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ORIGINAL RESEARCH

Short-Term Circadian Disruption Impairs Bile Acid and Lipid Homeostasis in Mice

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SUMMARY

Short-term circadian disruption disturbs bile acid and lipid homeostasis in mice, in part via transcriptional occupancy of the *Cyp7a1* promoter by HNF4α and Dbp. Coupled with Western diet, free fatty acids are increased, and hepatic clock gene expression is altered.

BACKGROUND & AIMS: Bile acids are physiologic detergents that also activate nuclear receptors to regulate glucose and lipid homeostasis. Cholesterol 7α-hydroxylase (Cyp7a1), the rate-limiting enzyme that converts cholesterol to bile acids, is transcriptionally regulated by bile acids and circadian rhythms. Fasting, nutrients, and the circadian clock critically control hepatic bile acid and lipid homeostasis, and circadian misalignment is associated with the metabolic syndrome in humans. To delineate these interactions, we employed a sleep disruption model to induce circadian disruption and examined hepatic metabolism with respect to bile acids, lipids, and clock gene expression.

METHODS: B6xC57 mice were maintained on chow or Western diet and were sleep disrupted for 6 hours/day for 5 days. Mice were sacrificed at 4-hour intervals over 24 hours. Hepatic metabolic genes were examined, and bile acid pool and lipid profiles were measured over 24 hours.

RESULTS: Sleep disruption significantly suppressed circadian expression of core clock genes, genes involved in lipid metabolism, and key regulators of *Cyp7a1* as well as *Cyp7a1* expression itself. Sleep disruption abolished the peak in serum cholesterol and increased liver and serum free fatty acids. Bile acid pool size was increased while liver bile acids were decreased. Chromatin immunoprecipitation assay revealed that hepatocyte nuclear factor 4α (HNF4α) and D-site binding protein (Dbp) occupancies were suppressed at the Cyp7a1 promoter in sleep-disrupted mice. When coupled with Western diet, sleep disruption abolished liver clock rhythms and elevated free fatty acids.

CONCLUSIONS: Even short-term circadian disruption dramatically alters hepatic clock gene expression, bile acid metabolism, and lipid homeostasis to contribute to dyslipidemia. *(Cell Mol Gastroenterol Hepatol 2015;1:664–677; http://dx.doi.org/10.1016/j.jcmgh.2015.08.003)*

Keywords: Bile Acid Synthesis; Circadian Rhythm; Lipid Metabolism.

Many cellular and physiologic reactions occur at specific times of the day and follow a circadian rhythm (*circa*: approximate; *diem*: day), which allows for timing of biochemical and behavioral processes that are synchronized to the external environment. The environmental light/dark cycle is the predominant Zeitgeber, or entraining agent, to the hypothalamic biological clock (the suprachiasmatic nucleus), which is the master synchronizer that coordinates timing of central and peripheral rhythms, including the sleep-wake cycle, the fasting-feeding cycle, and body temperature.[1] On a cellular level, organisms are met with the need to preserve glucose and lipid homeostasis over 24 hours. In peripheral organs such as the liver and white adipose tissue, neural and hormonal circadian signals serve to temporally segregate metabolic reactions. This, coupled with circadian regulation of activity, allows for advantageous metabolic responses to changes in the external environment such that individual organs and tissues are operating under maximal efficiency.

In mammals, core molecular clockwork is present in nearly all cell types and consists of rhythmically expressed clock genes that produce an autoregulatory feedback loop. The products of these clock genes transcriptionally activate and repress clock gene activity, such that the core molecular clock oscillates with a period of ∼24 hours. Among these genes, circadian locomotor output cycles kaput (*Clock*) and brain and muscle Arnt-like 1 (*Bmal1*) represent the forward limb of the regulatory loop, whose proteins heterodimerize and drive transcription of period (*Per*) and cryptochrome (*Cry*) genes. PER and CRY enter the nucleus and subsequently inhibit CLOCK/BMAL1 activity, thus reducing their own transcription. The molecular clock is further regulated by an additional feedback loop in which the nuclear receptors reverse-erythroblastosis α (*Rev-erbα*) and retinoic acid-related orphan receptor α (*RORα*) negatively and

Abbreviations used in this paper: **BMAL1, brain and muscle Arnt-like 1; CCG, clock-controlled genes; ChIP, chromatin immunoprecipitation; CLOCK, circadian locomotor output cycles kaput; CYP7A1, cholesterol 7α-hydroxylase; DBP, D-site binding protein; HNF4α, hepatocyte nuclear factor 4α; Per, period; Rev-erbα, reverse-erythroblastosis α; RORα, retinoic acid-related orphan receptor α; Shp, small heterodimer partner; Srebp-1, sterol regulatory element-binding protein-1; ZT, Zeitgeber time.**

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positively, respectively, regulate *Bmal1* transcription by competing for retinoic acid-related orphan receptor response elements in the *Bmal1* promoter.[2] These interlocking regulatory loops allow for temporal management of many metabolic processes, including glucose and cholesterol homeostasis.

Recent studies indicate approximately 10% of genes within the liver transcriptome are rhythmically expressed,[3] up to 20% of the mouse liver proteome is under circadian control,[4] and the phases of gene and protein expression can be used as reporters of liver clock time. One such gene is the rate-limiting enzyme involved in the conversion of cholesterol to bile acids, cholesterol 7α-hydroxylase (*Cyp7a1*), which exhibits a circadian rhythm of expression of mRNA, protein, and activity in rodents and humans.[5,6] Bile acids are amphipathic molecules that aid in absorption of dietary lipids and regulate the gut microbiome population. They are also natural ligands for several nuclear receptors that regulate metabolic pathways. Conversion to bile acids represents the main catabolic pathway for cholesterol in humans;[7] thus, bile acids and *Cyp7a1* are crucial to homeostatic maintenance of lipid metabolism.

Growing research evidence suggests disruptions in circadian rhythms negatively impact human health. Disturbances such as sleep deprivation, shift work, and the 20th-century phenomenon of increased exposure to light at night are associated with increased incidence of cardiovascular events,[8] gastrointestinal disorders,[9] cancer,[10] and metabolic syndrome.[11,12] Synchronization between internal physiology and external environment is crucial, but in modernized populations this fundamental relationship may be increasingly disturbed. Recently, it was reported that sleep restriction altered transcriptional regulation of white adipose tissue in mice[13] and the *Clock*$^{\Delta 19}$ mutation in mice may contribute to the deregulation of lipid accumulation or mobilization.[14] Studies in humans demonstrate an association between shift work or chronodisruption and the development of obesity.[15,16]

Given these facts and the implications for bile acids in the regulation of glucose and lipid homeostasis, we employed a sleep-deprivation protocol in mice to examine the circadian and epigenetic regulation of bile acid and liver metabolism under disrupted circadian conditions.

Materials and Methods

Animals and Diets

Cohorts of 2- to 4-month-old female wild-type mice (B6xC57 background) were bred in-house, maintained on a standard chow diet and water ad libitum, and were housed in a temperature-controlled room with a 12/12 light/dark cycle, Zeitgeber time (ZT) 12 = lights off (6:00 PM). Mice were individually housed, and sleep-disruption techniques were performed using gentle stimulation (physical contact with a soft artist's paintbrush and light puffs of air) for 6 hours/day for 5 days during the middle of the light phase (ZT 2 – ZT 8). Body weight and daily food intake were recorded, and the mice were visually monitored continuously for signs of sleep during the procedure. The control mice were monitored and were not manipulated. After the sleep-disruption procedure had been completed on day 5, the mice were deeply anesthetized with isoflurane vapor and were sacrificed (in the fed-state) by cervical dislocation in 4-hour intervals (ZT 2, 6, 10, 14, 18, 22; n = 5–6 mice per time point) followed by rapid tissue extraction. The samples were stored at −80°C until analysis.

Additional cohorts of female wild-type mice were maintained on a high-fat, high-cholesterol Western diet (42% kcal from fat, 0.2% cholesterol, cat. no. TD.88137; Harlan Laboratories; Indianapolis, IN), and their body weight growth was monitored. Growth plateaued after approximately 4 months of dietary treatment, after which the mice were subjected to the previously described sleep-disruption protocol for 5 days (n = 4 mice per time point) and were sacrificed at the previously mentioned intervals.

All animal procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committee of Northeast Ohio Medical University (protocol #13-023).

Experimental Procedures

Quantitative Polymerase Chain Reaction. Total RNA was isolated using TRI-Reagent (Sigma-Aldrich, St. Louis, MO) from mouse livers harvested at the described ZTs, followed by centrifugation with chloroform and isopropanol. Reverse transcription and quantitative polymerase chain reaction (qPCR) were performed using Taqman probes and primers (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Relative mRNA levels were calculated using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method with values normalized to mouse glyceraldehyde 3-phosphate dehydrogenase expression as an internal control.

Lipid Analysis. Lipids were isolated by homogenizing liver tissue in 7:11:0.1 chloroform/isopropanol/NP-40 followed by evaporation at 60°C. Commercially available kits and reagents were used to quantify the total cholesterol (BioVision Incorporated, Milpitas, CA), triglycerides (Sekisui Diagnostics, Lexington, MA), and free fatty acids (Wako Chemicals, Richmond, VA) in liver and serum.

Bile Acid Analysis. Bile acids were isolated from 100-mg liver samples or whole intestine and gallbladder by a series of extractions and centrifugations in ethanol followed by incubation overnight at 60°C. Content was analyzed by kit (Diazyme, Poway, CA) according to the manufacturer's instructions. The bile acid pool size was calculated by summing the bile acid content in the liver, gallbladder, and intestine.

Chromatin Immunoprecipitation Assay. Effects of sleep disruption on transcription factor recruitment of Cyp7a1 promoter chromatin were investigated using a commercially available chromatin immunoprecipitation (ChIP) kit (EMD Millipore, Billerica, MA). Briefly, nuclei were isolated from 200 mg of pooled frozen liver tissue at each time point over 24 hours. Formaldehyde (37%) was used to crosslink protein to DNA, after which the nuclei were centrifuged and washed. Approximately 1×10^6 nuclei were