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Report

Urinary Bile Acid Shows Diurnal Fluctuation and Phase Shift with Daytime-Restricted Feeding in Rats

Hiroshi Kawai,[*,a] Ai Kurokawa,[b] Takuya Ishibashi,[c] Reiko Iwadate,[a] Naomi Kudo,[a] Yoichi Kawashima,[a] and Atsushi Mitsumoto[b]

[a]*Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan;* [b]*Faculty of Pharmaceutical Sciences, Josai International University, 1 Gumyo, Togane, Chiba 283-8555, Japan;* [c]*Global Innovation Research Organization, Ritsumeikan University, 1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan*

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Dysregulation of the biological clock disrupts the homeostasis of physiological functions, which may lead to the development of various disorders. To investigate the relationship between biological rhythms and disorders, an efficient monitoring method of the biological clock is necessary. In this study, we analyzed the circadian rhythmicity profile of bile acids in urine and investigated whether urinary bile acid (UBA) could reflect the circadian rhythm in liver physiology. Male Wistar-Hannover rats were maintained in metabolic cages under *ad lib* feeding and later subjected to restricted feeding (in which rats were fed only during light periods). Excreted urine was collected in each session, and bile acid contents were analyzed via an enzyme-based total bile acid assay. UBA content showed diurnal fluctuations under both *ad lib* and restricted feeding conditions and reached a peak during the dark period of *ad lib* feeding. In contrast, with restricted feeding, the peak was observed during light periods. Restricted feeding induced an 8-12 h phase shift. Diurnal fluctuation and phase shift by restricted feeding are distinctive features controlled by biological clocks in peripheral tissues. Since bile acids are synthesized in the liver, we propose that UBA might reflect the circadian fluctuation in liver physiology.

Key words circadian rhythm, bile acid, liver, urine, restricted feeding

INTRODUCTION

The importance of circadian rhythms for physiological conditions has been increasingly recognized recently. Physiological functions are optimized by the biological clock to work efficiently under the 24 h light/dark cycle of the earth. Dysregulation of this clock disrupts the homeostasis of the physiological functions, leading to the development of various diseases such as metabolic syndrome and cancer.[1,2] To investigate the relationships between the biological clock and physiological functions, efficient *in vivo* monitoring method for the biological clock is necessary. Since continuous long-term monitoring is often necessary to analyze the circadian rhythm and its disruption, noninvasive methods are preferable to prevent physical damage to either animals or human subjects.

Bile acids are synthesized in the liver and secreted to the duodenum via the bile duct.[3,4] Most of the bile acids are circulated in the enterohepatic circulations, but small portions flow into the systemic circulation and are eliminated in urine.[3,4] Bile acid content in the blood are usually maintained at low concentrations, but increase when the liver and/or biliary tract are damaged. Several studies revealed that the elevation of serum or urinary bile acids indicates liver disorder.[5–7] Bile acid composition in the serum and urine can be an indicator of liver state.

The rate-limiting enzyme in bile acid biosynthesis, CYP7A1, is regulated by the liver clock.[8,9] CYP7A1 shows circadian expression and activity, peaking during dark periods in rats and mice.[10–14] Bile acid content in liver also show diurnal fluctuation similar to CYP7A1.[15–18] Although there are no reports about the diurnal fluctuation of bile acids in urine, urinary bile acid (UBA) content might also show circadian fluctuation according to the circadian rhythm in liver physiology. In this study, we analyzed the diurnal fluctuation of UBA in rats, and analyzed the potential of UBA as an indicator of the circadian rhythm in liver physiology.

MATERIALS AND METHODS

Animals Six- to 7-week-old male Wistar-Hannover rats (150-200 g) were obtained from CLEA Japan Inc. (Tokyo, Japan), and were maintained in an air-conditioned room at 24 ± 2°C with a 12/12 h light/dark cycle (lights on at 07:00). As the time of lights on is defined as zeitgeber time (ZT) 0, rats were maintained under lights during ZT0 to ZT12. The rats had free access to food and water unless otherwise described. Animal maintenance and treatments were in accordance with the Helsinki Guidelines for the care and use of laboratory animals. All procedures were approved by the Institutional Animal Care and Use Committee of Josai International University (approval number: 32).

Urine Collection Rats were individually kept in a metabolic cage for 20 d. Rats were maintained with *ad lib* feeding for 10 d (AF session) followed by restricted feeding for

*To whom correspondence should be addressed. e-mail: hkawai@josai.ac.jp

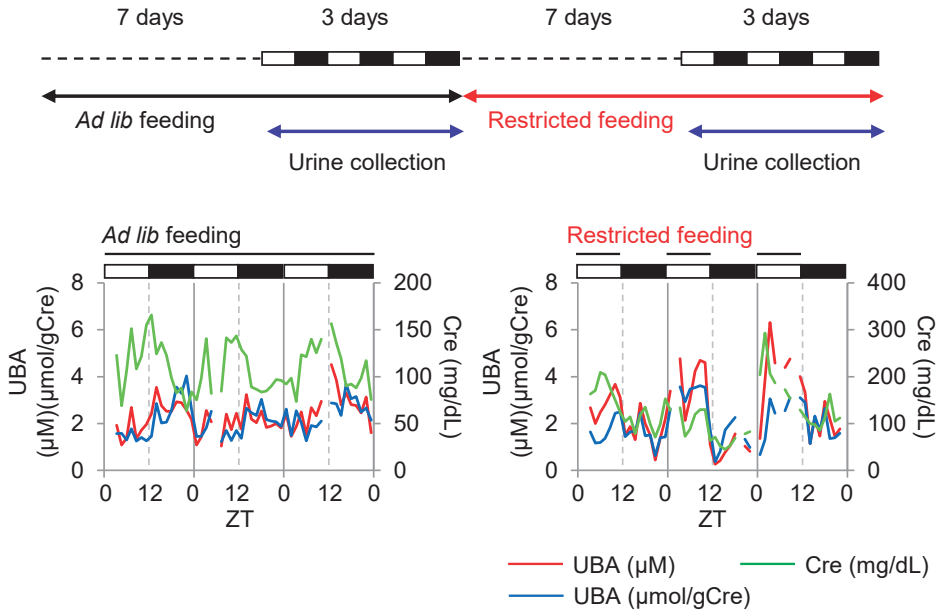

Fig. 1. Experimental Schedule and Representative Results of 3-Day Measurement

The UBA (red line), creatinine (green line), and normalized UBA (blue line) in *ad lib* and restricted feeding sessions are shown. White and black bars above each figure represent light and dark periods, respectively. Lines above the light/dark bars represent feeding period in each session.

10 d (RF session) wherein they were fed only during the light period, ZT0-12. Excreted urine was collected every 80 min in a tube containing 0.1 mL of 2 M HCl during the last 3 d of each session. Collected urine was adjusted to pH 7-9 with 8 M NaOH. Urine samples were frozen and kept at –80°C until analysis.

Quantification of Urinary Bile Acids (UBA) UBA content was measured by using a total bile acid test kit (DIAZYME, Hannover, Germany) according to the manufacturer's instructions with slight modifications as previously described.[7] Two microliters of urine sample mixed with 67.5 μL of thio-NAD solution was prewarmed at ambient temperature for 5 min. Next, 22.5 μL of 3-α-hydroxysteroid dehydrogenase (3αHSD) solution was added to the mixture, and the formation of thio-NADH was monitored by absorbance at 405 nm at ambient temperature. Bile acid content was determined by the change in the absorbance over a period of 30 min.

To normalize the UBA values, we used urine creatinine concentration. The amount of urinary compounds are normalized with creatinine, which has been revealed to be useful for UBA analysis.[6,7] Urine creatinine concentration was measured by the Jaffé method by using a commercially available kit (Wako, Tokyo, Japan) according to the manufacturer's instructions. The normalized UBA values were expressed in micromoles per gram of creatinine (μmol/g Cre).

Statistical Analysis Data are expressed as means ± SEM. Difference in diurnal fluctuation between *ad lib* and restricted feeding was analyzed with two-way repeated measures ANOVA (Fig. 2). Circadian rhythms were analyzed by Cosinor analysis with a single Cosinor method according to Tong (1976) and Cornelissen (2014) by setting the period length as 24 h (Fig. 3 and Table 1).[19,20] In Cosinor analysis, a regression cosine curve based on a data time series is obtained by calculating three curve parameters. Acrophase is the time at which the regression curve is at a maximum. Mesor is the mean regression curve level. Amplitude is the regression curve height from the mesor at the acrophase. Difference between *ad lib* and restricted feeding in each rhythm parameter was analyzed with paired *t*-test (Table 1). All statistical analyses were performed with R (The R Foundation for Statistical Computing, Vienna, Austria). The significance level was set as $p < 0.05$.

RESULTS

Diurnal Fluctuation of UBA in *ad lib* and Restricted Feeding Figure 1 shows the experimental schedule and representative raw data of UBA, creatinine (Cre), and normalized UBA (UBA/Cre) in a rat. UBA, creatinine, and normalized UBA showed diurnal fluctuation. Figure 2 shows the diurnal fluctuation of normalized UBA in 4 rats. Each rat shows fluctuation with a peak during the dark period in *ad lib* feeding session. The peak was shifted in the restricted feeding session. Two-way repeated measures ANOVA detected the interaction effect between ZT and feeding condition ($p < 0.001$) and the main effect of ZT ($p < 0.001$). No significant effect was detected in the main effect of feeding condition ($p = 0.529$).

Circadian Rhythm of UBA Figure 3 and Table 1 show the result of Cosinor analysis of the normalized UBA. The rhythm profile was different between the *ad lib* and restricted feeding sessions. All rats show significant circadian rhythms in *ad lib* session ($R^2 = 0.329$ - 0.495, $p < 0.001$). The mean value of the acrophase was ZT21.2. In the restricted feeding session, the rhythm was unstable and R^2 values were lower than that in *ad lib* session ($R^2 = 0.056$ - 0.255, $p = 0.004$ - 0.295). Although the rhythm was not as robust as that in the *ad lib* session, the mean value of the acrophase in restricted feeding session was ZT9.3. Restricted feeding induced 8-12 h phase shift. Paired *t*-test detected significant difference in acrophase between *ad lib* and restricted feeding ($p = 0.004$).

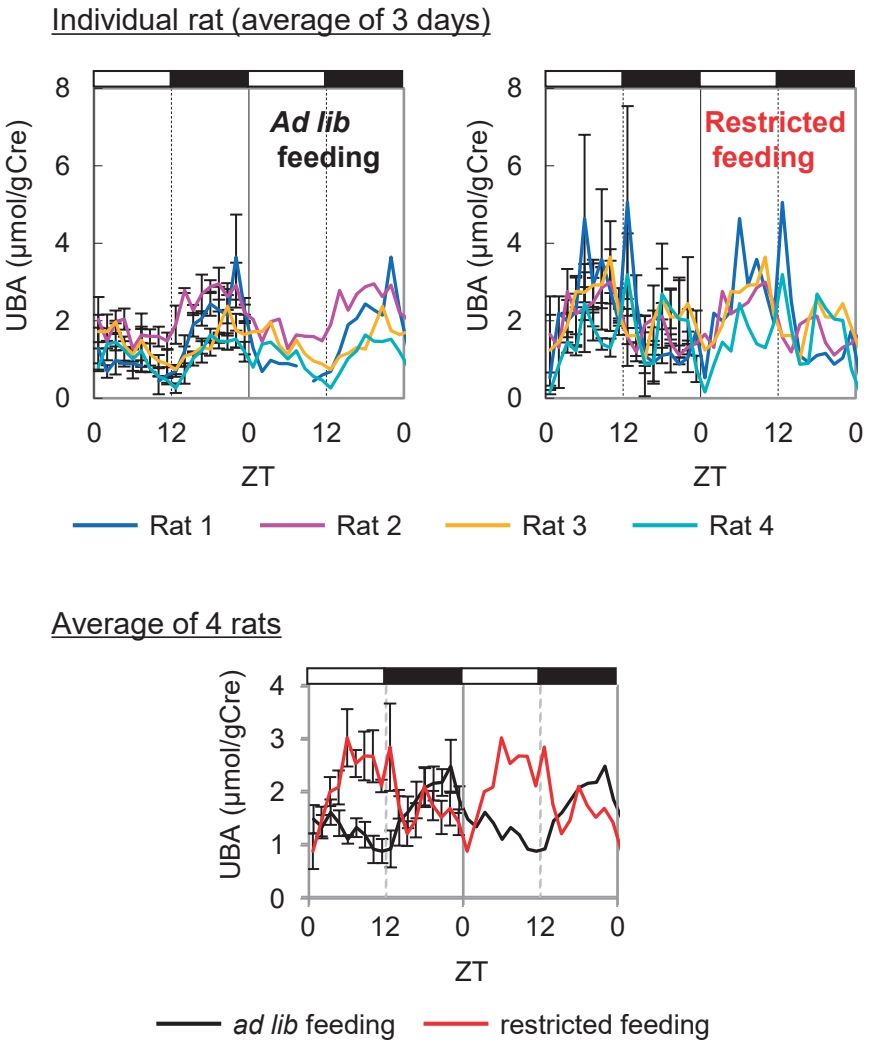

Fig. 2. Diurnal Fluctuation of the UBA

Diurnal fluctuation of the normalized UBA in *ad lib* and restricted feeding sessions are shown as average value for 3 days in each rat (upper panels; n = 1-3) and average of 4 rats (lower panel; n = 4). The second 24 h mention in each graph is duplication of the first 24 h mention, but without error bars. White and black bars above each figure represent light and dark periods, respectively.

DISCUSSION

In this study, we detected circadian fluctuations of UBA and phase shift of the UBA rhythm in daytime-restricted feeding sessions. These circadian profiles are similar to those previously reported in CYP7A1 and bile acids in the liver.[10–18] UBA might reflect the circadian rhythm of CYP7A1 and bile acids in the liver.

Circadian clock systems exist in almost all mammalian cells. The master clock, located in the hypothalamic suprachiasmatic nucleus (SCN), orchestrates the peripheral clocks in other tissues.[21] All clocks are synchronized under natural conditions; however, external stimuli, such as restricted feeding could induce desynchronization of peripheral clocks. Restricted feeding alters the phase of circadian clocks in peripheral tissues including the liver but not the master clock in the SCN.[12,22,23] Therefore, the physiological events that show diurnal fluctuation and phase shift by restricted feeding are considered to be regulated by the peripheral clocks. Biosynthesis of bile acid is regulated by CYP7A1 in the liver, and the expression of CYP7A1 is regulated by several transcriptional factors, which are regulated by the liver clocks.[8,9,13,14] CYP7A1 typically shows a circadian rhythm in its expression and activity with a peak during the active phase (dark period in the nocturnal rodents), and the phase of the rhythm is reversed by daytime-restricted feeding.[13,14] Bile acid content in the liver shows similar rhythm profiles to CYP7A1.[15–17] As shown in Fig. 2 and 3, UBA shows diurnal fluctuations and a phase shift similar to those shown by CYP7A1 and liver bile acids demonstrated in previous studies.[8,9,13–17] Since CYP7A1 and liver bile acid fluctuate according to the liver clocks, UBA rhythm might reflect the circadian rhythm in liver physiology. UBA might be a noninvasive rhythm indicator.

Although several studies have analyzed the bile acid rhythm in the liver and bile, only a few studies have reported on the bile acid rhythm in blood, and there are inconsistencies among these studies. Serum bile acid showed diurnal fluctuations with a peak during late light to dark period under *ad lib* feeding, and daytime-restricted feeding shifted the peak to the light period.[13,16,18] These profiles are consistent with the bile acid rhythm in the liver and bile, as reported by previous studies, and also consistent with the UBA rhythm in the present study. However, another study reported that plasma bile acid concentrations did not show a circadian rhythm in *ad lib* feed-

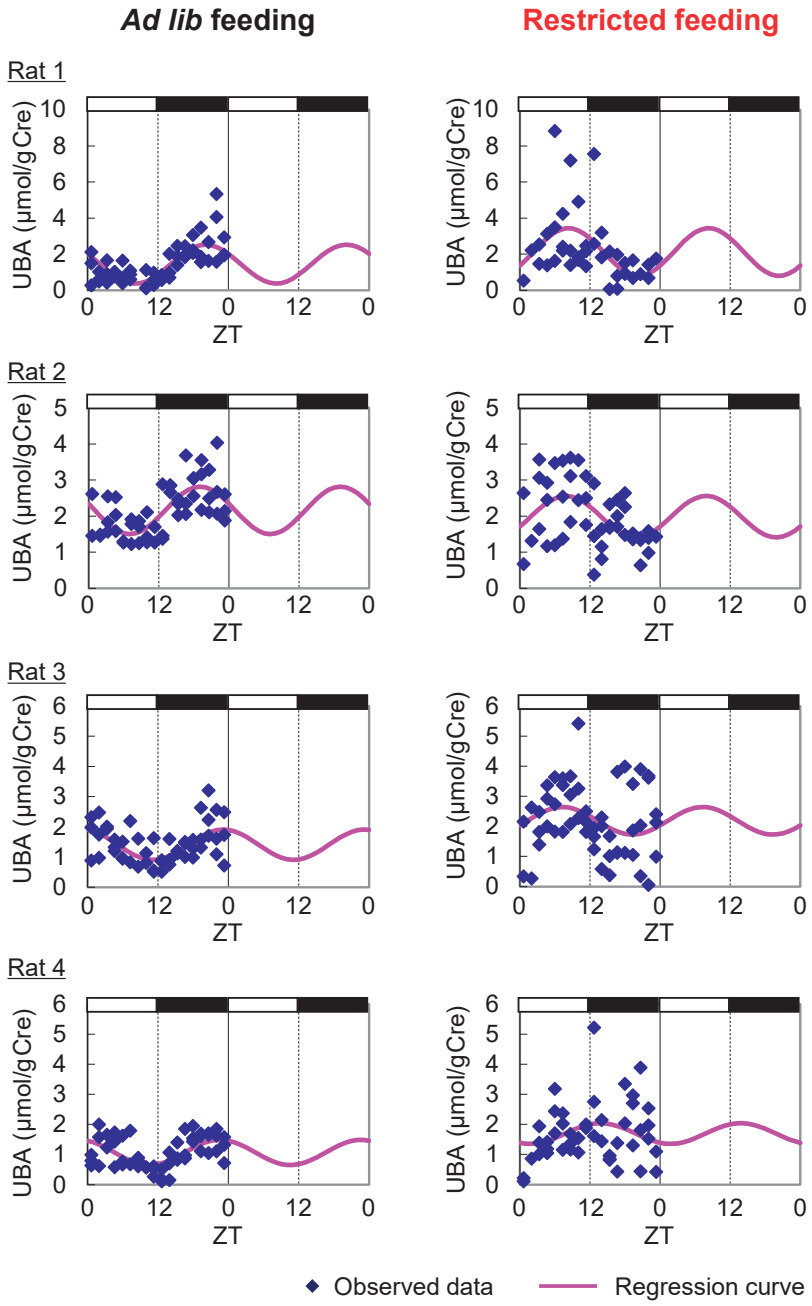

Fig. 3. Circadian Rhythm of the UBA Fluctuation

Each plot represents an observed value of normalized UBA during 3 days in *ad lib* (left panels) and restricted feeding sessions (right panels), and a curved line represents the regression cosine curve determined by Cosinor analysis. White and black bars above each figure represent light and dark periods, respectively.

Table 1. Circadian Rhythm Parameters of UBA Determined by the Cosinor Analysis

	Rat 1	Rat 2	Rat 3	Rat 4	Mean ± SEM
Ad lib feeding					
Mesor	1.44	2.16	1.41	1.07	1.52 ± 0.23
Amplitude	1.08	0.66	0.50	0.42	0.66 ± 0.15
Acrophase (ZT)	20.1	19.1	23.1	22.6	21.2 ± 0.97
p	< 0.001	< 0.001	< 0.001	< 0.001	
R^2	0.495	0.459	0.329	0.325	
Restricted feeding					
Mesor	2.12	1.98	2.19	1.68	2.00 ± 0.11
Amplitude	1.32	0.57	0.46	0.34	0.68 ± 0.22
Acrophase (ZT)	8.3	7.9	7.3	13.8	9.3 ± 1.5 **
p	0.005	0.004	0.135	0.295	
R^2	0.255	0.226	0.080	0.056	

** p < 0.01vs *ad lib* feeding by paired *t*-test.

ing and that daytime-restricted feeding induced a circadian rhythm of plasma bile acid with a peak during the dark period in rats.[14] We could not explain this inconsistency between the results of previous studies. Hence, further studies on the circadian regulation of metabolism and excretion of bile acid are necessary to clarify the mechanism that determines the circadian rhythmicity of bile acid in blood and urine.

The UBA analysis might be useful for long-term continuous monitoring of the circadian rhythm in liver physiology in rats as described above. One of the limitations of the UBA analysis as a rhythm indicator is its applicability to humans, as there are species differences in bile acid composition. In rats, sulfated bile acid is less than 1% of the total bile acid in urine, whereas 85% of total bile acids are sulfated in human urine.[24] Most sulfoconjugation occurs at the 3-hydroxyl group.[24] The present study used a bile acid-measuring kit with 3αHSD, which cannot detect 3-sulfated bile acid without desulfation. The UBA measurement in humans might be more difficult to accomplish than in rats with the present detection method. Urinary sulfated bile acid (USBA) might be an alternative to UBA for analyzing human samples. The USBA analysis kit available is useful to diagnose hepatic disorders.[25,26] Further analysis of UBA and USBA would clarify the utility of these urinary bile acids as a liver rhythm indicator in humans.

Several groups have developed unique methods to estimate *in vivo* profiles of the biological clocks. Clock gene analysis of hair follicle cells can predict the profile of clock gene expression rhythms.[27] This method only requires a plucked hair as a sample and can evaluate the rhythm of the biological clocks in human hair tissue. The other method analyzes blood metabolites by metabolomics-based detection of hundreds of small molecules that show circadian oscillation in blood, further estimating the phase of the internal body time by analyzing relative contents of these circadian metabolites.[28] Compared to previous methods, UBA rhythm analysis in this study was not robust, and significant amounts of sample were necessary to estimate rhythm profiles. However, UBA analysis is less invasive than by previous methods and can be conducted by performing a simple assay with commercially available kits. Noninvasive simple measurement and specificity to liver rhythm might be useful characteristics of UBA analysis.

Conflict of interest The authors declare no conflict of interest.

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