Research Paper

Eicosapentaenoic Acid Reverses the Oleic Acid-induced Reduction in *Per1* mRNA Expression in Cultured Rat Hepatocytes

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Abstract

The aim of the present study was to examine the conditions and chemicals that affect the expression of circadian clock-related genes in obesity. We investigated the expression of *Per1* mRNA in H35 cultured rat hepatoma cells. Incubation with 100 nM dexamethasone for 2 h increased *Per1* mRNA expression successfully in H35 rat hepatoma cells after 19 h or 22 h of stimulation. Further, 50 mM oleic acid (OA) loading resulted in marked accumulation of triglycerides in H35 cells and decreased the elevated levels of dexamethasone-induced *Per1* mRNA after 19 h or 22 h of stimulation. Resveratrol and niacin did not show any effect on *Per1* mRNA expression in H35 cells. However, eicosapentaenoic acid (EPA) reversed the OA-induced reduction in *Per1* mRNA after dexamethasone stimulation in the cultured cells. These data suggest the possible role of EPA as an agent to modify the circadian clock oscillation.

Key words: Per1, clock gene, eicosapentaenoic acid.

Introduction

Prevalence of obesity is increasing worldwide. Obesity is frequently associated with many cardiovascular anomalies, such as diabetes mellitus, hyperlipidemia, and hypertension. It is a major reason behind morbidity and mortality among patients affected with cardiovascular diseases. Obesity is induced by disturbances in the energy homeostasis whose components, including sleeping, thermogenesis, feeding, and glucose and lipid metabolism, are under the control of circadian regulation. Circadian rhythms are regulated by a multioscillator system. The central clock is located within the suprachiasmatic nucleus (SCN) of the brain, which produces a rhythmic output controlled by neural and hormonal signals. The SCN clock is reset by external light and triggered through the ganglion cells of the retina. Circadian oscillators are in fact present in all the tissues, where they are synchronized by unidentified signals to regulate transcriptional activity throughout the day[1-3]. Circadian gene expression is maintained through a transcription-translation negative feedback loop. The primary loop is composed of the basic helix-loop-helix transcription factors: circadian locomoter output cycles protein kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL1). These transcription factors drive expression of Period (*Per1*, *Per2*) and Cryptochrome (*Cry1*, *Cry2*) genes[4,5]. CLOCK and BMAL1 also dimerize to form the CLOCK-BMAL1 complex, which subsequently induces the expression of a large number of output genes. Interplay between metabolism and the circadian clock has been

suggested by several lines of evidence[6-8].

We previously investigated the association between the expression profile of clock genes and obesity using human peripheral blood mononuclear cells (PBMCs). The expression profiles of these genes in obese subjects were apparently different from those in non-obese subjects. Because *Per1* among the several clock genes examined in the study showed the most apparent difference between obese and non-obese subjects[9], we focused on *Per1* in this study. However, the reason behind the altered clock gene expression profile in obese subjects is not completely clear yet. To understand the mechanism of this alteration, in vitro examination of the clock gene expression profile is needed. Importantly, fatty liver is one of the common complications of obesity. Since cultured hepatocytes seem to be useful in evaluating the influence of fat or glucose loading agents on clock gene expression, in this study, we also tried to determine the conditions that alter the expression of clock genes in cultured hepatocytes.

Methods

Determination of mRNA expression

For in vitro experiments, H35 cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum (FCS). Total RNA was isolated and reverse-transcribed to cDNA as described previously[9]. Real-time quantitative PCR (RTq-PCR) was performed with four times diluted cDNA using TaqMan Universal Master Mix (Applied Biosystems, Foster city, CA, USA) and ABI 7500 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. Assay-on-demand primers and probes were purchased from Applied Biosystems (*Per1*, Applied Biosystems). 18S rRNA expression (Applied Biosystems) was quantified for sample normalization. RT-qPCR data were analyzed using a standard curve. In all these cases, correlation coefficients for the standard curves were > 0.90.

Synchronization of H35 cells

To synchronize the clock gene oscillation, we tried stimulating the H35 cells with high concentration of horse serum and dexamethasone. After 24 h of culture with DMEM containing 0.5% FCS, H35 cells were subcultured in DMEM containing 50% horse serum or 100 nM dexamethasone for 2 h. Next, the cells were transferred to DMEM containing 15% FCS for 19 h or 22 h. *Per1* mRNA levels were quantified by RT-qPCR before and after 19 h or 22 h of synchronization.

To examine the effect of glucose on *Per1* mRNA expression pattern after synchronization, H35 cells were incubated with 50 mM or 100 mM glucose. Next,

Per1 mRNA was quantified by RT-qPCR before and after 18 h and 22 h of synchronization.

To determine the most effective stimulation to induce triglyceride accumulation, H35 cells were incubated with 50 mM fatty acids Eicosapentaenoic acid (EPA), arachidonic acid (AA), oleic acid (OA), or palmitic acid (PA) or 50 mM cholesterol for 24 h or 72 h. After incubation with lipids, *Per1* mRNA was quantified by RTq-PCR.

To examine the effect of OA on *Per1* mRNA expression pattern after synchronization, H35 cells were incubated with 400 µM OA. *Per1* mRNA was quantified by RTq-PCR before and after 18 h and 22 h of synchronization.

To find a condition that can reverse the OA-induced decrease of *Per1* mRNA expression, H35 cells were treated with 20 µM resveratrol, 500 µM niacin, and 50 µM or 110 µM eicosapentaenoic acid. Further, cells were treated with 100 nM dexamethasone for 2 h, and *Per1* mRNA was quantified by RTq-PCR before and after 18 h and 22 h of synchronization.

Statistical analysis

Results are shown as the mean ± standard error of the mean (SEM). The significance of differences between mean values was evaluated by Student's t-test for unpaired data. A p value less than 0.05 was considered significant.

Results

Evaluation of synchronization of Per1 mRNA oscillation

To find a suitable stimulation to synchronize *Per1* mRNA oscillation, we evaluated the effect of 50% horse serum stimulation on *Per1* mRNA. However, *Per1* mRNA levels did not change after 50% horse serum stimulation. We also evaluated the effect of dexamethasone on *Per1* mRNA levels. Dexamethasone (100 nM) addition led to a six-fold increase in Per1 mRNA levels at 18 h and 22 h in comparison to those before the treatment.

Effect of high glucose on Per1 mRNA levels in H35 cells

We evaluated the effect of high glucose on *Per1* mRNA in H35 cells. *Per1* mRNA levels were not affected by treatment with 50 mM glucose and 100 mM glucose after dexamethasone synchronization.

Effects of fatty acids and cholesterol on triglyceride accumulation in H35 cells

Incubation with EPA, AA, OA, PA, or cholesterol resulted in significant increase in the cellular triglycerides after 24 h. Similarly, incubation with

EPA, AA, OA, or PA caused significant increase in the cellular triglycerides after 72 h. Incubation with only OA for 72 h led to maximum accumulation of triglycerides in H35 cells.

Influence of lipid accumulation on dexamethasone-induced Per1 mRNA expression

Incubation with 400 µM OA decreased dexamethasone-induced *Per1* mRNA oscillation after 18 h and 22 h.

Influence of resveratrol, niacin, and EPA on OA-induced reduction of Per1 mRNA peaks after dexamethasone treatment

OA-induced decrease in *Per1* mRNA levels was not reversed by treatment with 20 µM resveratrol and 500 µM niacin after dexamethasone treatment. Furthermore, 50 µM EPA could also not increase the *Per1* mRNA levels, but 110 µM EPA significantly increased *Per1* mRNA in OA-loaded cells after 18 h and 22 h of dexamethasone treatment.
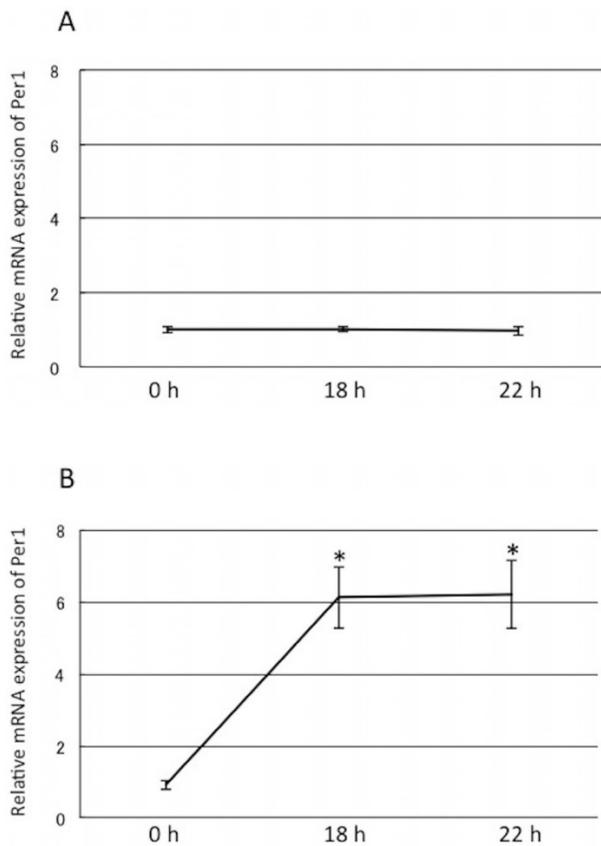

Figure 1. Dexamethasone but not horse serum stimulation increased Per1 mRNA expression A: 50% horse serum stimulation did not change Per1 mRNA levels. **B:** Dexamethasone (100 nM) increased Per1 mRNA levels by six folds at 18 h and 22 h compared to time 0. Y-axis represents mRNA expression of *Per1* with respect to that of 18s. *: p<0.05 vs 0 h, mean±S.E
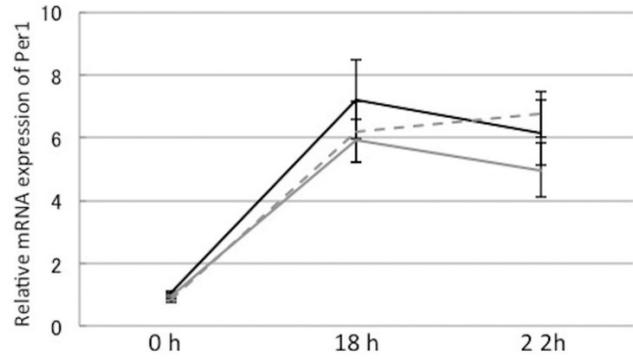

Figure 2. High glucose did not alter *Per1* mRNA levels in H35 cells. Gray line: control, Dashed line: 50 mM glucose, Solid line: 100 mM glucose. Y-axis represents mRNA expression of *Per1* with respect to that of 18s. Mean±S.E.
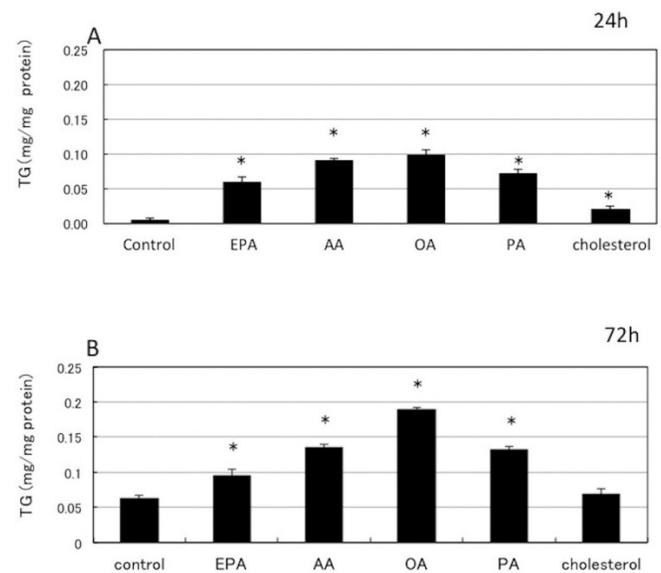

Figure 3. Incubation with fatty acids and cholesterol resulted in significant increase in the cellular triglycerides. A: 24 h incubation with fatty acids and cholesterol resulted in significant increase in the cellular triglycerides. **B:** 72h incubation with fatty acids and cholesterol resulted in significant increase in the cellular triglycerides. EPA (eicosapentaenoic acid), AA (arachidonic acid), OA (oleic acid), PA (palmitic acid). Mean±S.E, *: p<0.05 vs control.
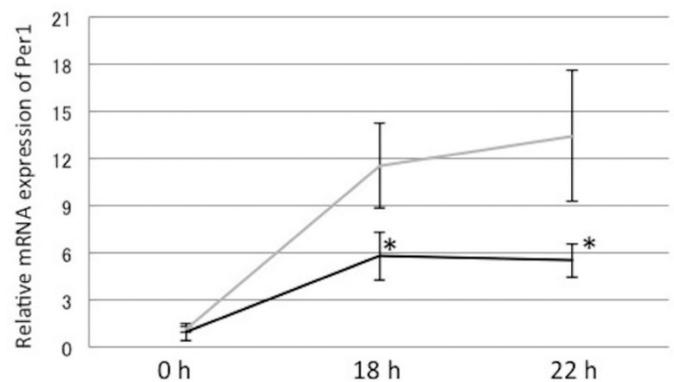

Figure 4. Incubation with 400 µM OA decreased dexamethasone-induced *Per1* mRNA oscillation after 18 h and 22 h. Gray line: control; Solid line: 400 µM oleic acid, Y-axis represents mRNA expression of *Per1* with respect to that of 18s. *: p<0.05 vs control, mean±S.E
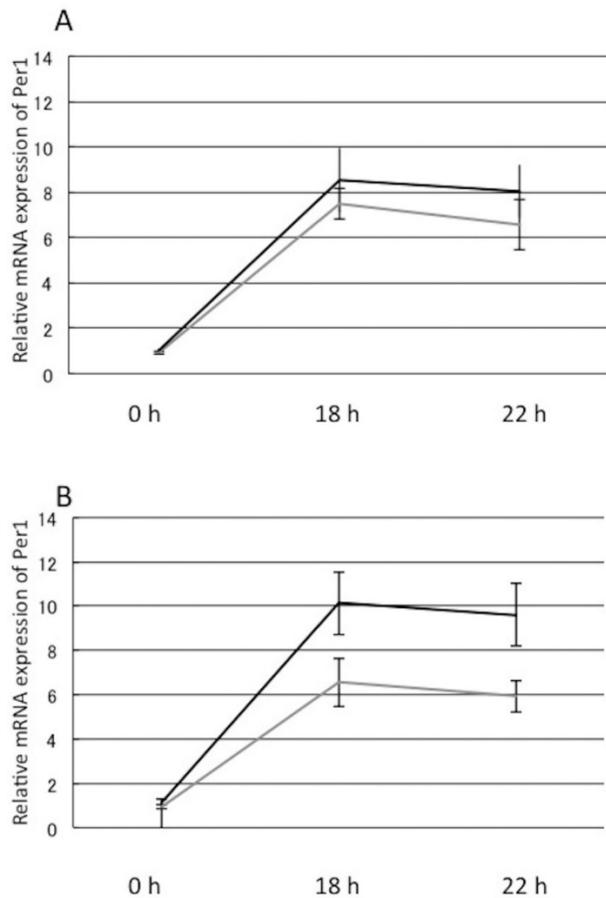
A

B

Figure 5. OA-induced decrease in *Per1* mRNA levels was not reversed by treatment with 20 μM resveratrol and 500 μM niacin after dexamethasone treatment. A: OA-induced decrease in *Per1* mRNA levels was not reversed by treatment with 20 μM resveratrol. Gray line: control, Solid line: 20 μM resveratrol, mean±S.E **B:** OA-induced decrease in *Per1* mRNA levels was not reversed by treatment with 500 μM niacin. Gray line: control, Solid line: 500 μM niacin, mean±S.E Y-axis represents mRNA expression of *Per1* with respect to that of 18s.
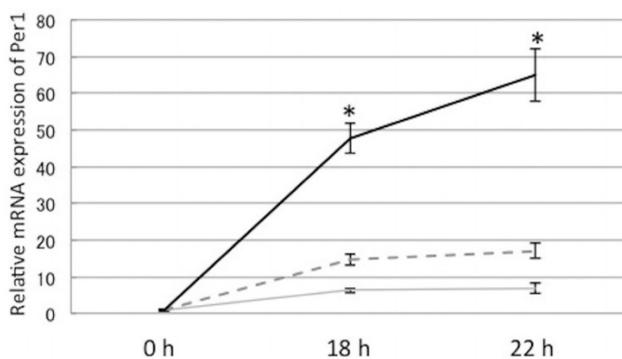

Figure 6. 110 μM EPA significantly increased *Per1* mRNA in OA-loaded cells after 18 h and 22 h of dexamethasone treatment. Gray line: control, Dashed line: 50 μM EPA, Solid line: 110 μM EPA, *: $p < 0.05$ vs. control, mean±S.E Y-axis represents mRNA expression of *Per1* with respect to that of 18s.

Discussion

In our previous study, we evaluated the mRNA expression profile of the genes implicated in circadian

regulation using PBMCs of the obese subjects. Oscillation pattern of mRNA expression of circadian clock-related genes in the obese subjects was apparently different from that in the healthy subjects. Among the circadian clock-related genes, difference of oscillation pattern between obese and non-obese subjects was most evident in the case of *Per1* gene.

For further examination of the influence of obesity on clock gene expression, in vitro study is a fast and informative method. Balsalobre *et al.* reported that serum shock induces circadian clock gene oscillation in cultured fibroblasts and hepatocytes[10]. Dexamethasone also induces circadian rhythm of clock genes in culture cells by type 0 phase resetting[11]. Because fatty liver is the most common and important complication of obesity, we used cultured hepatocytes to evaluate the effect of fat accumulation on *Per1* gene oscillation. Horse serum loading (50%) did not affect *Per1* mRNA expression. However, dexamethasone stimulation increased *Per1* mRNA expression in H35 hepatoma cells.

To evaluate the influence of fat accumulation on *Per1* mRNA expression, we first tried to find a suitable condition for accumulation of triglycerides in H35 cells. We examined several fatty acids and cholesterol and found that OA loading was the most effective to increase triglyceride storage in H35 cells. Dexamethasone-induced *Per1* mRNA expression was decreased in the cells loaded with oleic acid. Because high blood glucose is another frequently associated complication of obesity, we examined the effect of high glucose on dexamethasone-induced *Per1* mRNA expression. However, high glucose levels did not affect *Per1* mRNA expression. In our previous study, *Per1* mRNA oscillation was decreased in the obese subjects. The same trend was observed in the triglyceride-accumulated cultured hepatocytes. These findings suggest that the accumulation of triglycerides in hepatocytes possibly decreases the circadian clock oscillation.

Resveratrol mimics the effects of calorie restriction via activation of sirtuin 1, which is an important player in the regulation of cellular energy homeostasis. Sirtuins are NAD-dependent deacetylases, which regulate glucose and lipid metabolism and participate in the pathophysiology of hepatic steatosis[12]. We evaluated the effect of resveratrol on *Per1* mRNA reduction induced by oleic acid loading. However, resveratrol did not increase *Per1* mRNA expression. These findings suggest that obesity-induced reduction in *Per1* mRNA is not directly linked with the energy homeostasis.

Niacin is effective in the regulation of glucose metabolism and NAD-sirtuin pathway, which may be related to the altered mitochondrial biogenesis[13,14]. We

examined the effect of niacin on OA-induced *Per1* mRNA reduction. However, niacin did not show any significant effect on *Per1* mRNA expression.

EPA loading successfully improved OA-induced *Per1* mRNA reduction. Furutani et al. reported that fish oil facilitates restriction feeding-induced entrainment of the peripheral clock. The entrainment by fish oil supplementation was attenuated in G protein-coupled receptor 120 (GPR120)-deficient mice[15]. GPR 120 is the putative receptor for omega-3 fatty acid including EPA and docosahexaenoic acid (DHA). It has also been reported that easily digestible carbohydrates facilitate restriction feeding-induced peripheral clock entrainment[16,17]. Our results along with these previous findings indicate that EPA might improve obesity-induced circadian clock dysfunction through GPR120 signaling.

In this study, we successfully evaluated clock gene expression in cultured rat hepatocytes. As the circadian clock system is orchestrated by the interaction of the central clock and peripheral clocks, evaluation of the peripheral clock in cultured cells is convenient and quick method. However, evaluation should also be performed in vivo to estimate the interaction of the central and peripheral clocks.

In conclusion, oleic acid loading decreased *Per1* mRNA expression in cultured hepatocytes. Among several agents examined in this study, only EPA reversed the OA-induced reduction in *Per1* mRNA expression in the cultured cells. These data suggest that EPA can be employed as an agent to modify circadian clock oscillation.

Competing Interests

The authors have declared that no competing interest exists.

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