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Clock gene dysfunction in patients with obstructive sleep apnea syndrome

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Running title: Dysfunction of *Period1* in OSAS

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Abstract

Clock genes regulate mammalian circadian rhythms, and dysfunction of clock genes can contribute to various disorders. To investigate whether obstructive sleep apnea syndrome (OSAS) influences clock gene function, we examined *Period1* (*Per1*) mRNA expression *in vitro* and *in vivo*.

In 8 healthy subjects and 8 OSAS patients, we measured plasma noradrenaline, serum IL-6, and high-sensitivity CRP (hs-CRP), and *Per1* mRNA expression in peripheral whole blood. Expression of *Per1* mRNA in cultured cells was examined under IL-6 or noradrenaline stimulation *in vitro*. After noradrenaline was administered to mice *in vivo*, *Per1* mRNA expression in the brain was examined.

The concentrations of serum IL-6, hs-CRP and plasma noradrenaline were elevated in OSAS patients, but improved by CPAP therapy. *Per1* mRNA expression in the peripheral blood at 2:00 h significantly decreased by CPAP in OSAS patients. The stimulation with IL-6 did not directly induce *Per1* mRNA *in vitro*. The administration of noradrenaline induced *Per1* mRNA in cerebral cortex of mice *in vivo*.

We first revealed that OSAS caused clock gene dysfunction, and CPAP helped to improve it. Sympathetic activation and elevation of the plasma noradrenaline concentration in OSAS may be one of the factors involved in disorders of *Per1* mRNA expression.

Key words: clock gene, CRP, Interleukin-6, noradrenaline, *Period1*, sleep apnea

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Introduction

Obstructive sleep apnea syndrome (OSAS) is an important disease that contributes to excessive daytime sleepiness, mood disturbances and cardiovascular disease. Moreover, OSAS is thought to cause proinflammatory conditions, abnormal sympathetic activation, and intermittent hypoxia during apnea. A number of previous studies have examined the expression of various inflammatory factors in OSAS patients. The serum concentrations of cytokines such as TNF-α, interleukin-6 (IL-6) and IL-8 have been reported to be elevated in OSAS patients and to be improved by CPAP therapy [1-3]. IL-6 can induce C-reactive protein (CRP) in the mammalian liver, which contributes to cardiovascular disease [4]. Moreover, sympathetic activation in patients with OSAS is associated with hypertension [5].

In mammals, the circadian master clock resides in the suprachiasmatic nucleus (SCN) and entrains peripheral clock function in various tissues through neural and humoral signaling [6]. Abnormal central clock gene expression in the SCN can cause circadian sleep disorders, mood disturbances and some psychiatric diseases. Moreover, dysfunction of peripheral clock genes has been investigated in various human disorders, including tumor genesis, obesity, and cardiovascular disease [7-9]. Although detail molecular mechanism of the circadian oscillator in rodents that are nocturnally active has been revealed, there are few reports in diurnally active human. Recently, human peripheral blood cells have been used as a surrogate to estimate the mRNA expression of clock genes in other peripheral tissues [10-14].

Human *Period1* (h*Per1*) is one of the important clock genes. Previous studies have shown that the *Per1* promoter contains several enhancers such as cAMP response

element (CRE) [15-17] and glucocorticoid response element (GRE) [18-20] in its 5'-upstream region. The expression of *Per1* can be reportedly induced by treatment of glucocorticoids, IL-6 and α-adrenoceptor or β-adrenoceptor agonists [21-24]. The plasma noradrenaline and/or adrenaline and serum IL-6 concentrations are reportedly elevated in patients with OSAS [3, 25]. Some papers also revealed that cortisol concentrations are elevated in severe OSAS [26]. OSAS may potentially influence h*Per1* mRNA expression. However, the influence of OSAS on human clock genes has not yet been reported. In this study, to investigate whether OSAS influences clock gene function, we examined h*Per1* mRNA expression *in vitro* and *in vivo*. In human, it is difficult to analysis the induction of clock genes in organs. We used human peripheral blood cells as a surrogate to estimate the mRNA expression of clock gene.

Subjects and Methods

Subjects and patients

Eight diurnally active (~ 06:30 h to ~ 23:00 h) healthy subjects and eight patients with OSAS participated in this study (Table 1). Age and weight of two groups were matched. The healthy subjects had no cardiovascular disease. The healthy subjects and patients generally ate breakfast between 07:00 - 08:00 h, lunch between 12:00 - 14:00 h, and dinner between 18:00 - 20:00 h, although they were not strictly restricted to this time schedule. No food was allowed between meals, and no caffeine-containing beverages were permitted. Local authorities approved the study protocol. Informed consent was obtained from all participants.

Sleep studies

All participants had undergone a diagnostic polysomnography (PSG) during the night in the hospital prior to the study. The signals of EEG (C3-A2, C4-A1), electro-oculograms, submental electromyograms, ECG, oxyhemoglobin saturation by a finger pulse-oximeter (SpO_2), a snoring microphone, nasal and/or mouth flow using pressure cannula, and both chest wall and abdominal efforts were recorded by PSG (SomnoTrac Ω, SensorMedics, Yorba Linda, CA). Two independent physicians each categorized the stage of consciousness on the EEG records, using the criteria of Rechtschaffen and Kales [27]. Criteria for apnea were defined as a cessation of air flow at the nose and/or mouth for at least 10 sec, while that for hypopnea was defined as an airflow > 50% reduction for at least 10 sec followed with a fall of at least 3 % in the SpO_2 or arousal. The apnea-hypopnea index (AHI) was calculated as the sum of all apneas and hypopneas divided by the total sleep time (TST), and were expressed as per hour. When AHI was above 5/h, we diagnosed the patient as having OSAS. However, only OSAS patients with an AHI > 30/h were included in this study. Patients with OSAS were treated by CPAP (REMstar Auto, Respironics Japan, Tokyo, Japan) after titration. After 3 months of CPAP therapy, patients with OSAS were also examined with PSG during the night using CPAP. Exclusion criteria were central apnea syndrome, Cheyne-Stokes respiration, major facial or pharyngeal anatomic abnormalities, previous treatment with CPAP, and sedative or hypnotic therapy.

Measurement of serum cortisol, IL-6, hs-CRP and plasma noradrenaline concentrations in participants

We collected 10 ml of venous blood samples from both 8 healthy subjects and 8 patients with OSAS at 18:00, 2:00, 6:00, and 14:00 h during the study day without medication. After 3 months using CPAP in patients with OSAS, we also collected 10-ml of venous blood samples at each time point with CPAP therapy in the hospital. The serum and plasma samples were immediately stored at -80°C for further analysis.

The serum cortisol concentrations were determined by a radioimmunoassay based on ^{125}I-labeled cortisol (TFB, Tokyo, Japan). The serum IL-6 concentrations were measured by ELISA (High Sensitivity IL-6 Biotrak ELISA, Amersham Bioscience, Piscataway, NJ). The plasma noradrenaline concentrations were measured by high performance liquid chromatograph (HPLC) (CA test, TOSOH, Tokyo, Japan). The serum high-sensitivity CRP (hs-CRP) concentrations were measured using a nepherometer (N-Latex CRP II, Dade Behring, Tokyo, Japan).

Per1 mRNA expression in human peripheral blood cells

Total RNA was isolated from peripheral white blood cells in 2.5 ml of each whole blood sample using the PAXgene Blood RNA kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. The cDNA was synthesized using the QuantiTect Reverse Transcription kit (QIAGEN). Real-time polymerase chain reactions (PCR) were performed using the LightCycler system (Roche Diagnostics, Basel, Switzerland) with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) [16, 28]. Specific primer pairs used were as follows: h*β-actin* (191 bp), 5'-AGCATCCCCCAAAGTTCACA-3' and 5'-AAGCAATGCTATCACCTCCC-3'; h*Per1* (132 bp), 5'-CTGAGGAGGCCGAGAGGAAAGAA-3' and 5'-AGGAGGAGGAGGCACATTTACGC-3'. The amplifications were performed using

the following protocol: initial denaturation at 95°C for 10-min to activate the FastStart Taq DNA polymerase, 45 cycles consisting of 95°C for 15-sec, 55°C for 5-sec and 72°C for 10-sec (transition rates of 20°C/sec). The fluorescence value of each capillary was measured at 530 nm. The expression of h*Per1* mRNA was determined relative to the *β-actin* mRNA expression.

Cell culture

Mouse embryonic fibroblast NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). To determine whether IL-6 or noradrenaline can induce m*Per1* mRNA expression *in vitro*, the NIH3T3 cells were treated with various concentrations (10, 100 and 1000 pg/ml) of mouse IL-6 (R&D System, Minneapolis, MN) or noradrenaline (10, 25 and 50 ng/ml) (Sigma -Aldrich St. Louis, MO) for 24 h. After the treatment, the mRNA levels of m*Per1* were assessed by RT-PCR as described below.

For the quantification of the relative RNA levels, the cDNAs of m*Per1,* and *GAPDH* were synthesized and amplified by using the Superscript one-step RT-PCR system (Invitrogen, Carlsbad, CA). The specific primer pairs used were as follows: m*Per1* (403 bp), 5'-CCAGGCCCGGAGAACCTTTTT-3' and 5'-CGAAGTTTGAGCTCCCGAAGTG-3', and mouse *GAPDH* (178 bp), 5'-GACCTCAACTACATGGTCTACA-3' and 5'-ACTCCACGACATACTCAGCAC-3'. To evaluate the quantitative reliability of RT-PCR, a kinetic analysis of the amplified products was done to ensure that signals were derived only from the exponential phase of amplifications. The exponential phase of *GAPDH* amplification in all experimental conditions occurred between the 26th and the

28th cycles, and the exponential phases of a target gene (m*Per1*) occurred between the 27th and the 30th cycles. The amplification efficiencies of the *GAPDH* and target gene were comparable. Therefore, the amplification products were collected and quantified at the 27th or 28th cycle. The ratio of the amplified target to the amplified internal control (calculated by dividing the value of each m*Per1* by that of *GAPDH*) was compared among the groups.

Experimental Animals

We used male ICR mice 4-6 weeks of age (Tokyo experimental animals Co., Tokyo, Japan) were used for the general experiments. The animals were housed in a room controlled at a temperature of 22 ± 2 °C, humidity at 60 ± 5%, and a 12-hr light/12-hr dark cycle (LD; lights on from 6:00 to 18:00 h). ZT0 and ZT12 were the lights-on and lights-off times, respectively. The light intensity at the surface of the cages was approximately 100-lx. The mice were fed a standard diet and water was available *ad libitum*. An osmotic mini-pump (model 2001, ALZET; Palo Alto, CA) was implanted under the skin of mice, and was used for the continuous administration of noradrenaline (1.5 μg/hr) or saline for 6 days.

Total RNA from mouse tissues was extracted as follows: the brain was quickly removed at ZT6 and ZT18. Coronal brain slices (500 μm) were prepared using a rodent brain matrix (RBM-2000C; ASI Instruments, Inc., Warren, MI), and the SCN was punched out bilaterally from the brain slices. To obtain an adequate amount of RNA from the SCN, three mice's SCN in each group were combined and extracted. The extraction of total RNA from the cerebral cortex of an individual mouse was carried out separately by using the RNeasy mini kit (QIAGEN). The mRNA levels of m*Per1* were assessed by

RT-PCR as described above. Experimental animal care was conducted under permission from the Committee for Animal Experimentation in the Division of Pharmaceutical Sciences at Kyusyu University.

Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM) for all samples. The statistical differences among groups were analyzed by one-way ANOVA, and multiple comparisons were performed by Fisher's protected least significant difference (PLSD) or Dunnett's test (StatView, SAS Institute Inc., Cary, NC). A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Efficacy of CPAP therapy

After using CPAP for 3 months, we again examined the night PSG in patients with OSAS at the hospital. The indices of AHI and Epworth sleepiness scale (ESS) were significantly decreased and improved by CPAP. The other indices were also clearly improved by CPAP therapy (Table 2). The CPAP device can record the usage automatically, and compliance of CPAP therapy was objectively calculated. The mean time of CPAP use was 5.2 ± 0.4 h per night. Average percentage of CPAP use, which was calculated by dividing cumulative day when patients used CPAP device by total days, was 75 ± 3.6 %.

Serum cortisol concentration

Figure 1 shows the daily variations of serum cortisol concentrations in the healthy subjects, the patients with OSAS, and OSAS patients treated with CPAP therapy for 3 months. In each group, the serum concentrations of cortisol were significantly lowest at 2:00 h and consistently highest in the early morning at 6:00 h using Fisher's PLSD test. No statistically significant differences in the serum cortisol concentrations at each time point were found between the 3 groups. The 24-h mean serum cortisol levels were 9.9 ± 1.7 (μg/dl) in the normal subjects, 10.1 ± 1.8 in the OSAS patients and 11.4 ± 2.0 in the OSAS patients treated with CPAP. No statistically significant differences in the 24 h mean serum cortisol concentrations were found. Individually, all subjects showed normal daily variation and phasing of cortisol secretion.

Serum IL-6 and hs-CRP

The serum IL-6 concentrations in the healthy subjects were 1.6 ± 0.1 (pg/ml, mean ± SEM) at 18:00 h, 1.5 ± 0.2 at 2:00 h, 1.7 ± 0.3 at 6:00 h, and 1.6 ± 0.2 at 14:00 h. In the patients with OSAS, the serum IL-6 concentrations were 9.1 ± 2.7 (pg/ml) at 18:00 h, 7.0 ± 1.8 at 2:00 h, 6.6 ± 1.9 at 6:00 h, and 4.0 ± 0.7 at 14:00 h. After CPAP therapy for 3 months, the serum IL-6 concentrations were 3.8 ± 0.5 (pg/ml) at 18:00 h, 5.4 ± 1.0 at 2:00 h, 2.8 ± 0.4 at 6:00 h, and 2.4 ± 0.3 at 14:00 h in the patients with OSAS treated with CPAP. The daily mean serum concentrations of IL-6 were 1.6 ± 0.3 (pg/ml) in the healthy subjects, 6.7 ± 1.2 in the patients with OSAS and 3.6 ± 0.6 in the OSAS patients treated with CPAP. A significant difference was found between the 3 groups by one-way ANOVA ($p < 0.05$). The serum concentrations of IL-6 were significantly decreased by CPAP therapy (Fig. 2A, $p < 0.005$).

The serum hs-CRP concentrations in the healthy subjects were 255 ± 63 (ng/ml, mean ± SEM) at 18:00 h, 237 ± 61 at 2:00 h, 188 ± 40 at 6:00 h, and 245 ± 59 at 14:00 h. In the patients with OSAS, the serum hs-CRP concentrations were 1011 ± 475 (ng/ml) at 18:00 h, 985 ± 479 at 2:00 h, 1008 ± 522 at 6:00 h, and 1240 ± 636 at 14:00 h. After CPAP therapy for 3 months, the serum hs-CRP concentrations were 487 ± 82 (ng/ml) at 18:00 h, 424 ± 62 at 2:00 h, 473 ± 70 at 6:00 h, and 475 ± 73 at 14:00 h in the patients with OSAS treated with CPAP. The daily mean serum concentrations of hs-CRP were 231 ± 41 (ng/ml) in the healthy subjects, 1061 ± 188 in the patients with OSAS and 465 ± 82 in the OSAS patients treated with CPAP. A significant difference was found between the 3 groups by one-way ANOVA ($p < 0.05$). The serum concentration of hs-CRP was significantly decreased by CPAP therapy (Fig. 2B, $p < 0.04$).

Levels of plasma noradrenaline

The plasma noradrenaline concentrations in the supine position at 2:00 h were 144 ± 11 (pg/ml) in the healthy subjects, 251 ± 35 in the patients with OSAS and 158 ± 22 in the OSAS patients treated with CPAP. The plasma noradrenaline concentrations were significantly different between the 3 groups by one-way ANOVA ($p < 0.05$), and significantly decreased by CPAP therapy ($p < 0.04$, Fisher's PLSD) (Fig. 3).

***Per1* mRNA expression in peripheral whole blood cells by real-time PCR analysis**

Figure 4 shows the h*Per1* mRNA expression in peripheral whole blood cells according to real-time PCR analysis. The value for h*Per1* mRNA expression at each time point was divided by each peak value. The significant daily variations of h*Per1* mRNA expression in peripheral whole blood cells from the healthy subjects and OSAS patients

treated with CPAP were found by repeated measure one-way ANOVA ($p < 0.05$ and $p < 0.03$, respectively). However, no significant daily variation of h*Per1* mRNA expression was found in OSAS patients. In this study, we confirmed that h*Per1* mRNA expression in normal subjects at 6:00 was significantly higher than that at 2:00 or at 14:00 by multiple comparisons using Fisher's PLSD (Fig. 4A, $p < 0.02$ and $p < 0.03$, respectively). The highest h*Per1* transcription in peripheral blood was previously observed in healthy human subjects at times experienced as morning [10, 11, 13, 14]. Our result was consistent with these previous reports.

Especially, the relative values at 2:00 h were significantly different between the 3 groups ($p < 0.04$, one-way ANOVA). In the OSAS patients treated with CPAP, the relative value for h*Per1* mRNA expression at 2:00 h was significantly lower than that at 2:00 h in the OSAS patients by Fisher's PLSD ($p < 0.02$). CPAP therapy significantly improved the alteration in h*Per1* mRNA expression in the OSAS patients.

Per1* mRNA expression in NIH3T3 cells by stimulating with IL-6 and noradrenaline *in vitro

Treatment of NIH3T3 cells with IL-6 resulted in no significant induction of m*Per1* mRNA by one-way ANOVA, although a high concentration (1000 pg/ml) of IL-6 slightly induced m*Per1* mRNA (Fig. 5A). On the other hand, treatment of NIH3T3 cells with noradrenaline caused an induction of m*Per1* mRNA in a concentration-dependent manner by one-way ANOVA ($p < 0.003$, Fig. 5B). Significant inductions of m*Per1* mRNA were observed when the cells were treated with 25 and 50 ng/ml noradrenaline using Dunnett's test ($p < 0.05$, respectively, Fig. 5B).

Per1* mRNA expression in the mouse brain during the administration of noradrenaline *in vivo

Male ICR mice were exposed to 3 weeks of light/dark cycles for every 12 hours. Following this, noradrenaline was administered continuously to 3 mice by an osmotic pump for 6 days. In the SCN, m*Per1* mRNA was not induced by noradrenaline during both the light (ZT6) and dark periods (ZT18). However, m*Per1* mRNA in the cerebral cortex was significantly induced during the light period when the mouse was asleep and not active ($p<0.05$, Fig. 6). Briefly m*Per1* mRNA in the central biological clock was not influenced by noradrenaline, but m*Per1* mRNA expression was induced in the peripheral tissues.

Discussion

We first revealed that the daily variation of h*Per1* mRNA expression in the peripheral whole blood cells from OSAS patients was different from that in healthy subjects. In the OSAS patients, no significant daily variation of h*Per1* mRNA expression in the peripheral whole blood cells was found. CPAP therapy significantly improved the alteration in h*Per1* mRNA expression in the OSAS patients. We examined the factors that influenced *Per1* mRNA expression. Treatment of NIH3T3 cells with noradrenaline induced m*Per1* mRNA in a concentration-dependent manner *in vitro*. Moreover, the administration of noradrenaline induced m*Per1* mRNA in the cerebral cortex of mice *in vivo*. On the other hand, prolonged stimulation with IL-6 could not induce m*Per1* expression *in vitro*. Sympathetic activation in OSAS patients may be one of the factors for the dysfunction of *Per1* mRNA expression during sleep. Moreover, CPAP is useful

for improving the serum CRP, IL-6, and plasma noradrenaline concentrations, and dysfunction of the clock gene, h*Per1*. The *Per1* mRNA expression in the peripheral blood cells may be a new index to evaluate the efficacy of CPAP therapy in OSAS patients.

Glucocorticoid signals of the mammalian clock gene, *Per1*

Previous reports have shown that circadian expression of *Per1* could be elicited by multiple signaling pathways such as epidermal growth factor, equine serum in high concentration, forskolin, phorbol ester, glucocorticoids and sympathetic neuron-related factors [6, 15]. Glucocorticoid is a particularly potent signal to elicit rhythmic mRNA expression in peripheral clock genes. Several reports suggested that the increase in *Per1* mRNA accumulation is caused by glucocorticoid signaling via the GRE consensus sequences in *Per1* [18-20]. Rat *Per1* mRNA expression in cultured rat-1 fibroblasts was reportedly induced by a glucocorticoid hormone analogue, dexamethasone [21]. We also previously confirmed that stimulation with dexamethasone strongly induced h*Per1* mRNA in cultured human bronchial epithelial cells, BEAS-2B [22]. Moreover, we have reported that injection of prednisolone *in vivo* markedly induced h*Per1* mRNA expression in human peripheral blood mononuclear cells after 1-hr of dosing [28].

Some papers reported that cortisol concentrations were elevated in severe OSAS [26]. However, another reported that OSAS was not associated with any change in the level of salivary or plasma cortisol rhythmicity [29]. In this study, since no statistically significant differences in the serum cortisol concentrations at each time point were found between healthy subjects, severe OSAS patients, and severe OSAS patients treated with CPAP, the mechanism to induce h*Per1* mRNA at 2:00 h in OSAS patients may not be related to the signaling pathway via GRE.

Effect of stimulation of IL-6 for *Per1* mRNA expression

We found that the serum concentrations of both IL-6 and hs-CRP in the OSAS patients were significantly higher than those in the healthy subjects, and were decreased by CPAP in this study. It has been reported that hypoxia can activate various transcriptional factors of NF-κB and NF-IL-6, and can increase the production of IL-6 [30]. IL-6 is an important proinflammatory cytokine that has been implicated in the pathogenesis of atherosclerosis. The plasma concentrations of IL-6 are reportedly correlated with the mortality rate in patients with unstable coronary artery disease and with the risk of myocardial infarction [31]. Serum CRP concentrations are elevated in patients with OSAS [3], because IL-6 induces the synthesis of all acute-phase proteins, including CRP [4].

A previous report suggested that stimulation with high concentration of IL-6 could induce h*Per1* mRNA in cultured cells [23]. To investigate whether IL-6 can directly induce *Per1* mRNA *in vitro*, we stimulated cultured NIH3T3 cells with IL-6 for 24-hr. Treatment of NIH3T3 cells with IL-6 resulted in no significant induction of m*Per1* mRNA, although a high concentration (1000 pg/ml) of IL-6 slightly induced m*Per1* mRNA. We postulated that IL-6 elevation might not directly induce h*Per1* mRNA.

Effect of noradrenaline to induce *Per1* mRNA

OSAS causes an increase in sympathetic activity [25]. Urinary excretion of noradrenaline and/or adrenaline in OSAS patients is higher than in normal subjects [32]. The elevated circulating noradrenaline concentrations or elevated urinary noradrenaline excretion are considered to be a marker of severity of OSAS [33]. We found that the plasma noradrenaline concentration increased in patients with OSAS.

Adrenoceptor agonists can reportedly induce the expression of *Per1* by signal pathways of cAMP - protein kinase A (PKA) - cAMP response element binding protein (CREB) or MAPK-CREB [16,24,34]. We examined whether noradrenaline could induce *Per1* mRNA *in vitro and in vivo*. We found that treatment of NIH3T3 cells with noradrenaline caused an induction of m*Per1* mRNA in a concentration-dependent manner. Moreover, we administered noradrenaline to 3 mice by osmotic pump for 6 days *in vivo*. We found that m*Per1* mRNA in the SCN was not induced by noradrenaline during both the light and dark periods, but m*Per1* mRNA in the cerebral cortex was significantly induced during the light period when the mice were not active. We confirmed that stimulation with noradrenaline induced *Per1* mRNA expression *in vitro and in vivo*. However, since it is still unclear whether the induction of *Per1* mRNA in cerebral cortex may have an influence on emotion, additional study is needed to clarify its actual physiologic impacts.

OSAS causes intermittent hypoxia that activates hypoxia inducible factor 1 (HIF-1) -mediated transcription. The transcriptional activator HIF-1 is regarded as a master regulator of oxygen homeostasis during hypoxia that regulates the expression of many genes [35]. Moreover, Ryan et al. recently indicated that intermittent hypoxia activated the inflammatory pathways of nuclear factor-κB (NF-κB) and might be associated with a specific role for the pathophysiology of cardiovascular complications in OSAS [36]. Further studies will be needed to investigate whether intermittent hypoxia can induce h*Per1* mRNA *in vitro* and *in vivo*.

In conclusion, sympathetic activation and elevation of the plasma noradrenaline may be one of the factors that increase *Per1* mRNA expression in patients with OSAS during sleep. CPAP therapy is useful for improving the abnormal changes in the serum

IL-6, CRP, plasma noradrenaline concentrations, and h*Per1* mRNA expression in patients with OSAS. In addition, peripheral blood cells provide an easier way to evaluate changes in the expression of peripheral clock genes. Human peripheral blood cells may be used as a surrogate for clock gene mRNA expression. However, the actual physiologic impacts are still unclear, and further studies will be needed to determine the relationship between the dysfunction of the clock gene in OSAS patients and the clinical symptoms such as excessive daytime sleepiness and mood disturbances.

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***Conflict of Interest Statement*:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Figure Legend

Fig. 1 **Serum cortisol concentrations in healthy subjects, patients with OSAS, and OSAS patients treated by CPAP therapy.**

The significant daily variations of serum cortisol concentrations were found in each group using repeated measure one-way ANOVA ($p < 0.0001$, respectively). However, no statistically significant differences in the serum cortisol concentrations at each time point were found between the 3 groups. The values were presented as mean ± SEM (n = 8).

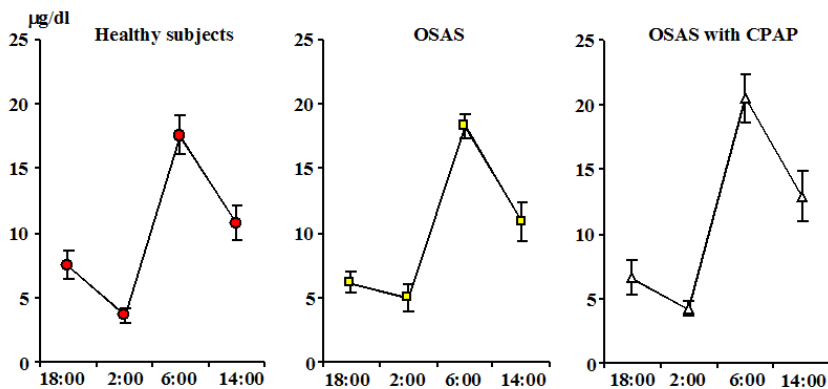

Fig. 1
Burioka N

Fig. 2 **Serum IL-6 and hs-CRP before and after CPAP therapy in patients with OSAS.**

(A) The serum IL-6 concentration was significantly decreased by CPAP therapy in patients with OSAS ($p < 0.005$). (B) The serum hs-CRP concentration was also

significantly decreased by CPAP therapy in patients with OSAS ($p < 0.04$). Paired t-test was used to compare the values before and after CPAP therapy in patients with OSAS. Open squares show the values at 18:00 h. Open triangles show the values at 2:00 h. Open circles show the values at 6:00 h. Closed circles show the values at 14:00 h.

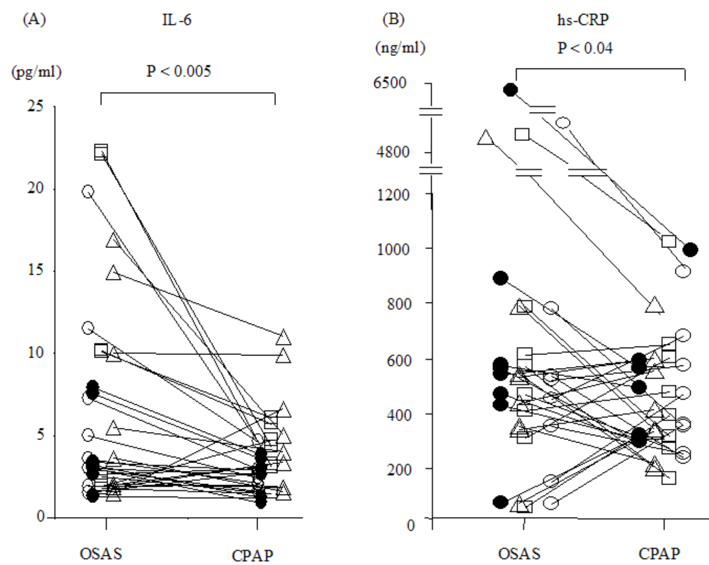

Fig. 3 **Plasma noradrenaline concentration at 2:00 in healthy subjects, patients with OSAS and OSAS treated with CPAP.**

The variation in the plasma noradrenaline concentrations in the supine position between the 3 groups was significant by one-way ANOVA ($p < 0.05$). Plasma noradrenaline concentration in the OSAS patients was significantly higher than that in the normal subjects ($p < 0.04$, Fisher's PLSD). CPAP therapy significantly decreased the plasma noradrenaline concentration ($p < 0.04$).

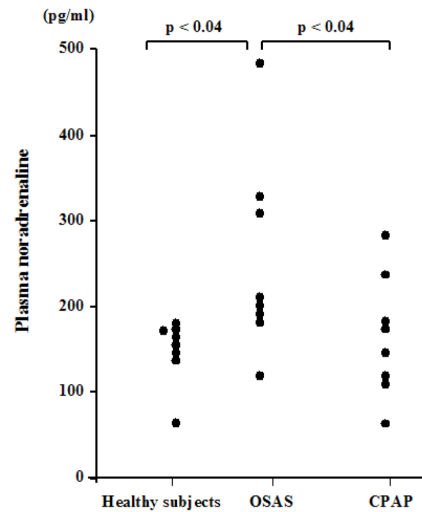

Fig.3
Burioka N

Fig. 4 **Variation in the relative values of h*Per1* mRNA expression in human peripheral blood cells by real-time PCR analysis.**

The value for h*Per1* mRNA expression at each time point was divided by each peak value ($n = 8$, mean ± SEM). The expression of h*Per1* mRNA was determined relative to the *β-actin* mRNA expression using real-time PCR analysis. The variation in the relative values of h*Per1* mRNA expression at 4 time points was statistically significant in the healthy subjects and in the patients with OSAS during CPAP therapy by repeated measure one-way ANOVA ($p < 0.05$ and $p < 0.03$, respectively). Multiple comparisons were performed by Fisher's PLSD.

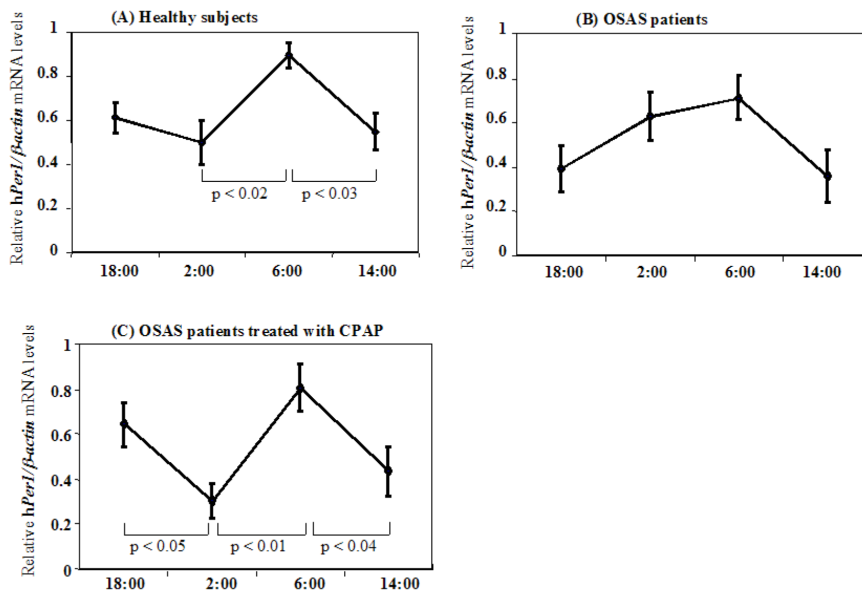

Fig.4
Burioka N

Fig. 5 **Mouse *Per1* mRNA expression in mouse NIH3T3 cells by stimulating with IL-6 or noradrenaline *in vitro.***

The expression of m*Per1* mRNA was determined relative to *GAPDH* mRNA expression using RT-PCR analysis (n = 3, mean ± SEM). Treatment of NIH3T3 cells with IL-6 resulted in no significant induction of m*Per1* mRNA (Fig. 5A). On the other hand, treatment of NIH3T3 cells with noradrenaline caused a significant induction of m*Per1* mRNA in a concentration-dependent manner by one-way ANOVA (P<0.003, Fig. 5B). Significant inductions of m*Per1* mRNA were observed when the cells were treated with 25 and 50 ng/ml noradrenaline using Dunnett's test ($p < 0.05$, respectively, Fig. 5B).

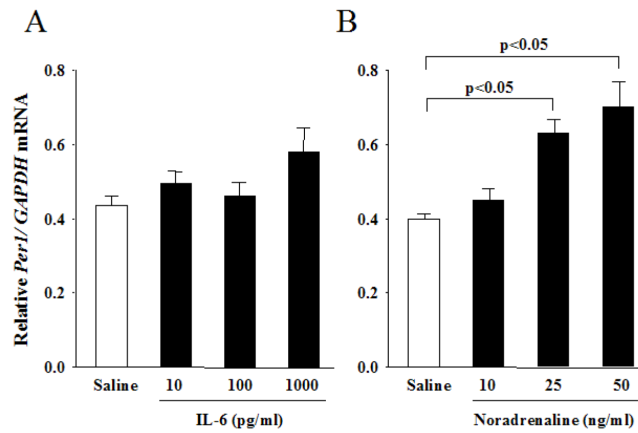

Fig.5
Burioka N

Fig. 6 ***Per1* mRNA expression in the mouse brain by administration of noradrenaline *in vivo.***

Noradrenaline was administered continuously to 3 mice by osmotic pump for 6 days (n=3, mean ± SEM). In the SCN, m*Per1* mRNA was not induced by noradrenaline during both the light (ZT6) and dark periods (ZT18). However, m*Per1* mRNA in the cerebral cortex was significantly induced during the light period ($p < 0.05$). ZT0 and ZT12 were the lights-on (6:00 h) and lights-off (18:00 h) times, respectively.

C: control (saline), N: noradrenaline, ZT: Zeitgeber time.

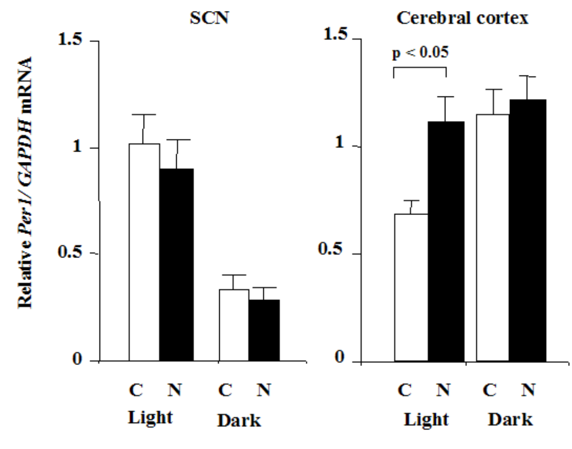

Fig.6
Burioka N

Table 1 Characteristics of normal subjects and patients with OSAS

	Normal	OSAS	p value
Male/Female	7/1	8/0	
Age, (yr)	43.1 ± 4.8	45.9 ± 4.3	0.43
Body mass index	24.6 ± 0.6	25.9 ± 0.6	0.12
AHI (no./hr)	3.8 ± 0.6	53.9 ± 6.2	0.003
ESS	5.9 ± 1.3	13.6 ± 1.2	0.006

BMI: body-mass index, AHI: apnea-hypopnea index, ESS: Epworth sleepiness scale. Body-mass index is defined as the weight in kilograms divided by the square of the height in meters.

Mann-Whitney U-test was used to compare the values between normal subjects and patients with OSAS.

Table 2 **Baseline characteristics of the patients and efficacy of CPAP**

	OSAS	CPAP	p value
AHI (no./hr)	53.9 ± 6.2	6.3 ± 1.1	0.0002
Arousal index (no./hr)	51.1 ± 6.9	23.6 ± 3.5	0.0067
ESS	13.6 ± 1.2	7.9 ± 1.1	0.0094
Total sleep time (min)	395 ± 27	420 ± 20	0.50
Sleep stage (%)			
Stage I sleep	19.3 ± 2.9	14.5 ± 1.7	0.096
Stage II sleep	60.3 ± 1.6	53.3 ± 2.2	0.0045
Stage III sleep	2.8 ± 1.6	4.0 ± 0.5	0.478
Stage IV sleep	1.6 ± 1.2	6.8 ± 1.2	0.01
REM sleep	16.1 ± 2.1	21.4 ± 1.6	0.022
Mean SpO_2 during sleep (%)	92.3 ± 1.1	96.3 ± 0.4	0.0035
Lowest SpO_2 during sleep (%)	73.6 ± 3.4	91 ± 0.5	0.0008
Rate of SpO_2 < 90% (%)	31.1 ± 9.6	0.8 ± 0.4	0.0145

AHI: apnea-hypopnea index, ESS: Epworth sleepiness scale, Rate of SpO_2 < 90%: time of SpO_2 < 90% divided by total sleep time.

Paired t-test was used to compare the values before and after CPAP therapy in patients with OSAS.