TG mice, I have analyzed the locomoter activity of fat-1 TG mice. After 10 days of 12 h: 12 h light and dark cycle conditioning, all WT and fat-1 TG mice went to constant dark condition to measure the endogenously generated free-running period (FRP). As expected all WT mice displayed period length shorter than 24 h period averaging about 23.48 h (Fig 26A left). However, fat-1 TG mice displayed ~.51 h of period lengthening ($p<0.001$) close to

24 h of FRP (Fig. 26B). The total wheel revolution per day were not significantly different between the two groups, however FRP close to 24 h in fat-1 mice demonstrates that it holds robust endogenously generated circadian rhythm.

Figure 18. 24 h *In vivo* imaging of Per2::luc and *fat-1*/Per2::luc mice. (A) *In vivo* luminescence imaging of Per2::luc and *fat-1*/Per2::luc mice 6 h interval throughout 24 hr period. Images were assessed simultaneously at each time point. The upper row displays the dorsal position representing images of kidney 8 mins after 15 mg/kg D-luciferin injection. The lower panel represents the images of submandibular gland and liver 10 mins after D-luciferin injection. (B) Normalized photon counts of kidney, liver and submandibular gland (Sub Gla). y-axis represents the recent change of bioluminescence throughout 24 hr period after daily average photon counts were determined for each individual organ. Mean±SEM; n=3.

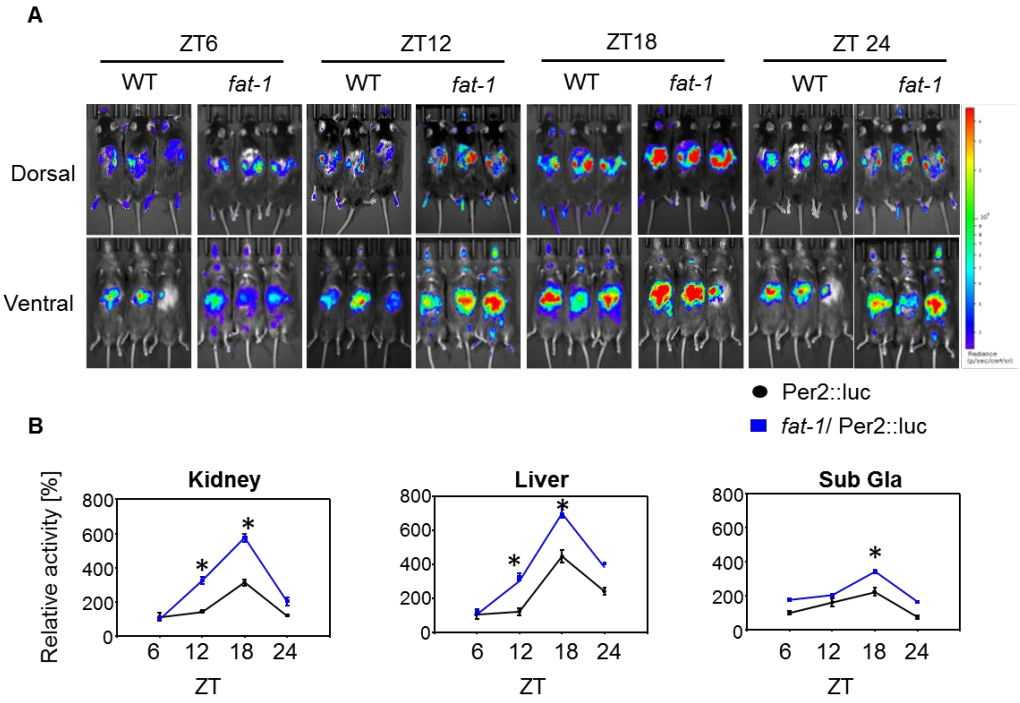

A
ZT6
ZT12
ZT18
ZT 24
WT
fat-1
WT
fat-1
WT
fat-1
WT
fat-1
Dorsal
Ventral
Per2::luc
fat-1/ Per2::luc
B
Kidney
Liver
Sub Gla
Relative activity [%]
800
600
400
200
0
6
12
18
24
ZT
*

Figure 18. 24 h *In vivo* imaging of Per2::luc and *fat-1*/Per2::luc mice. (A) *In vivo* luminescence imaging of Per2::luc and *fat-1*/Per2::luc mice 6 h interval throughout 24 hr period. Images were assessed simultaneously at each time point. The upper row displays the dorsal position representing images of kidney 8 mins after 15 mg/kg D-luciferin injection. The lower panel represents the images of submandibular gland and liver 10 mins after D-luciferin injection. (B) Normalized photon counts of kidney, liver and submandibular gland (Sub Gla). y-axis represents the recent change of bioluminescence throughout 24 hr period after daily average photon counts were determined for each individual organ. Mean±SEM; n=3.

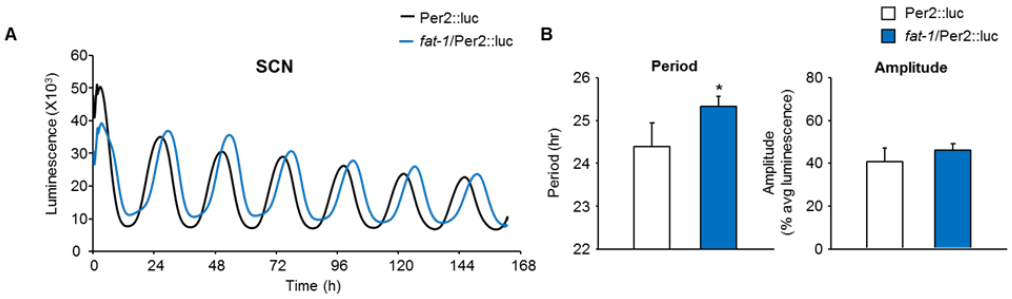

A
Per2::luc
fat-1/Per2::luc
SCN
Luminescence (X10³)
60
50
40
30
20
10
0
0
24
48
72
96
120
144
168
Time (h)
B
Per2::luc
fat-1/Per2::luc
Period
Period (hr)
26
25
24
23
22
*
Amplitude
Amplitude (% avg luminescence)
80
60
40
20
0

Figure 20. Comparison of clock gene expressions in the hypothalamus between WT and *fat-1* TG. (A) mRNA levels of *Bmal1, Clock, Per1 and Rev-erbα* in hypothalamic tissues of *fat-1* and WT mice. Each time point represents average of four individual mice Mean±SEM; n=4. (*, *P<0.05*).

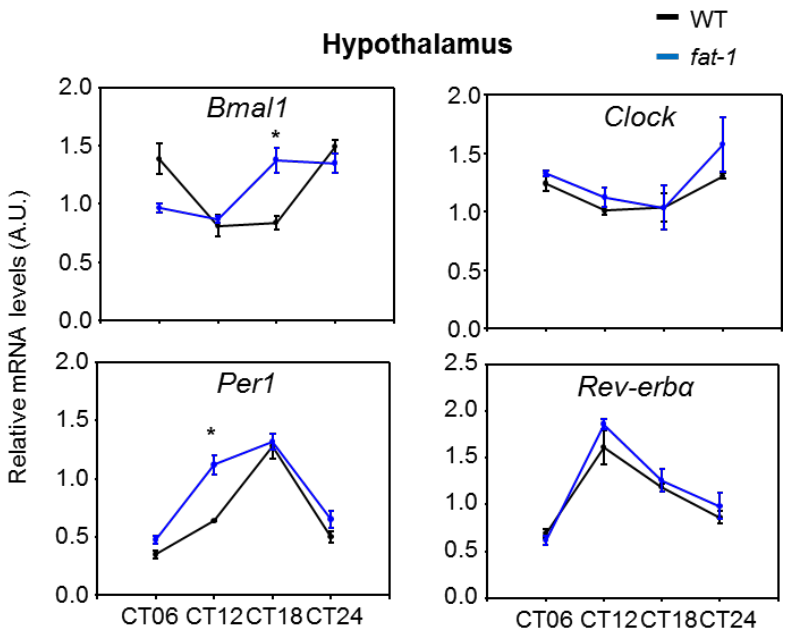

Hypothalamus
WT
fat-1
Bmal1
Clock
Per1
Rev-erbα
Relative mRNA levels (A.U.)
CT06 CT12 CT18 CT24
CT06 CT12 CT18 CT24

Figure 21. Hypothalamic neuropeptide expression between WT and *fat-1* TG Time-specific alterations of mRNA levels in hypothalamic appetite-regulating neuropepties *Nyp, Pomc, Agrp* and *Cart* at CT06 and CT18 of WT and *fat-1* mice hypothalamus. Mean±SEM; n=4. (*,*P<0.05*).

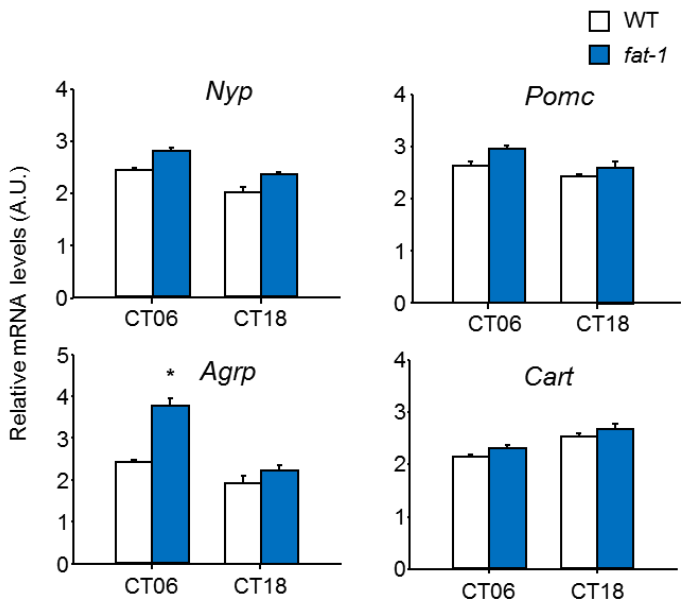

WT
fat-1
Relative mRNA levels (A.U.)
Nyp
Pomc
Agrp
Cart
*
CT06
CT18
0
1
2
3
4
5

Figure 22. Comparison of Per2 expression in *fat-1* WT liver tissue culture. (A) Representative records of bioluminescence showing PER2::LUC and *fat-1*/PER2::LUC liver. Photon counts are plotted against the LD cycle of 24 hrs. Shown are 168 hrs, equivalent of 7 days of continuous explant culture recording. (B) Bar charts summarizing changes in average period and amplitude. Peak-to-peak intervals during indicated time in an explant were averaged, and the mean period ± SEM (hrs) from independent sets of experiments were calculated. Mean±SEM; n=6

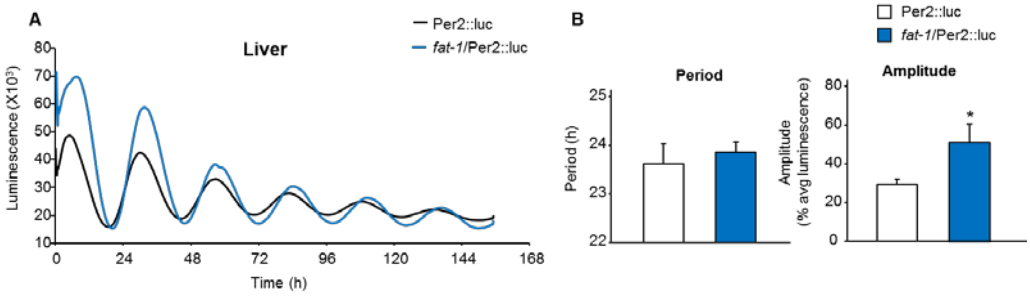

A
Liver
Per2::luc
fat-1/Per2::luc
Luminescence (X10³)
80
70
60
50
40
30
20
10
0
24
48
72
96
120
144
168
Time (h)
B
Period
Period (h)
25
24
23
22
Amplitude
(% avg luminescence)
80
60
40
20
0
*
Per2::luc
fat-1/Per2::luc

Figure 23. Comparison of clock gene expressions in the liver and kidney between WT and *fat-1* TG. *fat-1* and WT mice were sacrificed at indicated times and liver and heart tissues were extracted for mRNA analysis. *Bmal1, Clock, Per1* and *Rev-erbα* mRNA transcripts in (A) liver and (B) heart of *fat-1* and WT. Mean±SEM; n=4.

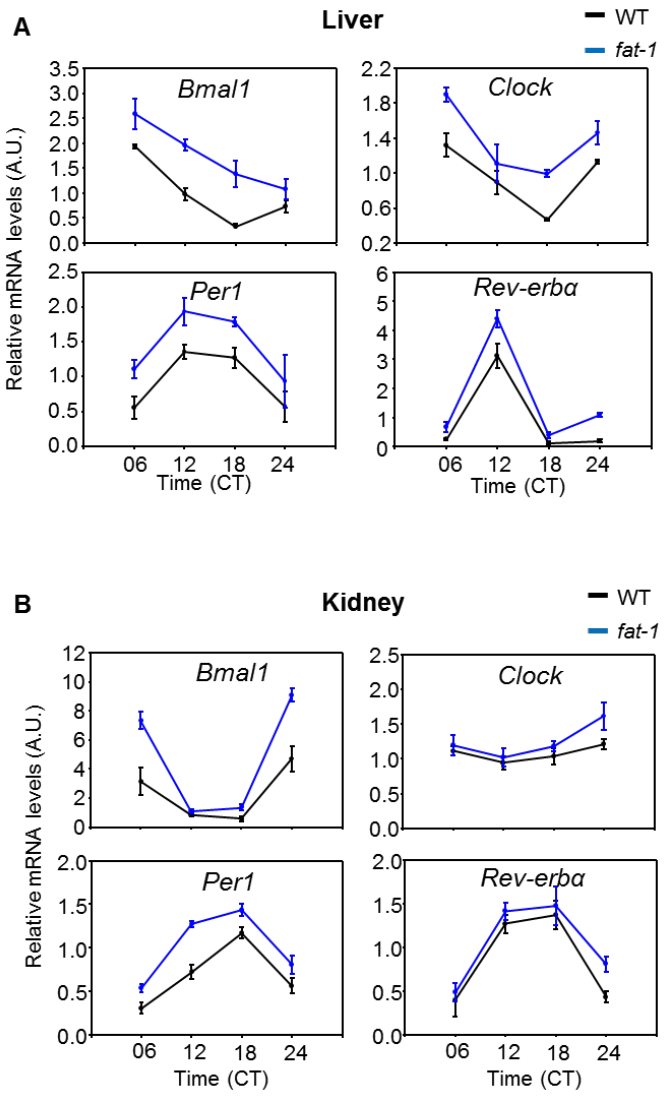

A
Liver
WT
fat-1
Bmal1
Clock
Per1
Rev-erbα
Relative mRNA levels (A.U.)
Time (CT)
06
12
18
24
B
Kidney
WT
fat-1
Bmal1
Clock
Per1
Rev-erbα
Relative mRNA levels (A.U.)
Time (CT)

Figure 24. BMAL1 stability in *fat-1* TG mice liver. WT and *fat-1* MEFs were either treated with cycloheximide (30 μg/ml) alone or together with MG132 (50 μM) for the indicated times. The level of BMAL1 was analyzed by immunoblotting with anti-BMAL1 antibodies and Actin was used as a loading control. Relative BMAL1 levels were quantified by densitometer where each time point consists of means ± SEM of three independent experiments.

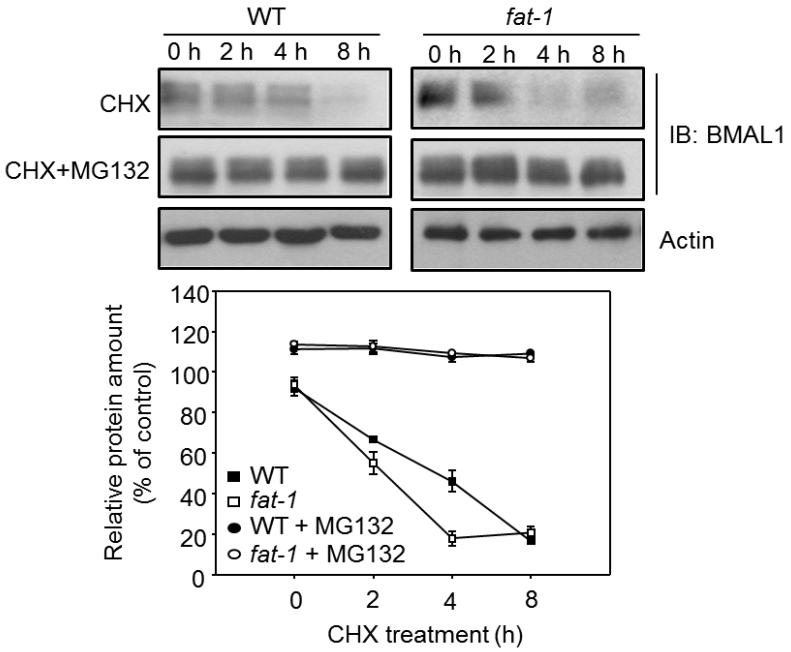

WT
fat-1
0 h 2 h 4 h 8 h
0 h 2 h 4 h 8 h
CHX
CHX+MG132
IB: BMAL1
Actin
Relative protein amount (% of control)
140
120
100
80
60
40
20
0
WT
fat-1
WT + MG132
fat-1 + MG132
0
2
4
8
CHX treatment (h)

Figure 25. Temporal changes of BMAL1 sumoylation and ubiquitination in cytoplasm and nucleus. (A) Representative circadian bioluminescence generated from PER2::LUC and *fat-1*/PER2::LUC MEFs. Cells were synchronized by 1 μM dexamethasone (DEX) for 2 h, and expressions were monitored for 72 hrs. (B) Changes in the abundance of modified BMAL1 by SUMO2/3 and Ub in cytoplasmic and nuclear fraction were analyzed after 6 h and 18 h after 1 μM DEX synchronization. Cells were treated with MG132 (50 μM) for 1 h before the indicated times. Fractions were analyzed by immunoprecipitation with anti-BMAL1 antibodies and immunoblotting with anti-SUMO2/3 and anti-Ub and anti-BMAL1 antibodies.

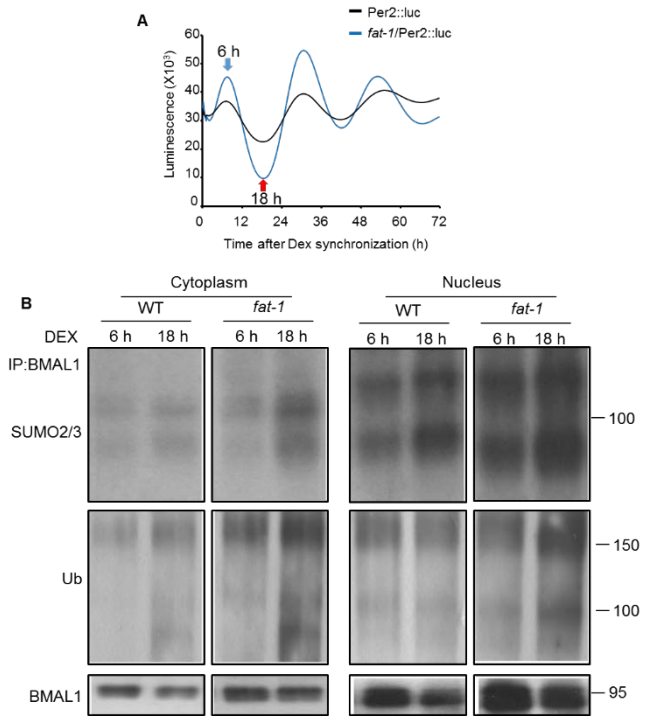
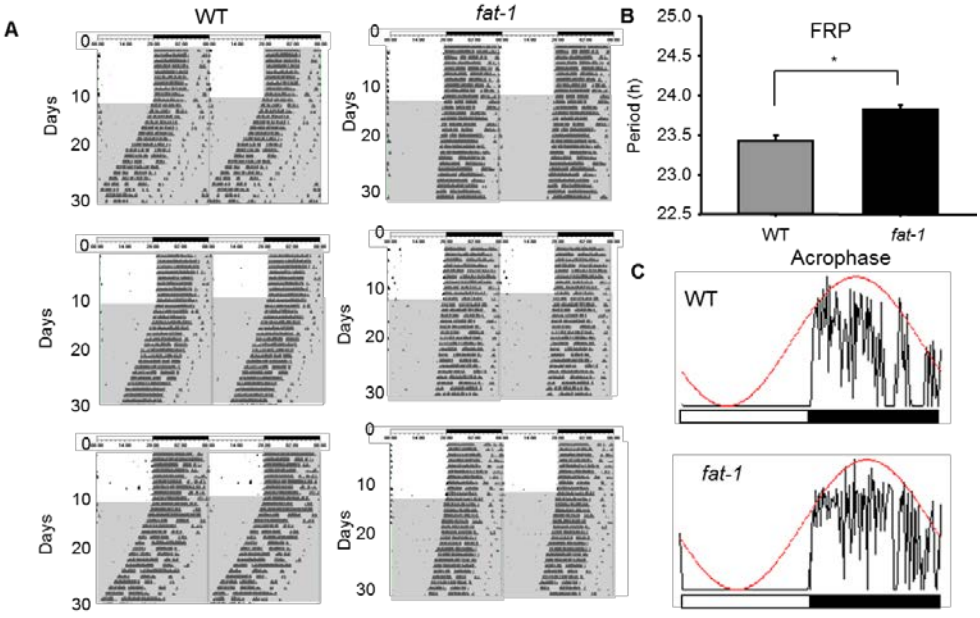

A
Per2::luc
fat-1/Per2::luc
6 h
18 h
Luminescence (X10³)
Time after Dex synchronization (h)
B
Cytoplasm
Nucleus
WT
fat-1
DEX
6 h
18 h
IP:BMAL1
SUMO2/3
Ub
BMAL1
100
150
100
95

Figure 26. Difference of free-running period between WT and *fat-1* TG mice. (A) Recordings of wheel-running activity of >10 weeks old male WT and *fat-1* TG mice entrained in LD cycle for 10 days following DD condition for 20 days. (B) Free-running periods (FRP) of each individual mice during DD (*tau)* condition were analyzed by Chi-square distribution and data represents mean±SEM of WT (n=6) and *fat-1* TG (n=8) mice. (C) Representative acrophase distribution between WT and *fat-1*TG mice.


A
WT
fat-1
Days
0
10
20
30
B
FRP
Period (h)
25.0
24.5
24.0
23.5
23.0
22.5
WT
fat-1
C
Acrophase
WT
fat-1

DISCUSSION

In the present study, I observed systemic effects of omega-3 FA on circadian clock system by utilizing *fat-1* TG mouse. *Fat-1* TG mouse displayed elevated clock gene expression in peripheral tissues including liver and kidney, and increased sumoylation and ubiquitination of core clock gene BMAL1, inducing its turnover. Although clock gene expression were not elevated in hypothalamic tissue, Per2 expression in the SCN and hypothalamic *Bmal1* mRNA transcripts displayed shifting of period. The period shifting of clock gene in the hypothalamic tissue coincided with shifting of the free-running period which were close to ~24 h as WT mouse had period around ~23.48 h.

Fat-1 TG mouse constitutes a novel genetic model to study influence of omega-3 FA in circadian clock. Previous studies have utilized *fat-1* TG mice to cross-breed with various established disease mouse model to investigate the influence of omega-3 FA on development and progression of disease (Kang et al., 2007). For example, Berquin and colleges have investigated genetic effects of omega-3 on prostate cancer by cross-mating *fat-1* TG mice with prostate-specific *Pten*-knockout mice, a prostate cancer animal model, generating $Pten^{P-/-}fat\text{-}1^{T/-}$ transgenic

mice (Berquin et al., 2007). *Pten*$^{P-/-}$*fat-1*$^{T/-}$ transgenic mouse displayed reduced prostate cancer development and increased longevity as compared to *Pten*-knockout mouse (Berquin et al., 2007). In the present study, I have utilized well-established circadian clock Per2 gene expressing animal model, Per2::luc KI mouse (Yoo et al., 2004), to investigate influence of omega-3 FA on global Per2 expression in Per2::luc KI mouse crossed with *fat-1* TG mouse, *fat-1*/Per2::luc. Clearly, our findings extends the previous observation of utilizing dietary supplementation of omega-3 FA on clock gene expression, and investigating *fat-1*/Per2::luc mouse allowed proper evaluation of effect of omega-3 FA on circadian clock gene expression.

Many studies have revealed that high fat diet (HFD) consisting large amount of saturated fatty acids disrupt circadian rhythm. HFD leads to desynchronization between systemic interactions of core clock and local clock regulation (Kohsaka et al., 2007), and also compromise molecular clock system residing in each local clocks. Treatment of palmitic acid to mHypoE-37, immortalized hypothalamic neuronal cell-line, significantly increased transcriptional expression of *Bmal1*, and also caused phase delay of 1.4 h in *Bmal1* and *Rev-erbα* mRNA transcript levels (Greco et al., 2014). In addition to palmitic acid treatment, Greco and colleges demonstrated that DHA treatment also altered *Bmal1* expression by increasing period length and phase advance *Bmal1*

expression different from palmitic acid treatment (Greco et al., 2014). Our study also observed altered hypothalamic clock gene expression in *fat*-1TG mice where *Bmal1* expression displayed phase advance and shifting of Per2 expression in the SCN (Fig. 2). In addition, Furutani and colleges recently demonstrated that administration of tuna oil or soybean oil containing of DHA and EPA in modified dietary animal chow promoted restrictive feeding (RF) induced phase shift in liver clock (Furutani et al., 2015). And mice fed with diet containing DHA and EPA displayed significant phase delay of liver clock and induced three-fold increase of *Per2* mRNA expression in the liver 120 min after RF feeding (Furutani et al., 2015). In *fat-1* TG mice, under normal condition provided with regular chow, I have also observed increased Per2 expression in both *in vivo* imaging (Fig. 1), liver explant cultures, and clock gene transcripts in liver and kidney tissue (Fig. 3). Therefore, results from my investigation clearly demonstrates that both SFA and PUFA, especially omega-3 FA, alters circadian gene expression, however they conduct distinct effects on systematic outcomes.

In particular, I revealed elevated clock gene expression in *fat-1* TG mice consisted of elevated sumoylation and ubiquitination of BMAL1 (Fig. 4 and Fig. 5). It has been reported that modification of BMAL1 by SUMO2/3 and ubiquitin is responsible for circadian transcription activation and degradation of CLOCK/BMAL1 heterodimer complex (Lee

et al., 2008). In *fat-1* MEFs, I observed sequential abundance of sumoylated and ubiquitinated BMAL1 in cytoplasm and nucleus, mostly prominent modification of BMAL1 showing in the nucleus (Fig. 5B). Elevated dual modification of BMAL1 in *fat-1* TG mice lead to increased degradation regulating stability of BMAL1 and its turnover (Fig. 4B). In accordance with previous study, treatment of MG132 stabilized BMAL1 degradation demonstrating sumoylated and ubiquitinated BMAL1 degradation in proteasome-dependent manner (Lee et al., 2008; Kwon et al., 2006). Therefore, from the molecular mechanistic study, I provided possible clues about enhanced clock gene expression involving presence of omega-3 FA.

In addition, I have observed that in DD condition followed by 12:12 LD cycle entrainment, *fat-1* TG mouse displayed lengthening of period close to ~24 h compared to WT mouse showing average period length of ~23.48 h (Fig. 6). Period lengthening in *fat-1* TG mosue under free-running condition correlates with the shifting and elongated clock gene expression in the SCN (Fig. 2) which dictates SCN regulation of entrainment and free-running rhythms under DD condition (Reppert and Weaver., 2002). Similar results were achieved in αMUPA TG mouse encoding overexpression of urokinase-type plasminogen activator (uPA) in the brain (Miskin et al., 1990). Some characteristics of αMUPA mice display spontaneously eating ~25% less and live ~20% longer compare

to WT (FVB/N) mouse (Miskin and Masos, 1997'; Miskin et al., 2005). Similar to my observation of *fat-1* TG mouse, αMUPA mouse exhibit higher amplitude in the circadian expression of the clock genes in the liver and also free-running period close to ~24 h in both 8 months and 18 months old mice (Froy et al., 2006; Gutman et al., 2011; Tevy et al., 2013). Although I did not observe the longevity of *fat-1* TG mice, correlation of enhanced clock gene expression and behavioral locomoter activity with αMUPA mice, *fat-1* TG mice may exhibit longer life-span. And further studies are needed to clarify this issue.

The present study, I investigated effects of omega-3 FA on circadian clock system. And provide evidence for role of omega-3 FA on molecular circadian clock oscillation, and illustrate possible benefits of omega-3 FA influencing pronounced circadian oscillation which may yield longer life

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국문초록

생체시계가 대사기능에 미치는 영향에 관한 연구

노한상

인간이 가지고 있는 생체시계는 주기적인 환경 변화에 적응을 할 수 있도록 시신경교차핵에 존재하는 중추시계나 여러 조직에 존재하는 말초시계에서 동일한 분자적인 조절을 하게 된다. 이러한 생체시계의 분자적 조절은 주기적으로 낮과 밤에 일어나는 현상 외에도 우리 몸에서 여러 가지의 생리적 작용에도 영향을 미친다. 그 중 생체 시계는 우리 몸에서 일어나는 대사 작용에 분자 수준이나 개체 수준에서 많은 영향을 주는 것으로 알려 져 있다.

AMP-activated protein kinase (AMPK)는 주된 에너지 센서로써 몸에서 에너지 양이 줄어들게 되면 활성화되어 대사 조절이나 당 조절을 통하여 에너지 양을 다시 높이는 작용을 한다. 활성화된 AMPK는 이러한 작용 외에도 생체시계에 직접적인 영향을 준다. PER2나 CRY1의 경우 활성화 된 AMPK로 인하여 인산화가 되어 세포 안에서 수준이 저하되게 된다. 여러 가지의 생리적 현상과 대사 작용에 영향을 미치는 AMPK는 비만이나 당뇨병 같은 대사 질환의 주된 표적으로 여

러 약물들이 개발되었다. 이러한 약물 중 당뇨병 치료제로 알려진 메트포민과 신규약물인 HL271이 어떻게 생체시계에 영향을 미치는지 알아보았다. 기존에 알려진 메트포민의 작용기작을 바탕으로 신규약물인 HL271의 작용기작을 연구한 결과 HL271은 메트포민 보나 매우 낮은 농도에서 AMPK를 활성화 시키고 생체 시계 유전자인 PER2와 CRY1의 번역 후 공정 과정을 촉진 시키는 것을 볼 수 있었다. 생리작용으로 체내의 당 수준과 인슐린 정도를 측정해 보았을 때 메트포민과 HL271은 다른 결과를 초래하는 것을 관찰 할 수 있었다. 메트포민의 경우 당 생성에 직접적으로 작용하는 *G6pase* 나 *Pck1*의 수준을 24시간을 주기로 간에서 억제하는 것을 알 수 있었지만 HL271의 경우 낮추지 못하는 것을 볼 수 있었다. 그러므로 인하여 메트포민과 HL271은 분자적으로 AMPK 활성화나 생체시계 유전자 조절에 비슷한 작용을 보이지만 개체 수준에서는 AMPK의 하위 단계에서나 직접적으로 당 수준을 조절하는 분자적 요소들에 다른 영향을 미치는 것을 시사한다.

체내의 지방 및 지방산은 우리 몸에서 중요한 에너지원으로 사용된다. 그 중 불포화 지방산 중 필수 지방 요소의 경우 우리 몸에서 자체적으로 만들 수 없고 섭식을 통하여 얻을 수 있다. 불포화 지방산 중에서도 오메가-3의 경우 뇌 기능 및 정상적인 성장 발달에 매우 중요한 인자로 알려 져 있다. 이렇게 중요한 역할을 하는 오메가-

3를 섭식 외에 체내에서 자체적으로 만들 수 있는 유전자 변형 쥐인, Fat-1 TG를 활용하여 오메가-3가 생체 시계 작용에 어떠한 작용을 미치는지 알아보았다.

Fat-1 TG와 생체 시계의 리포터 쥐로 유전자 변형된 Per2::Luc KI을 교배하여 오메가-3가 중추시계와 말초시계에서 미치는 작용을 실시간 발광 기법을 통하여 연구를 수행하였다. 그 결과 Fat-1쥐의 중추시계는 보통 쥐보다 주기가 빠른 것을 목격할 수 있었고 말초시계에서는 보다 높은 진폭으로 생체시계의 발진에 영향을 미치는 것을 알 수 있었다. 분자적으로 생체시계의 핵심 유전자인 BMAL1의 조절 기작을 연구 해본 결과 Fat-1 TG에서의 경우 BMAL1의 수준이 세포질이나 핵내에서 보통 쥐보다 높은 수준으로 존재하는 것을 확인할 수 있었고 BMAL1은 번역 후 과정 중 ubiquitination과 sumoylation된 형상으로 존재하는 BMAL1이 상대적으로 많은 수준에서 존재하는 것을 목격할 수 있었다. 그러므로 인하여 오메가-3는 중추시계나 말초시계에서 각각 다른 작용을 보이지만 결론적으론 생체시계의 핵심유전자인 BMAL1의 활성과 번역 후 공정 과정에 영향을 미침으로써 주기의 변화나 진폭을 높이는 것임을 밝힐 수 있었다. 더불어 오메가-3가 분자적으로 중추시계나 말초시계에서 미치는 영향을 행동학적으로 분석 해본 결과 보통 생쥐의 경우 내제적인 시계가 평균적

으로 약 23.5시간의 주기를 보이는 반면, Fat-1 TG의 경우 24시간에 가까운 주기성을 보이는 것을 목격할 수 있었다. 이러한 결과를 예전의 연구들과 비교해 보았을 때 Fat-1 TG에서 만들어 지는 오메가-3는 생체시계를 보다 튼튼하고 강건하게 조절을 하는 것으로 판단된다. 그러므로 본 연구를 통하여 생체시계와 대사 작용의 연관성과 조절 작용을 한 층 더 알아낼 수 있었고 앞으로 생체시계와 오메가-3의 작용 기전에 대한 이해 및 상호작용 연구에 많은 도움이 될 것으로 생각된다.

주요어: 일주기리듬, AMPK, 메트포민, HL271, 오메가-3, Fat-1 TG, Period2, Cryptochrome 1, Bmal1.

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