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Cinnamic acid shortens the period of the circadian clock in mice

Katsutaka Oishi[a,b,c,*,1], Saori Yamamoto[a], Hideaki Oike[a,d], Naoki Ohkura[e], Masahiko Taniguchi[f]

[a] Biological Clock Research Group, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, Japan
[b] Department of Applied Biological Science, Graduate School of Science and Technology, Tokyo University of Science, Noda, Chiba, Japan
[c] Department of Computational Biology and Medical Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba, Japan
[d] Division of Food Function Research, Food Research Institute (NFRI), National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki, Japan
[e] Molecular Physiology and Pathology, School of Pharma-Sciences, Teikyo University, Itabashi, Tokyo, Japan
[f] Division of Pharmacognosy, Osaka University of Pharmaceutical Sciences, Takatsuki, Osaka, Japan

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ABSTRACT

Cinnamic acid (CA) derivatives have recently received focus due to their anticancer, antioxidant, and antidiabetic properties. The present study aimed to determine the effects of cinnamic acid on the circadian clock, which is a cell-autonomous endogenous system that generates circadian rhythms that govern the behavior and physiology of most organisms. Cinnamic acid significantly shortened the circadian period of PER2::LUC expression in neuronal cells that differentiated from neuronal progenitor cells derived from PER2::LUC mouse embryos. Cinnamic acid did not induce the transient mRNA expression of clock genes such as *Per1* and *Per2* in neuronal cells, but significantly shortened the half-life of PER2::LUC protein in neuronal cells incubated with actinomycin D, suggested that CA post-transcriptionally affects the molecular clock by decreasing *Per2* mRNA stability. A continuous infusion of CA into mice via an Alzet osmotic pump under constant darkness significantly shortened the free-running period of wheel-running rhythms. These findings suggest that CA shortens the circadian period of the molecular clock in mammals.

1. Introduction

The central clock located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus governs various behavioral and physiological circadian rhythms in mammals such as sleep/wake cycles, body temperature and blood pressure [1]. The molecular mechanism of the circadian clock is cell autonomous and consists of a network of autoregulatory transcription-based feedback loops that drive the rhythmic expression of clock genes such as *Per1* and *Per2*, and these cell-autonomous and self-sustained oscillators are found not only in the SCN but also in peripheral tissues as well as dissociated cells [2,3]. Functional clock molecules are essential to maintain the normal periodicity of behavioral rhythms [4]. For example, two *Per2* knockout mouse lines have been generated, and the period shortened in both types of mice that subsequently became arrhythmic within 2–3 weeks under constant darkness [5,6]. A mutation in human *Per2* is associated with familial advanced sleep phase syndrome, an autosomal dominant condition with early morning awakening and early sleep times [7]. Deletion of other clock genes such as *Per1*, *Clock*, *Cry1*, and *Cry2* affects the free-running period of circadian behavior in a gene-specific manner, whereas *Bmal1* knockout results in behavioral arrhythmicity under constant darkness [4]. Acute induction of clock gene mRNA expression plays a critical role in phase shifting the circadian clock *in vivo* and in cells cultured *in vitro*. In addition to transcriptional feedback regulation, other essential roles of post-translational modifications such as phosphorylation and ubiquitination have been identified [8,9]. Perturbed clock function is implicated in numerous pathologies including sleep disorders associated with circadian rhythms, psychiatric conditions, hypertension, cardiovascular diseases, cancer and metabolic disorders [3].

Cinnamic acid (CA) and its derivatives are abundant in fruits. vegetables and flowers, and have attracted attention due to their anticancer, antimicrobial, antioxidant and antidiabetic properties [10,11]. Cinnamic acid derivatives such as ferulic acid and cinnamide derivatives also have neuronal effects including anticonvulsant, antidepressant, neuroprotective, analgesic, anti-inflammatory, muscle re-

* Correspondence to: Katsutaka OISHI, Ph.D. Biological Clock Research Group, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan.
E-mail address: k-ooishi@aist.go.jp (K. Oishi).
[1] K.O. and S.Y. contributed equally to this work.

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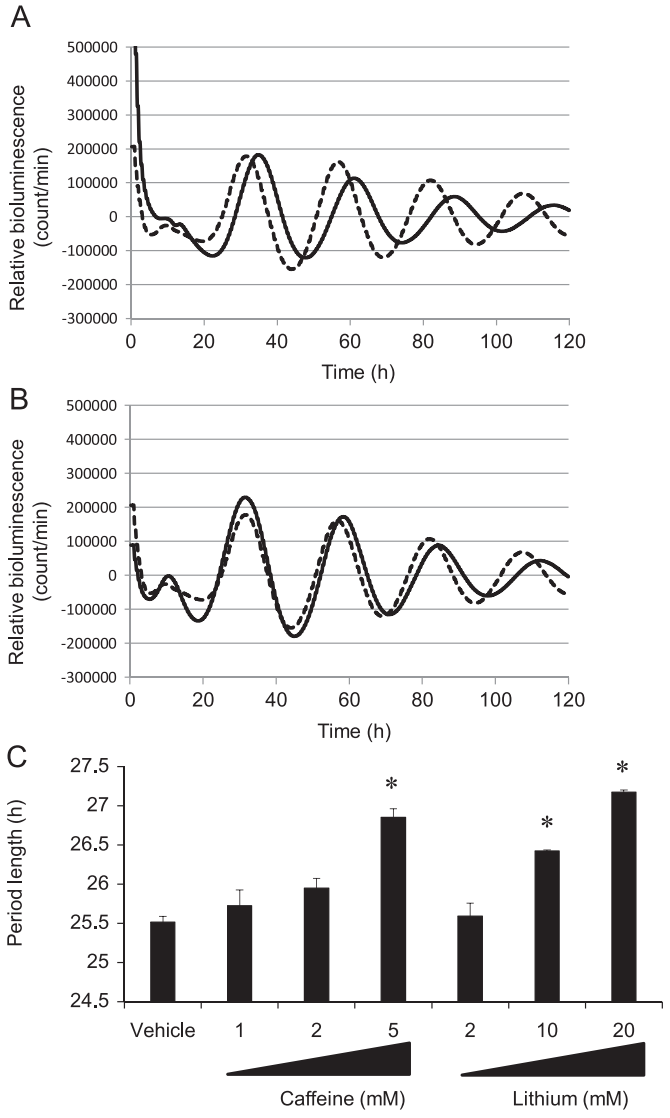

Fig. 1. Caffeine and lithium lengthen circadian period of PER2::LUC expression in neuronal cells. (A) Representative detrended data for PER2::LUC neuronal cells incubated with DMSO vehicle (dashed line) or 5 mM caffeine (solid line). (B) Representative detrended data for PER2::LUC neuronal cells incubated with DMSO vehicle (dashed line) or 20 mM lithium (solid line). (C) Effects of caffeine or lithium on period of rhythmic PER2::LUC expression in neuronal cells. Distance between peaks 1 and 3 was multiplied by 0.5 to determine period length. Circadian period of PER2::LUC bioluminescence oscillations was increased by either caffeine or lithium. $^{*}P < 0.01$ vs. vehicle; Dunnett's test. All values are expressed as means ± SEM (n=3 per group).

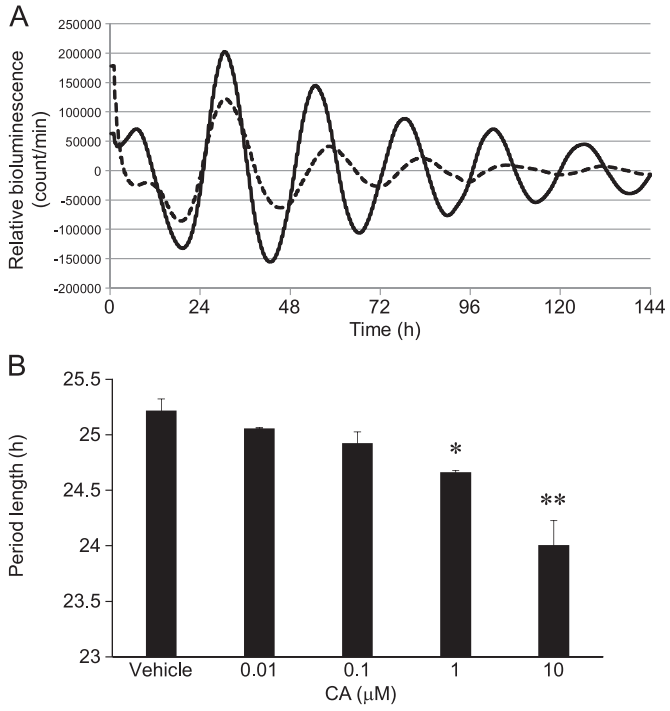

Fig. 2. Cinnamic acid (CA) shortens circadian period of PER2::LUC expression in neuronal cells. (A) Representative detrended data for PER2::LUC neuronal cells incubated with DMSO vehicle (dashed line) or 1 μM CA (solid line). (B) Effects of CA on period of rhythmic PER2::LUC expression in neuronal cells. Distance between peaks 1 and 3 was multiplied by 0.5 to determine period length. Circadian period of PER2::LUC bioluminescence oscillation was dose-dependently shortened by CA. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle; Dunnett's test. All values are expressed as means ± SEM (n=3 per group).

laxant and sedative properties [12–15]. The present study found that CA dose-dependently shortens the circadian period of the molecular clock by monitoring bioluminescence in neuronal cells derived from PER2::LUC mice. We also found that CA significantly shortened the free-running period of behavioral rhythms in mice.

2. Materials and methods

2.1. Chemicals

(E)-cinnamic acid (> 99% pure), luciferin and HEPES were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Caffeine (> 99% pure), lithium chloride (> 99% pure), forskolin, actinomycin D, cycloheximide, penicillin and streptomycin solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM/F12 and B27 were purchased from Life Technologies (Carlsbad, CA, USA).

2.2. Animals and behavioral measurements

Six-week-old male C57BL/6J mice (Japan SLC Inc., Hamamatsu, Japan) were individually housed in cages with SW-15 running wheels (Melquest Ltd., Toyama, Japan) under a 12 h light: 12 h dark cycle at a controlled ambient temperature of 24 ± 1 °C and provided with food and water *ad libitum*. Wheel-running activity was continuously recorded at 5-min intervals using the Chronobiology Kit® (Stanford Software Systems, Stanford, CA, USA) and activity data are displayed as actograms. The free-running period and amplitude of individual mice were estimated using χ^2 periodgrams.

We obtained neuronal cells expressing PER2::LUC protein from PER2::LUC C57BL/6J knock-in mice (The Jackson Laboratory, Bar Harbor, ME, USA) that were housed as described [16]. Endogenous PER2 protein is fused in-frame with a luciferase reporter in these mice, which allows real-time monitoring of PER2::LUC protein dynamics by recording bioluminescence [17].

This study proceeded in accordance with the guidelines for the Care and Use of Laboratory Animals at the National Institute of Advanced Industrial Science and Technology (AIST), and all procedures were approved by the Animal Care and Use Committee at AIST (Permissions #2013-054 and #2015-020).

2.3. Real-time reporter gene assays for neuronal cells

We prepared neuronal cells from day 14 PER2::LUC mouse embryos as described [16]. The cells were stimulated with 10 μM forskolin in differentiation medium (DMEM/F12 containing 2% fetal bovine serum (FBS), B27, 10 units/mL of penicillin and 100 μg/mL of streptomycin) supplemented with 0.1 mM luciferin, 10 mM HEPES