Clock gene dysfunction in patients with obstructive sleep apnoea syndrome

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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 apnoea syndrome (OSAS) influences clock gene function, the present authors examined *Period1* (*Per1*) mRNA expression *in vitro* and *in vivo*.

In eight healthy subjects and eight OSAS patients, plasma noradrenaline, serum interleukin (IL)-6, high-sensitivity C-reactive protein (hsCRP) and *Per1* mRNA expression in peripheral whole blood were measured. 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, hsCRP and plasma noradrenaline were elevated in OSAS patients, but improved by continuous positive airway pressure (CPAP) therapy. *Per1* mRNA expression in the peripheral blood significantly decreased at 02:00 h by CPAP in OSAS patients. Stimulation with IL-6 did not directly induce *Per1* mRNA *in vitro*. Administration of noradrenaline induced *Per1* mRNA in the cerebral cortex of mice *in vivo*.

The current study revealed that obstructive sleep apnoea syndrome caused clock gene dysfunction, and continuous positive airway pressure helped to improve it. Sympathetic activation and elevation of the plasma noradrenaline concentration in obstructive sleep apnoea syndrome may be one of the factors involved in disorders of *Period1* mRNA expression.

KEYWORDS: Clock gene, C-reactive protein, interleukin-6, noradrenaline, *Period1*, sleep apnoea

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Obstructive sleep apnoea syndrome (OSAS) is an important disease that contributes to excessive daytime sleepiness, mood disturbances and cardiovascular disease. Moreover, OSAS is thought to cause pro-inflammatory conditions, abnormal sympathetic activation and intermittent hypoxia during apnoea. A number of previous studies have examined the expression of various inflammatory factors in OSAS patients. The serum concentrations of cytokines, such as tumour necrosis factor-α, interleukin (IL)-6 and IL-8, have been reported to be elevated in OSAS patients and to be improved by continuous positive airway pressure (CPAP) therapy [1–3]. IL-6 can induce C-reactive protein (CRP) in the mammalian liver, a process which contributes to cardiovascular disease [4]. Additionally, 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 signalling [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 tumour genesis, obesity and cardiovascular disease [7–9]. Although a detailed molecular mechanism of the circadian oscillator has been revealed in rodents that are nocturnally active, there have been few studies in diurnally active humans. Recently, human peripheral blood cells have been used for representative estimations of the mRNA expression of clock genes in other peripheral tissues [10–14].

Human (h)*Period1* (*Per1*) is one of the important clock genes. Previous studies have shown that the *Per1* promoter contains several enhancers, such as the cAMP response element (CRE) [15–17] and the glucocorticoid response element (GRE) [18–20], in its 5′-upstream region. The expression of *Per1* can reportedly be induced by treatment with 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]. It has also been 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 the present study, to investigate whether OSAS influences clock gene function, h*Per1* mRNA expression was examined *in vitro* and *in vivo*. In humans, it is difficult to analyse the induction of clock genes in organs. Therefore, human peripheral blood cells were used to estimate the mRNA expression of clock genes.

SUBJECTS AND METHODS
Subjects and patients
Eight diurnally (~06:30–23:00 h) active healthy subjects and eight patients with OSAS participated in the present study (table 1). The age and weight of the two groups were matched. The healthy subjects had no cardiovascular disease. The healthy subjects and OSAS patients generally ate breakfast between 07:00 and 08:00 h, lunch between 12:00 and 14:00 h and dinner between 18:00 and 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 (Yonago, Japan) approved the study protocol. Informed consent was obtained from all participants.

Sleep studies
All participants had undergone diagnostic polysomnography (PSG) during the night in the hospital prior to the study (at either Hitachi Memorial Hospital, Yasugi, Shimane, or Tottori University Hospital, Yonago, Japan). Signals from electroencephalograms (EEG; C3-A2 and C4-A1), electro-oculograms, submental electromyograms, ECG, arterial oxygen saturation measured by a finger pulse oximeter (S_{P,O_2}), a snoring microphone, nasal and/or mouth flow using a pressure cannula, and both chest wall and abdominal efforts were recorded by PSG (SomnoTrac; SensorMedics, Yorba Linda, CA, USA). Two independent physicians each categorised the stage of consciousness on the EEG records, using the criteria of RECHTSCHAFFEN and KALES [27]. Apnoea was defined as a cessation of air flow at the nose and/or mouth for $\geqslant 10$ s, while hypopnoea was defined as an airflow reduction of $>50\%$ for $\geqslant 10$ s, followed by a fall of $\geqslant 3\%$ in the S_{P,O_2} or arousal. The apnoea/hypopnoea index (AHI) was calculated as the sum of all apnoeas and hypopnoeas divided by the total sleep time, and was expressed as events·h^{-1}. When the AHI was >5 events·h^{-1}, the patient was diagnosed as having OSAS. However, only OSAS patients with an AHI >30 events·h^{-1} were included in the current study. The patients with OSAS were treated by CPAP (REMstar Auto; Respironics Japan, Tokyo, Japan) after titration. After 3 months of CPAP therapy, the patients with OSAS were also examined with PSG during the night while using CPAP. Exclusion criteria were: central apnoea syndrome; Cheyne–Stokes respiration; major facial or pharyngeal anatomic abnormalities; previous treatment with CPAP; and previous sedative or hypnotic therapy.

Measurement of serum cortisol, IL-6, high-sensitivity CRP and plasma noradrenaline concentrations
Venous blood samples of 10 mL were collected from eight healthy subjects and eight patients with OSAS at 18:00, 02:00, 06:00 and 14:00 h during the study day without medication. After 3 months using CPAP, 10-mL venous blood samples were also collected from the patients with OSAS at each CPAP therapy time-point. The serum and plasma samples were immediately stored at -80°C until further analysis.

Serum cortisol concentrations were determined by a radio-immunoassay based on ^{125}I-labelled cortisol (TFB Inc., Tokyo, Japan). Serum IL-6 concentrations were measured by ELISA (High-Sensitivity IL-6 Biotrak ELISA; Amersham Bioscience, Piscataway, NJ, USA). The plasma noradrenaline concentrations were measured by HPLC (catecholamine test; TOSOH, Tokyo, Japan). The serum high-sensitivity CRP (hsCRP) concentrations were measured using a nephelometer (N-Latex CRP II assay; 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 synthesised using the QuantiTect Reverse Transcription kit (QIAGEN). Real-time PCR was 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'-AAGCAATGCT-ATCACCTCCC-3'; h*Per1* (132 bp) 5'-CTGAGGAGGCCGAG-AGGAAAGAA-3' and 5'-AGGAGGAGGAGGCACATTTAC-GC-3'. The amplifications were performed using the following protocol: initial denaturation at 95°C for 10 min to activate the FastStart Taq DNA polymerase, then 45 cycles consisting of 95°C for 15 s, 55°C for 5 s and 72°C for 10 s (transition rates of 20°C·s^{-1}). The fluorescence value of each capillary was measured at 530 nm. The expression of h*Per1* mRNA was determined relative to the h*β-actin* mRNA expression.

Cell culture
Mouse embryonic fibroblast NIH3T3 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% foetal bovine serum. To determine whether IL-6 or noradrenaline induced mouse (m)*Per1* mRNA expression *in vitro*, the

TABLE 1	Characteristics of normal subjects and patients with obstructive sleep apnoea syndrome (OSAS)		
	Normal	**OSAS**	**p-value**
Sex M/F n	7/1	8/0	
Age yrs	43.1±4.8	45.9±4.3	0.43
BMI	24.6±0.6	25.9±0.6	0.12
AHI events·h^{-1}	3.8±0.6	53.9±6.2	0.003
ESS	5.9±1.3	13.6±1.2	0.006

Data are presented as mean±SEM, unless otherwise stated. Body mass index (BMI) is defined as the weight (kg) divided by the square of the height (m). The Mann–Whitney U-test was used for comparisons between normal subjects and patients with OSAS. M: male; F: female; AHI: apnoea/hypopnoea index; ESS: Epworth sleepiness scale.

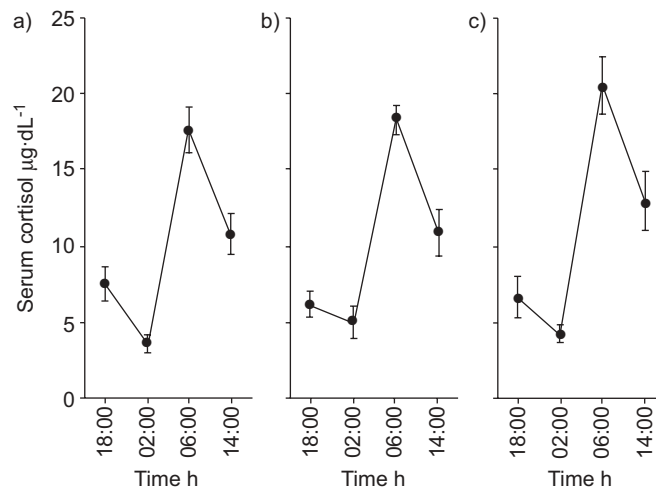| TABLE 2	Baseline characteristics of the obstructive sleep apnoea syndrome (OSAS) patients and efficacy of continuous positive airway pressure (CPAP)		
	OSAS	**CPAP**	**p-value**
AHI events·h^{-1}	53.9 ± 6.2	6.3 ± 1.1	0.0002
Arousal index events·h^{-1}	51.1 ± 6.9	23.6 ± 3.5	0.0067
ESS	13.6 ± 1.2	7.9 ± 1.1	0.0094
TST min	395 ± 27	420 ± 20	0.50
Sleep stage %			
I	19.3 ± 2.9	14.5 ± 1.7	0.096
II	60.3 ± 1.6	53.3 ± 2.2	0.0045
III	2.8 ± 1.6	4.0 ± 0.5	0.478
IV	1.6 ± 1.2	6.8 ± 1.2	0.01
REM	16.1 ± 2.1	21.4 ± 1.6	0.022
Mean S_{P,O_2} during sleep %	92.3 ± 1.1	96.3 ± 0.4	0.0035
Min S_{P,O_2} during sleep %	73.6 ± 3.4	91 ± 0.5	0.0008
% of TST when S_{P,O_2} $<$90%	31.1 ± 9.6	0.8 ± 0.4	0.0145

Data are presented as mean$\pm$SEM. Paired t-tests were used to compare the values before and after CPAP therapy in patients with OSAS. AHI: apnoea/hypopnoea index; ESS: Epworth sleepiness scale; TST: total sleep time; REM: rapid eye movement; S_{P,O_2}: arterial oxygen saturation measured by pulse oximetry.


FIGURE 1. Serum cortisol concentrations in a) healthy subjects, b) patients with obstructive sleep apnoea syndrome (OSAS) and c) OSAS patients treated with continuous positive airway pressure therapy. Data are presented as mean$\pm$SEM, with n=8 in each group. The daily variations of serum cortisol concentration were found to be significant in each group using repeated-measure one-way ANOVA (p$<$0.0001 in each case). However, no statistically significant differences in the serum cortisol concentrations at each time-point were found between the three groups.

NIH3T3 cells were treated for 24 h with 10, 100 or 1,000 pg·mL^{-1} of mouse IL-6 (R&D Systems Inc., Minneapolis, MN, USA) or 10, 25 or 50 ng·mL^{-1} of noradrenaline (Sigma-Aldrich Co., St Louis, MO, USA). After treatment, the mRNA levels of m*Per1* were assessed by RT-PCR.

For quantification of the relative RNA levels, the cDNAs of m*Per1* and the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene were synthesised and amplified using the SuperScript^{TM} One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA). Specific primer pairs used were as follows: m*Per1* (403 bp) 5'-CCAGGCCCGGAGAACCTTTTT-3' and 5'-CGAAGTTTGAGCTCCCGAAGTG-3'; m*GAPDH* (178 bp) 5'-GACCTCAACTACATGGTCTACA-3' and 5'-ACTCCACGACATACTCAGCAC-3'. To evaluate the quantitative reliability of RT-PCR, kinetic analysis of the amplified products was carried out, in order 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 ratios of the amplified target to the amplified internal control (calculated by dividing each m*Per1* value by that of *GAPDH*) were compared between the groups.

Experimental animals

Male ICR mice aged 4–6 weeks (Tokyo Experimental Animals Co., Tokyo, Japan) were used. The animals were housed at 22 ± 2°C, 60 ± 5% humidity, with a 12-h light/12-h dark cycle (lights on from 06:00 to 18:00 h). Zeitgeber time (ZT)0 and ZT12 were the lights-on and lights-off times, respectively. The light intensity at the surface of the cages was ~100 lx. The mice were fed a standard diet and water was available *ad libitum*. An osmotic miniature pump (model 2001; ALZET, Palo Alto, CA, USA) was implanted under the skin of the mice, and was used for the continuous administration of noradrenaline (1.5 µg·h^{-1}) or saline for 6 days.

Total RNA from mouse tissues was extracted as follows. The brain was quickly removed halfway through the light or dark phase (at ZT6 or ZT18, respectively). Coronal brain slices (500 µm) were prepared using a rodent brain matrix (RBM-2000C; ASI Instruments, Inc., Warren, MI, USA), and the SCN was punched out bilaterally from the brain slices. To obtain an adequate amount of RNA from the SCN, SCN material from three mice in each group was combined and extracted. The extraction of total RNA from the cerebral cortex of an individual mouse was carried out separately using the RNeasy Mini kit (QIAGEN). The mRNA levels of m*Per1* were assessed by RT-PCR, as described in the previous section. Experimental animal care was conducted under permission from the Committee for Animal Experimentation in the Division of Pharmaceutical Sciences at Kyushu University (Fukuoka, Japan).

Statistical analysis

The results are presented as mean$\pm$SEM for all samples. The statistical differences between groups were analysed by one-way ANOVA, and multiple comparisons were performed by Fisher's protected least significant difference (PLSD) or Dunnett's test. A value of p$<$0.05 was considered to indicate statistical significance.
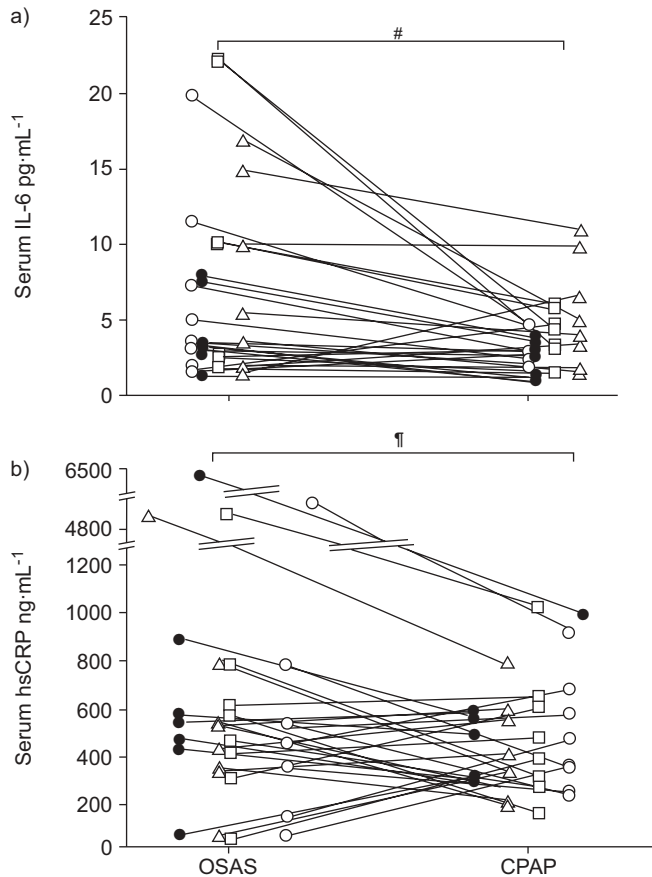
FIGURE 2. Serum interleukin (IL)-6 and high-sensitivity C-reactive protein (hsCRP) concentrations before and after continuous positive airway pressure (CPAP) therapy in patients with obstructive sleep apnoea syndrome (OSAS). a) The serum IL-6 concentration was significantly decreased by CPAP therapy in patients with OSAS. b) The serum hsCRP concentration was also significantly decreased by CPAP therapy in patients with OSAS. Paired t-tests were used to compare the values before and after CPAP therapy in patients with OSAS. Values were measured at 18:00 h ($\square$), 02:00 h ($\triangle$), 06:00 h ($\bigcirc$) and 14:00 h ($\bullet$). $^{\#}$: $p < 0.005$; ¶: $p < 0.04$.

RESULTS

Efficacy of CPAP therapy

After using CPAP for 3 months, a repeat night-time PSG was performed in patients with OSAS. The AHI and Epworth sleepiness scale index were significantly decreased and improved by CPAP. The other indices were also clearly improved by CPAP therapy (table 2). The CPAP device can record usage automatically, and the compliance of CPAP therapy was objectively calculated. The mean $\pm$ SEM time of CPAP use was 5.2 ± 0.4 h·night^{-1}. The average proportion of CPAP use was $75 \pm 3.6\%$, calculated by dividing the number of days when patients used the CPAP device by the total number of days of the study.

Serum cortisol concentration

Figure 1 shows the daily variations of serum cortisol concentrations in healthy subjects, 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 02:00 h and consistently highest at 06:00 h, using
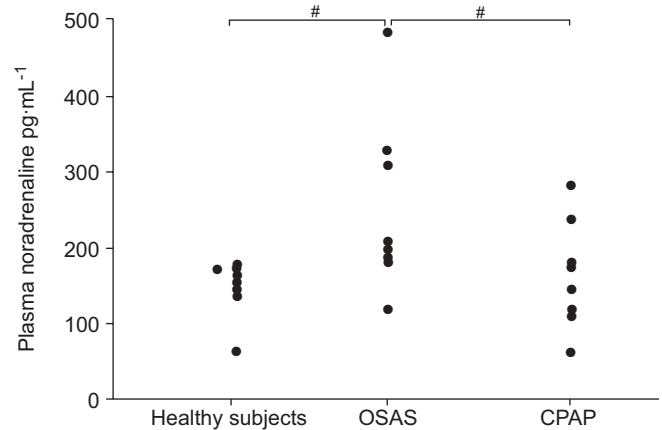
FIGURE 3. Plasma noradrenaline concentration at 02:00 h in healthy subjects, patients with obstructive sleep apnoea syndrome (OSAS) and OSAS treated with continuous positive airway pressure (CPAP). The variation in the plasma noradrenaline concentrations in the supine position between the three groups was significant by one-way ANOVA. The plasma noradrenaline concentration in the OSAS patients was significantly higher than that in the healthy subjects, and CPAP therapy significantly decreased the plasma noradrenaline concentration, as measured by Fisher's protected least significant difference. $^{\#}$: $p < 0.04$.

Fisher's PLSD test. No statistically significant differences in the serum cortisol concentrations at each time-point were found between the three groups. The 24-h mean $\pm$ SEM serum cortisol levels were 9.9 ± 1.7 µg·dL^{-1} in the normal subjects, 10.1 ± 1.8 µg·dL^{-1} in the OSAS patients and 11.4 ± 2.0 µg·dL^{-1} 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 hsCRP

The mean $\pm$ SEM serum IL-6 concentrations in the healthy subjects were 1.6 ± 0.1, 1.5 ± 0.2, 1.7 ± 0.3 and 1.6 ± 0.2 pg·mL^{-1} at 18:00, 02:00, 06:00 and 14:00 h, respectively. In the patients with OSAS, the serum IL-6 concentrations at the same time-points were 9.1 ± 2.7, 7.0 ± 1.8, 6.6 ± 1.9 and 4.0 ± 0.7 pg·mL^{-1}. After CPAP therapy for 3 months, the serum IL-6 concentrations in the OSAS patients were 3.8 ± 0.5, 5.4 ± 1.0, 2.8 ± 0.4 and 2.4 ± 0.3 pg·mL^{-1} at 18:00, 02:00, 06:00 and 14:00 h, respectively. The 24-h mean serum concentrations of IL-6 were 1.6 ± 0.3 pg·mL^{-1} in the healthy subjects, 6.7 ± 1.2 pg·mL^{-1} in the patients with OSAS and 3.6 ± 0.6 pg·mL^{-1} in the OSAS patients treated with CPAP. A significant difference was found between the three groups by one-way ANOVA ($p < 0.05$). The serum concentrations of IL-6 were significantly decreased by CPAP therapy ($p < 0.005$; fig. 2a).

The mean $\pm$ SEM serum hsCRP concentrations in the healthy subjects were 255 ± 63, 237 ± 61, 188 ± 40 and 245 ± 59 ng·mL^{-1} at 18:00, 02:00, 06:00 and 14:00 h, respectively. In the patients with OSAS, the serum hsCRP concentrations at the same time-points were $1,011 \pm 475$, 985 ± 479, $1,008 \pm 522$ and $1,240 \pm 636$ ng·mL^{-1}. After CPAP therapy for 3 months, the serum hsCRP concentrations in the OSAS patients were 487 ± 82, 424 ± 62, 473 ± 70 and 475 ± 73 ng·mL^{-1} at 18:00, 02:00, 06:00 and 14:00 h, respectively. The 24-h mean serum
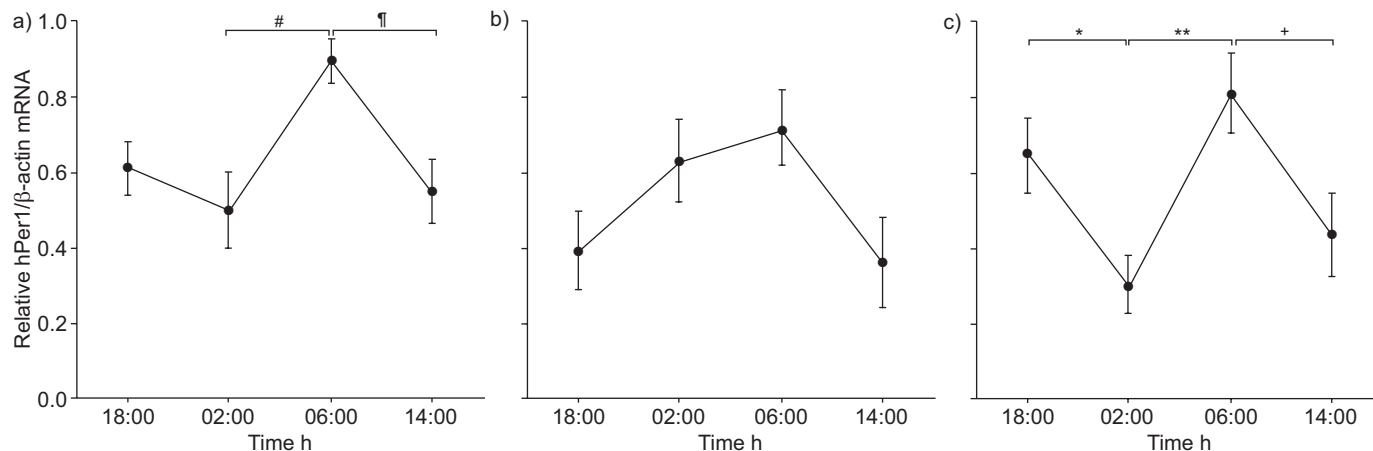
FIGURE 4. Variation in the relative values of human *Period1* (h*Per1*) mRNA expression in human peripheral blood cells, as determined by real-time PCR analysis in a) healthy subjects, b) patients with obstructive sleep apnoea syndrome (OSAS) and c) OSAS patients treated with continuous positive airway pressure (CPAP) therapy. Data are presented as mean±SEM, with n=8 in each group. The value for h*Per1* mRNA expression at each time-point was divided by each peak value. The expression of h*Per1* mRNA was determined relative to the *β-actin* mRNA expression. The variation in the relative values of h*Per1* mRNA expression at four time-points was statistically significant in the healthy subjects and in the patients with OSAS during CPAP therapy, by repeated-measure one-way ANOVA. Multiple comparisons were performed by Fisher's protected least significant difference. [#]: $p < 0.02$; [¶]: $p < 0.03$; [+]: $p < 0.04$; [*]: $p < 0.05$; [**]: $p < 0.01$.

concentrations of hsCRP were 231 ± 41 ng·mL^{-1} in the healthy subjects, $1,061 \pm 188$ ng·mL^{-1} in the patients with OSAS and 465 ± 82 ng·mL^{-1} in the OSAS patients treated with CPAP. A significant difference was found between the three groups by one-way ANOVA ($p < 0.05$). The serum concentration of hsCRP was significantly decreased by CPAP therapy ($p < 0.04$; fig. 2b).

Levels of plasma noradrenaline
The plasma noradrenaline concentrations in the supine position at 02:00 h were 144 ± 11 pg·mL^{-1} in the healthy subjects, 251 ± 35 pg·mL^{-1} in the patients with OSAS and 158 ± 22 pg·mL^{-1} in the OSAS patients treated with CPAP. The plasma noradrenaline concentrations were significantly different between the three groups by one-way ANOVA ($p < 0.05$), and significantly decreased after CPAP therapy (Fisher's PLSD; $p < 0.04$; 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. 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. It was confirmed that h*Per1* mRNA expression in healthy subjects at 06:00 h was significantly higher than at 02:00 h or at 14:00 h, by multiple comparisons using Fisher's PLSD ($p < 0.02$ and $p < 0.03$, respectively; fig. 4a). The highest h*Per1* transcription in peripheral blood was previously observed in healthy human subjects at times experienced as morning [10, 11, 13, 14]. The current result was consistent with these previous reports.

In particular, the relative values at 02:00 h were significantly different between the three groups ($p < 0.04$; one-way ANOVA). In the OSAS patients treated with CPAP, the relative value for h*Per1* mRNA expression at 02:00 h was significantly lower than at 02:00 h in the OSAS patients before treatment, as determined 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 ($1,000$ pg·mL^{-1}) of IL-6 slightly induced m*Per1* mRNA (fig. 5a). Conversely, treatment of NIH3T3 cells with noradrenaline caused an induction of m*Per1* mRNA in a concentration-dependent manner (one-way ANOVA; $p < 0.003$; fig. 5b). Significant inductions of m*Per1* mRNA were observed using Dunnett's test when the cells were treated with 25 and 50 ng·mL^{-1} noradrenaline ($p < 0.05$ in each case; fig. 5b).

Per1 *mRNA expression in the mouse brain during the administration of noradrenaline in vivo*
Male ICR mice were exposed for 3 weeks to a 12-h light/12-h dark cycle. Following this, noradrenaline was administered continuously to three mice *via* an osmotic pump for 6 days. In the SCN, m*Per1* mRNA was not induced by noradrenaline during either the light (ZT6) or dark (ZT18) periods. However, m*Per1* mRNA was significantly induced in the cerebral cortex 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.
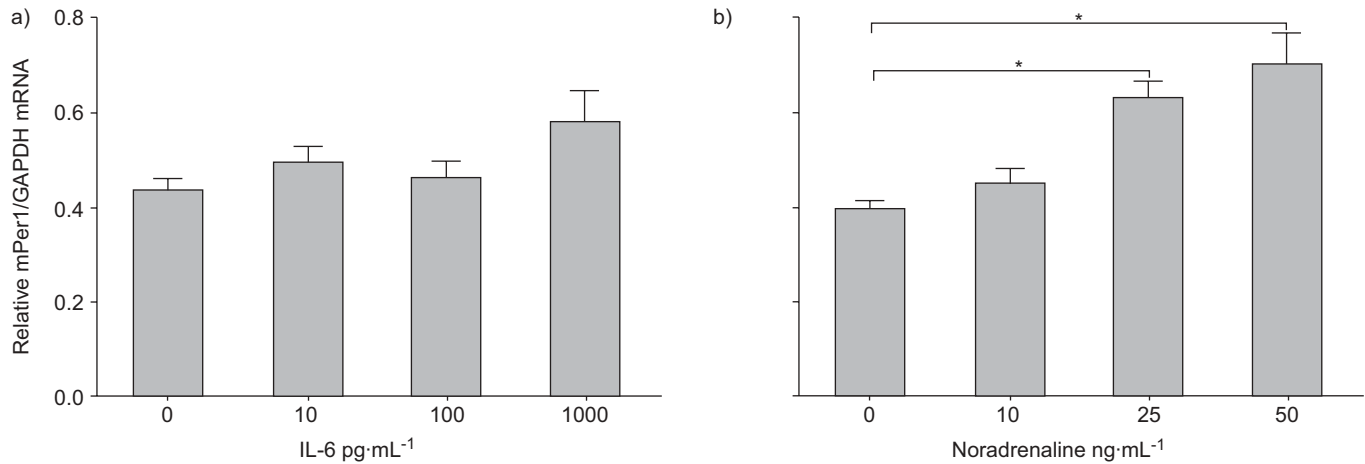
FIGURE 5. Mouse *Period1* (m*Per1*) mRNA expression in mouse NIH3T3 cells by stimulating with a) interleukin (IL)-6 or b) noradrenaline *in vitro*. The expression of m*Per1* mRNA was determined relative to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) mRNA expression using RT-PCR analysis. Data are presented as mean±SEM, with n=3 for each concentration. Treatment of NIH3T3 cells with IL-6 resulted in no significant induction of m*Per1* mRNA. Conversely, treatment of NIH3T3 cells with noradrenaline caused a significant induction of m*Per1* mRNA in a concentration-dependent manner, as determined by one-way ANOVA. Significant inductions of m*Per1* mRNA were observed using Dunnett's test when the cells were treated with 25 and 50 ng·mL^{-1} noradrenaline. *: $p < 0.05$.
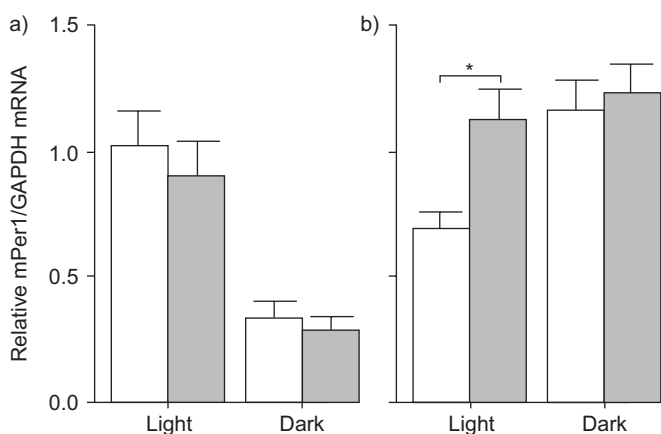
DISCUSSION

The current study 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. The factors that influenced *Per1* mRNA expression were examined. 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*. However, prolonged stimulation with IL-6 did not induce m*Per1* expression *in vitro*.

FIGURE 6. Mouse *Period1* (m*Per1*) mRNA expression in the mouse brain during administration of noradrenaline *in vivo*. Noradrenaline was administered continuously to three mice by osmotic pump for 6 days. Data are presented as mean±SEM. a) In the suprachiasmatic nucleus, m*Per1* mRNA was not induced by noradrenaline (■) during either the light or dark periods, compared with control saline administration (□). b) In the cerebral cortex, m*Per1* mRNA was significantly induced by noradrenaline during the light period. *: $p < 0.05$.

Sympathetic activation in OSAS patients may be one of the factors involved in the dysfunction of *Per1* mRNA expression during sleep. In addition, CPAP was useful for improving the serum CRP, IL-6 and plasma noradrenaline concentrations, and dysfunction of the clock gene, h*Per1*. *Per1* mRNA expression in the peripheral blood cells may be a new index for evaluating the efficacy of CPAP therapy in OSAS patients.

Glucocorticoid signals to the mammalian clock gene Per1

Previous reports have shown that circadian expression of *Per1* can be elicited by multiple signalling factors, 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 that elicits rhythmic mRNA expression in peripheral clock genes. Several reports have suggested that the increase in *Per1* mRNA accumulation is caused by glucocorticoid signalling *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]. It has also been previously confirmed that stimulation with dexamethasone strongly induced h*Per1* mRNA in cultured human bronchial epithelial cells, BEAS-2B [22]. Moreover, it has been reported that injection of prednisolone *in vivo* markedly induced h*Per1* mRNA expression in human peripheral blood mononuclear cells after 1 h [28].

One study has 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 the present 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 of induction of h*Per1* mRNA at 02:00 h in OSAS patients may not be related to the signalling pathway *via* GRE.

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

In the present study, the serum concentrations of both IL-6 and hsCRP in OSAS patients were significantly higher than those in healthy subjects and were found to be decreased by CPAP. It has been reported that hypoxia can activate various transcriptional factors of nuclear factor (NF)-κB and NF-IL-6, and can increase the production of IL-6 [30]. IL-6 is an important pro-inflammatory cytokine that has been implicated in the pathogenesis of atherosclerosis. 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 has suggested that stimulation with a high concentration of IL-6 could induce h*Per1* mRNA in cultured cells [23]. To investigate whether IL-6 could directly induce *Per1* mRNA *in vitro*, cultured NIH3T3 cells were stimulated with IL-6 for 24 h. Treatment of NIH3T3 cells with IL-6 resulted in no significant induction of m*Per1* mRNA, although a high concentration (1,000 pg·mL^{-1}) of IL-6 slightly induced m*Per1* mRNA. The current authors postulated that IL-6 elevation might not directly induce h*Per1* mRNA.

Effect of noradrenaline on induction of 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 markers of severity of OSAS [33]. The present study found that the plasma noradrenaline concentration increased in patients with OSAS.

Adrenoceptor agonists can reportedly induce the expression of *Per1* by the cAMP–protein kinase A–CRE-binding protein or mitogen-activated protein kinase–CRE-binding protein signal pathways [16, 24, 34]. The current authors examined whether noradrenaline could induce *Per1* mRNA *in vitro* and *in vivo*. Treatment of NIH3T3 cells with noradrenaline caused an induction of m*Per1* mRNA in a concentration-dependent manner. In addition, noradrenaline was administered to three mice by osmotic pump for 6 days *in vivo*. In the SCN, m*Per1* mRNA was not induced by noradrenaline during either the light or dark periods, but m*Per1* mRNA was significantly induced in the cerebral cortex during the light period when the mice were not active. Thus, it was 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 the cerebral cortex may have an influence on emotion, additional research is needed to clarify its actual physiological impacts.

OSAS causes intermittent hypoxia, which activates hypoxia-inducible factor (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.* [36] recently indicated that intermittent hypoxia activated the NF-κB inflammatory pathway and might be associated with a specific role in the pathophysiology of cardiovascular complications in OSAS. 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 plasma noradrenaline may be one of the factors that increase *Period1* mRNA expression during sleep in patients with obstructive sleep apnoea syndrome. Continuous positive airway pressure therapy is useful for improving the abnormal changes in the serum interleukin-6, C-reactive protein and plasma noradrenaline concentrations, and human *Period1* mRNA expression in patients with obstructive sleep apnoea syndrome. 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 to make representative estimations of clock gene mRNA expression. However, the actual physiological impacts are still unclear, and further studies will be needed to determine the relationship between the dysfunction of the clock gene in obstructive sleep apnoea syndrome patients and clinical symptoms, such as excessive daytime sleepiness and mood disturbances.

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