Cacao polyphenols regulate the circadian clock gene expression and through glucagon-like peptide-1 secretion

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(Received 17 March, 2020; Accepted 24 March, 2020)

Energy metabolism and circadian rhythms are closely related together, i.e., the timing of nutrient intake affects metabolism under the regulation of circadian rhythms. Previously, we have reported that cacao liquor procyanidin (CLPr) promotes energy metabolism, resulting in preventing obesity and hyperglycemia. However, it is not unclear whether CLPr regulates clock gene expression. In this study, we investigated whether the administration timing of CLPr affected clock gene expression and found that CLPr regulated the circadian clock gene expression through the glucagon-like peptide-1 (GLP-1) signaling pathway. CLPr administration at Zeitgeber time 3 increased the expression level of Per family and Dbp in the liver. At the same administration timing, CLPr increased GLP-1 and insulin concentration in the plasma and phosphorylation of AMPK in the liver. It was noteworthy that an antagonist for GLP-1 receptor Exendin (9-39) canceled CLPr-induced GLP-1 and insulinemia alteration. Thus, CLPr is a possible functional food material for prevention and/or amelioration of metabolic disorders through preventing circadian disruption through GLP-1 and AMPK pathways.

Key Words: circadian clock, glucagon like peptide-1, cacao liquor procyanidin, AMPK, liver

All animals and plants have 24-h circadian rhythm to synchronize biologicalfunctions for adapting to the environmental changes. The molecular oscillator of circadian clock is regulated by the transcription/translation feedback loops consisting of heterodimeric transcription factors, brain and muscle arnt-like 1 (BMAL1), circadian locomotor output cycles kaput (CLOCK) and their repressors, cryptochromes (CRY) and period (PER). In mammalian, central clock is located in the hypothalamic suprachiasmatic nucleus that receives light and regulates peripheral clocks in both healthy and obese rats. It is recently reported that (-)-epigallocatechin-3-gallate, a major catechin in green tea, ameliorates diet-induced metabolic syndrome associated with the circadian clock. Palmitate could alter the clock genes through GLP-1 secretion in GLUTag L-cells. These results prove that polyphe nols have a potential to affect metabolisms through altering the rhythms of peripheral clocks. It is, however, poorly reported that the effects of ingestion timing of polyphenols on clock gene expression. Ingestion of caffeine at night, but not at morning, delayed the rhythms, though its underlying mechanism is still unclear.

In this study, we focused on cacao polyphenols which are reported to have various health beneficial effects. Cacao liquor procyanidin (CLPr), which is an extract from cacao liquor containing peptide-1 (GLP-1) secretion from intestinal L-cells after food ingestion also has circadian rhythms. Binding of GLP-1 to its receptor in pancreas, resulting in the induction of insulin secretion to maintain the blood glucose homeostasis. In contrast, insulin and GLP-1 also regulate the clock gene expression. For example, insulin is possible to reset clock phase in cultured hepatocytes. Insulin also promotes Akt-mediated phosphorylation of BMAL1 at Ser42, which leads to the reduction of BMAL1 nuclear accumulation to reset hepatic circadian rhythms. Food intake and insulin injection cause the up-regulation of Per2 mRNA and down-regulation of Rev-erba mRNA within 2 h in normal mice, whereas these changes are not observed in streptozotocin-treated insulin-deficient mice. Therefore, insulin is a key factor for regulating circadian rhythm, but it is still unclear how hyperinsulinemia alters the expression of clock genes in the liver. It is also reported that a GLP-1 receptor agonist Exendin-4 affects the Per1 clock gene mRNA level in the liver 12 h after the administration, though the underlying molecular mechanism is unclear yet. These hormones and expression of peripheral clock genes affect each other, i.e., circadian clock genes and metabolism have reciprocal relationship. Therefore, clock genes play an important role in the regulation of metabolism related to maintaining homeostasis.

Not only nutrients but also food factors, especially polyphenols, have been noted to affect clock genes. Recent reports have demonstrated that polyphenols can adjust and/or regulate the circadian rhythm. For example, resveratrol affects the circadian rhythm of Sirt1 expression to restore the clock genes in Rat-1 fibroblast cells. In mice, resveratrol restores the circadian rhythmic disorder of lipid metabolism induced by the intake of high-fat diet. Proanthocyanidins have the ability to modulate peripheral molecular clocks in both healthy and obese rats. It is recently reported that (-)-epigallocatechin-3-gallate, a major catechin in green tea, ameliorates diet-induced metabolic syndrome associated with the circadian clock. Palmitate could alter the clock genes through GLP-1 secretion in GLUTag L-cells. These results prove that polyphenols have a potential to affect metabolisms through altering the rhythms of peripheral clocks. It is, however, poorly reported that the effects of ingestion timing of polyphenols on clock gene expression.

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doi: 10.3164/jcbn.20-38
taining (+)-catechin, (-)-epicatechin and procyanidins abundantly, prevents hyperglycemia by stimulating glucose uptake and glucose transporter type 4 translocation through AMP-activated protein kinase (AMPK) pathway in muscle cells.[25,26] CLPr increases energy metabolism and prevents obesity and hyperglycemia through AMPK pathway.[26] Moreover, CLPr activates enteroendocrine GLP-1/insulin pathway to reduce the postprandial hyperglycemia. Oral intake of cinnamtannin A2, a tetrameric procyanidin contained in CLPr, increases the GLP-1 and insulin secretion in mice.[27] From these results, we hypothesize that CLPr regulates the circadian clock gene through GLP-1/insulin and AMPK pathways. In this study, we investigated the effects of CLPr on the expression of circadian clock genes in mice by focusing on the function of GLP-1.

Materials and Methods

Reagents. Plasma glucose, GLP-1, insulin and adiponectin levels were measured using the corresponding commercial assay kit [Mouse Insulin Elisa Kit (RTU) (Akrim-011RU) and GLP-1 (Active) Elisa Kit (Akmgp-011)] were purchased from FUJIFILM Wako Shibayagi Co. (Gunma, Japan), while Lab assay Glucose kit was from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Exendin (9-39) was obtained from R&D Systems, Inc. (Minneapolis, MN). Antibodies against AMPK, p-AMPK liver kinase B1 (LKB1), p-LKB1 and p-CaMKII/calmodulin-dependent protein kinase kinase (CaMKK) 2 and β-actin (Cell Signaling Technology Inc., Beverly, MA) were used in this study. Antibody against CaMKK2 was purchased from Abcam (Hercules, CA). All other reagents used were of the highest grade available from commercial sources.

Polyphenol composition of CLPr. CLPr was prepared from cacao liquor as previously reported and kindly provided from Meiji Holdings Co., Ltd. (Tokyo, Japan).[28] Polyphenols in CLPr were quantified by a high performance liquid chromatography and liquid chromatography-mass spectrometry as previously described.[28,29] Polyphenol composition of CLPr is shown in Table 1 and the amounts of individual polyphenol are represented as epicatechin equivalent. The total amount of polyphenol was separately measured by the Prussian blue method.[30]

Animal treatment. All animal experiments were approved by the Institutional Animal Care and Use Committee (Permission #27-05-08) and carried out according to the guidelines for animal experiments at Kobe University. Male C57BL/6N mice (5 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan), and kept in a temperature-controlled room (22 ± 2°C) with a 12:12-h light/dark cycle [lights on at 8:00 am: equal to Zeitgeber time (ZT) 0]. Mice were acclimatized for 7 days with free access to a D10102M (AIN-93M base) diet (Research Diets, Inc., New Brunswick, NJ) and tap water.

Experiment 1: After acclimatization, C57BL/6N mice were randomly divided into 8 groups of 5 each. The mice were orally administrated CLPr at 150 mg/kg body weight or water (5 ml/kg body weight) as a control at ZT-3, 3, 9 and 15. After 3 h-administration, the mice were sacrificed under anesthesia using sevoflurane as an inhalational anesthetic and sodium pentobarbital as an analgesic. Liver, skeletal muscle and adipose tissue were taken and used to measurement of the expression level of clock genes by RT-PCR.

Experiment 2: Forty C57BL/6N mice were also randomly divided into 8 groups of 5 each. Twenty mice of them were orally administrated CLPr at 150 mg/kg body weight or water (5 ml/kg body weight) at ZT 3. A GLP-1 receptor antagonist Exendin (9-39) was intraperitoneally injected to the mice at 200 nmol/kg body weight 5 min before the CLPr administration. After 1 h-CLPr administration, the mice were sacrificed under the same anesthesia conditions and blood was collected from cardiac puncture in a heparinized microtube. Plasma was obtained by centrifugation at 5,000 × g for 10 min at 4°C. Plasma level of GLP-1 and insulin was measured by using corresponding commercial kit according to the manufacturer’s instructions. Liver was collected to use for measurement of phosphorylation of LKB1 and AMPK by western blotting. Another twenty mice were also dived into the same groups and given the combination of CLPr and/or Exendin (9-39). They were sacrificed 3 h after the CLPr administration and liver was collected to measure the expression level of clock genes and energy metabolism-related genes by RT-PCR.

Analysis of mRNA by RT-PCR. Isolation and purification of mRNA and preparation of cDNA were performed as previously described.[26] cDNA was subjected to RT-PCR amplification using SYBR Green premix Taq (Takara Bio). Relative gene expression level was calculated by the comparative CT method, using the expression of the Gapdh gene as an internal control.

Western blot analysis. Liver lysate was prepared according to the previous report.[30] After protein concentration in the lysate was quantified by a Lowry’s method,[31] the lysate was subjected

| Gene           | Forward primer          | Reverse primer          |
|----------------|-------------------------|-------------------------|
| Gapdh          | catgctccctctgctgctctta | cctgctccacccctcttga    |
| Per1           | gcttggagggcagctaacct   | tggcttagcggcagttggt    |
| Per2           | caaacaggagagagcatacct  | tctgctcccttcacacac     |
| Per3           | ggaaggcaggaagggcgagca  | tggagagaagaagaagcttcttg |
| Cry1           | atcctgcagctcctacatac   | tccgctcaggtctctgtg     |
| Cry2           | gcaagctcctcctcacacatc  | gcggctcagggtctctgtg    |
| Clock          | ccagctcagctcctccatc    | tggctcctactgcttgacaaa  |
| Bmal1          | tcaagctgacaggcttctatcc | cggctcacttcaccacaaa    |
| Rev-erbα       | cccctgactcataaaaccac   | tgcagctcaggtctctgct    |
| Dbp            | gcattaagccatgagact     | ccagcttcctctcttcttg    |

Table 2. Primer sequences used for real-time PCR amplification

| Gene    | Forward primer          | Reverse primer          |
|---------|-------------------------|-------------------------|
| Catechin| 4.28                    |                         |
| Epicatechin| 6.12                |                         |
| Procyanidin B2| 3.6                  |                         |
| Procyanidin B5| 0.75                 |                         |
| Procyanidin C1| 2.28                 |                         |
| Cinnamoyllicin A2| 1.01              |                         |
| Total polyphenol| 69.8                |                         |
to Western blot analysis following the sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% gel. The proteins were transferred to a polyvinylidene difluoride membrane (Merck Millipore Ltd., Billerica, MA) and the membrane was treated with commercially available blocking solution (Blocking One, Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature. The membrane was incubated with antibodies against, p-AMPK (1:5,000), AMPK (1:5,000), p-LKB1 (1:5,000), LKB1, CAMKK and β-actin (1:10,000) overnight at 4°C, followed by the corresponding horseradish peroxidase-conjugated secondary antibody (1:20,000) for 90 min at 4°C. The proteins bands were visualized using Immuno Star LD (FUJIFILM Wako Pure Chemical Co.) and detected with a light-Capture II (ATTO Co., Tokyo, Japan). Density of the specific band was determined using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis. The data are presented as the means ± SE. Dunnett multiple comparison test (Fig. 1, 5 and 6) or Tukey-Kramer multiple comparison test (Fig. 2–4) was used to determine the significant difference. The level of statistical significance was set to p<0.05.

Results

Effect of single administration of CLPr on the expression of clock genes at four different time-points in the peripheral tissues. In the experiment 1, it was investigated whether a single oral administration of CLPr at ZT-3, 3, 9 and 15 affected the expression rhythm of clock genes in liver, skeletal muscle and adipose tissue. In the liver, CLPr administration at ZT3 increased the expression level of Per1, Per2, Per3 and gene for D-site of albumin promoter binding protein (Dbp) (Fig. 1A–C and I), while decreased that of Bmal1 (Fig. 1G). In addition, CLPr administration at ZT15 increased the expression level of Per1, Per2, Per3 and gene for D-site of albumin promoter binding protein (Dbp) (Fig. 1A–C and I), while decreased that of Bmal1 (Fig. 1G). In addition, CLPr administration at ZT3 increased the expression level of Per1, Per2, Per3 and gene for D-site of albumin promoter binding protein (Dbp) (Fig. 1A–C and I), while decreased that of Bmal1 (Fig. 1G). In addition, CLPr administration at ZT15 increased the expression level of Per1, Per2, Per3 and gene for D-site of albumin promoter binding protein (Dbp) (Fig. 1A–C and I), while decreased that of Bmal1 (Fig. 1G). In addition, CLPr administration at ZT3 increased the expression level of Per1, Per2, Per3 and gene for D-site of albumin promoter binding protein (Dbp) (Fig. 1A–C and I), while decreased that of Bmal1 (Fig. 1G).

CLPr regulates the circadian rhythm of clock gene expression through GLP-1 secretion in the liver. In the experiment 2, to evaluate whether CLPr increased GLP-1 and insulin secre-
tion, the mice were orally administered of CLPr at 150mg/kg
body weight at ZT 3 and measured the concentration of GLP-1 and
insulin in the plasma of the mice 1h after the CLPr administration.
As shown in Fig.4, plasma GLP-1 and insulin concentrations
were significantly increased. When GLP-1 receptor antagonist
Exendin (9-39) was pretreated to the mice 5min before the CLPr
administration, it completely canceled the CLPr-increased plasma
insulin concentration to the control level. On the other hand,
Exendin (9-39) did not affect plasma GLP-1 concentration in both
CLPr and control groups as expected because of the antagonist of
GLP-1 receptor, but not GLP-1 inhibitor.

We further examined whether Exendin (9-39) canceled CLPr-
affected the expression of clock genes in the liver 3 h after the CLPr
administration at ZT3. As shown in Fig. 5, significant changes in
the expression of Per1, Per2, Per3, Cry1 and Cry2 by the CLPr administration
were disappeared by pre-administration of Exendin (9-39).
These results indicated that CLPr-increased GLP-1 secretion was
involved in the altered expression of clock genes.

**Discussion**

Most organisms have circadian rhythm to adjust their metabolic
homeostasis for adaptation to the environment. Circadian rhythm
is regulated by clock genes, especially the core clock ones such as
Per, Cry, Bmal1 and Clock. Feeding behavior and light stimula-
tion regulate amplitude and phase of the clock gene expression. Disruption of the circadian rhythm lead to metabolic disorders
such as type 2 diabetes and obesity. Recently, it has been
reported that functional food ameliorates metabolic syndrome
associating with circadian clock. With regard to CLPr, it
prevents obesity and hyperglycemia. In this study, we found
that CLPr regulates the circadian clock gene expression through
GLP-1 signaling pathway in the liver using a GLP-1 receptor
antagonist Exendin (9-39) (Fig. 1 and 5). GLP-1 also regulated

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**Fig. 2.** Expression of clock genes and energy metabolism-related genes in the muscle 3 h after the administration of CLPr. Mice were orally administered CLPr at 150 mg/kg body weight or water (5.0 ml/kg body weight) at ZT-3, 3, 9 or 15. The expression level of clock genes was measured in the muscle and their mRNA level was normalized by gapdh. The polygonal line graphs for Per1 (A), Per2 (B), Per3 (C), Cry1 (D), Cry2 (E), Clock (F), Bmal1 (G), Rev-erba (H) and Dbp (I) are shown. Data are represented as the means ± SE (n = 5). *Significantly different from the control group at each time point (p<0.05; Dunnett’s test).
CLPr-induced activation of LKB1/AMPK pathway (Fig. 6). This is the first report that the GLP-1 receptor antagonist affected the expression level of clock genes and the GLP-1 was involved in phosphorylation of AMPK promoted by procyanidin-rich CLPr. These previous and current results suggest that the clock gene expression is deeply involved in the prevention mechanism of CLPr on metabolic disorders and that there exists an effective and suitable timing for an intake of procyanidin rich foods to get the health beneficial functions. Liver plays a central role in metabolism and energy utilization and regulates the physiological status of the whole body. It is known that the liver is the most vital zeitgeber organ for peripheral clocks. Results in this study showed that CLPr administration at ZT3 increased expression level of Per1, Per2, Per3 and Dbp in the liver (Fig. 1), indicating that the liver is responsible for the alteration of clock gene expression by CLPr administration. Clock genes in the muscle also weekly respond to CLPr (Fig. 2), but those in adipose tissue showed almost no response against CLPr (Fig. 3). Thus, the liver is the most susceptible tissue among examined three tissues after the intake of procyanidin rich foods. In the liver, it was reported that insulin directly regulates the phase entrainment of circadian oscillators, especially at the light phase: An injection of insulin at the light phase advanced the phase of circadian rhythm and increased the expression of Per1, Per2, Per3 and Dbp through mitogen-activated protein kinase, phosphoinositide 3-kinase and protein kinase Ca in the liver of mice. In the present study, it was confirmed CLPr administration increased plasma insulin and GLP-1 concentrations (Fig. 4) as the same manner as our previous report. In addition, a GLP-1 antagonist Exendin (9-39) completely inhibited CLPr-increased plasma insulin (Fig. 4) and attenuated CLPr-altered expression of Per1, Dbp and Bmal1. Therefore, the alteration of clock genes by CLPr is explainable due to the increased insulin through CLPr-increased secretion of GLP-1. These findings may be involved the improvement of hyperglycemia and energy metabolism by CLPr administration. However, we did not address whether obtained findings were dependent on the phase advance of circadian rhythm by insulin. To clarify this issue, it is necessary to perform more experiments in future.

Recently, it has been reported that GLP-1, which is released from the gut L cells, is involved in not only secretion of insulin from pancreatic β-cells, but also modulation of the metabolisms and energy expenditure. In this study, the GLP-1 receptor antagonist Exendin (9-39) completely canceled CLPr-caused phosphorylation of AMPK (Fig. 6). This finding supports previous one that an agonist of GLP-1 receptor liraglutide promoted.

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**Fig. 3.** Expression of clock genes and energy metabolism-related genes in the white adipose tissue 3 h after the administration of CLPr. Mice were orally administered CLPr at 150 mg/kg body weight or water (5.0 ml/kg body weight) at ZT-3, 3, 9 or 15. The expression level of clock genes was measured in the white adipose tissue and their mRNA level was normalized by gapdh. The polygonal line graphs for Per1 (A), Per2 (B), Per3 (C), Cry1 (D), Cry2 (E), Clock (F), Bmal1 (G), Rev-erba (H) and Dbp (I) are shown. Data are represented as the means ± SE (n = 5). *Significantly different from the control group at each time point (p<0.05; Dunnett’s test).
AMPK phosphorylation in the liver. AMPK is an important regulator of metabolism and circadian rhythm. It is reported that AMPK regulates the expression of several clock gene products in mammalian tissues, because AMPK phosphorylates CRY1 and CRY2 and stimulates their degradation. However, we could not observe CRY family expressions by CLPr administration. These results suggest that changes in the clock gene expression by the CLPr administration at ZT3 is independent of AMPK phosphorylation.

CLPr-increased plasma insulin and GLP-1 levels are involved in the decreased expression of Bmal1 at ZT3, because it is reported that the circadian rhythm of Bmal1 is in parallel with the plasma insulin and GLP-1 levels. Function of Bmal1 in the liver is important to buffering the circulating glucose level in a time-of-day dependent manner, and contributes to systemic glucose homeostasis. On the other hand, we previously reported that a single oral administration of CLPr suppressed postprandial hyperglycemia through both GLP-1 and AMPK pathways. It is known that AMPK and its downstream factor peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) are involved in the decrease in Bmal1 expression. Although PGC-1α expression increased at ZT3 (data not shown), AMPK pathway might not associate with CLPr-caused changes in the clock gene expression as above mentioned. It is, therefore, suggested that CLPr-caused down-regulation of Bmal1 may be involved in amelioration of postprandial hyperglycemia.

Currently, researches in the field of chrono-nutrition are progressing rapidly. It is increasing recognition on the importance of the interaction between food components and expression of circadian clock. Certain polyphenols such as resveratrol, nobiletin and proanthocyanidins possess chronobiological properties and can affect the expression of peripheral clock genes. For example,
resveratrol affects the circadian rhythms by changing the phase shift of Bmal1.\textsuperscript{11,12} Nobiletin protects against insulin resistance and disorders of lipid metabolism through regulation of Bmal1 expression in hepatocytes.\textsuperscript{13,14} The same report demonstrated that nobiletin regulates not only expression of Bmal1 but also phosphorylation of AMPK. Bmal1 and AMPK are the key regulators for metabolic fitness by regulating the hepatic mitochondrial function.\textsuperscript{15} In addition, certain nutrients increase Per2 expression through the insulin secretion.\textsuperscript{16-18} Taken together findings in this study and these previous reports suggest that CLPr acts as a clock-regulator to prevent and/or ameliorate metabolic disorders when administration of it at the suitable timing.

In conclusion, in this study, we found that CLPr affected the expression of circadian clock genes Per1, Per2, Per3 and Bmal1 and promoted secretion of GLP-1 and phosphorylation of AMPK. These findings indicate that CLPr is a possible functional food material for prevention and/or amelioration of metabolic disorders through preventing circadian disruption by activating GLP-1 and AMPK pathways.

**Fig. 6.** Effect of CLPr administration on the phosphorylation of AMPK and LKB1 after pretreatment with GLP-1 receptor antagonist. Mice were orally administered CLPr at 150 mg/kg body weight or water (5.0 ml/kg body weight) at ZT3. Exendin (9-39), a GLP-1 receptor antagonist, was pre-injected to the mice at 200 nmol/kg body weight 5 min before the CLPr administration. The liver was collected 1 h after the CLPr administration and phosphorylation of AMPK and LKB1 was measured by western blotting. Density of the phosphorylation protein was normalized by that of corresponding expression protein. Data are represented as the means ± SE (n = 5). Different letters indicate significant differences (p<0.05 by Tukey-Kramer test).

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| AMPK | AMP-activated protein kinase |
| BMAL1 | brain-muscle arnt like protein 1 |
| CaMKK | Ca\(^{2+}\)/calmodulin-dependent protein kinase |
| CLOCK | circadian locomotor output cycle kaput |
| CLPr | cacao liquor procyanidins extract |
| CRY | cryptochrome |
| DBP | D-element-binding protein |
| ELISA | enzyme-linked immunosorbent assay |
| GLP-1 | glucagon like peptide-1 |
| LKB | live kinase B1 |
| PER | period |
| Rev-erbα | reverse erythroblastosis virus alpha |
| ZT | zeitgeber time |

**Conflict of Interest**

No potential conflicts of interest were disclosed.

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