

Optimization of Dosing Schedule of Daily Inhalant Dexamethasone to Minimize Phase Shifting of Clock Gene Expression Rhythm in the Lungs of the Asthma Mouse Model

Naomi Hayasaka, Tsuyoshi Yaita, Tomoyuki Kuwaki, Sato Honma, Ken-ichi Honma, Takashi Kudo, and Shigenobu Shibata

Laboratory of Physiology and Pharmacology (N.H., T.Y., Ta.K., S.S.), School of Sciences and Engineering, Waseda University, Nishitokyo 202-0021, Japan; Department of Autonomic Physiology (To.K.), Chiba University Graduate School of Medicine, Chiba 260-8670, Japan; and Department of Physiology (S.H., K.H.), Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan

Glucocorticoid receptor agonists such as dexamethasone (DEXA) have been recommended for the treatment of asthma. An increased frequency of dosing with these drugs seems preferable for cases of severe or uncontrolled asthma. The purpose of this experiment was to find the appropriate dosing schedule (frequency and timing) for DEXA inhalation based on chronotherapeutic dosing to minimize phase shifts of clock function in the lungs of the ovalbumin-treated asthmatic mouse. The daily rhythm of clock gene expression was similar between control and ovalbumin-treated mice. Acute inhalation of DEXA significantly increased *mPer1* gene expression in the lungs but not the liver of mice. Daily exposure of DEXA at zeitgeber time 0 (lights on) or at zeitgeber time 18 (6 h after

lights off) for 6 d caused a phase advance or phase delay of bioluminescence rhythm in the lungs, respectively, similar to light-induced phase shifts in locomotor activity rhythm. Daily zeitgeber time 0 exposure to DEXA attenuated the expression level of the *mClca3* gene, which is associated with mucus overproduction, and there was a phase-advancing peak time of the *mClca3* rhythm. The present results denote the importance of selecting the most appropriate time of day for nebulizer administration of DEXA to minimize adverse effects such as the phase shifting of clock function in asthmatic lungs. This is the first report of a successful protocol that could obtain phase shifts of clock gene expression rhythm in isolated peripheral organs *in vivo*. (*Endocrinology* 148: 3316–3326, 2007)

NOCTURNAL SYMPTOMS AND overnight decrements in lung function are common to asthma sufferers (1). As many as 75% of asthmatic subjects are awakened by asthma symptoms at least once per week. An extensive body of research has demonstrated that nocturnal symptoms such as coughing and dyspnea are accompanied by circadian variations in airway inflammation and physiological variables such as airflow limitation and airway hyperresponsiveness (1, 2). Alterations in β_2 -adrenergic (3) and glucocorticoid (4) receptor function might play a role in modulating the nocturnal asthma phenotype. Inhaled corticosteroids are the mainstay of asthma therapy. Although treatment dosage and times depend on the severity of the asthma symptoms, a once-daily or twice-daily dosing schedule is often used (5–7). Twice-daily combination therapy of inhaled corticosteroids and long-acting β_2 -adrenoceptor agonists has now been established as a more effective treatment for moderate to severe asthma (8). Based on chronotherapeutic experiments, it is reported that the efficacy of glucocorticoids in asthmatic

patients changes with the time of administration (9, 10). Thus, the most effective dosing schedule (frequency, timing, and combination) of antiasthmatic drugs is now being considered in the clinical administration of medication to asthmatic patients.

The suprachiasmatic nucleus (SCN) includes a master pacemaker that regulates not only behavioral and physiological circadian rhythms such as locomotor activity, body temperature, and endocrine release but also peripheral clock function (11, 12) through the communication of timing information to a variety of peripheral tissues via neural (13) and humoral (14) connections. Recently several papers have suggested that dexamethasone (DEXA) can reset the rhythm of clock gene expression in the mouse liver as well as cell lines such as rat-1 (15–17). Thus, DEXA is known as the strongest of several chemicals that can reset circadian clock gene expression rhythm in peripheral tissue and cells. As mentioned above, glucocorticoids have also been recommended as a clinical medication to be administered once or twice daily to asthma patients. We investigated whether once-daily exposure of DEXA through a nebulizer apparatus could phase shift the clock gene expression rhythm in the lungs of ovalbumin (OVA)-treated mice (18) and produce a phase shift of daily lung function. To confirm whether the effect of DEXA on clock gene expression is common or specific, we also examined the effect of salbutamol (SAL), a bronchodilator drug used in the clinic, on clock function in the lungs of the OVA-treated asthma mouse model.

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Abbreviations: ACh, Acetylcholine; CT, circadian time; DEXA, dexamethasone; OVA, ovalbumin; SAL, salbutamol; SCN, suprachiasmatic nucleus; VT, tidal volume; ZT, Zeitgeber time.

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Materials and Methods

Experimental animals

In this experiment, we used male ICR mice 4–6 wk of age (Tokyo Experimental Animals Co., Tokyo, Japan) for general experiments. The *mBmal1*-luciferase mice were obtained from coworkers (Hokkaido University, Sapporo, Japan) (19) and reared in our animal quarters. The method for generating *mBmal1*-luciferase transgenic mice is described in detail by Nishide *et al.* (19). Environmental conditions of the animal room were controlled at a temperature of 22 ± 2 °C, humidity of $60 \pm 5\%$, and a 12-h light, 12-h dark cycle (lights on from 0600 to 1800 h). Zeitgeber time (ZT) 0 and ZT12 were the lights-on and lights-off times, respectively. Light intensity at the surface of the cages was approximately 100 lux. Mice were fed commercial chow (Oriental Yeast Co., Ltd., Tokyo, Japan) and water was available *ad libitum*. In all experiments, we examined the resetting action of DEXA on daily clock gene expression rhythm in the lungs of mice under light-dark but not constant dark conditions. Experimental animal care was conducted under permission from the Committee for Animal Experimentation in the School of Science and Engineering at Waseda University (permission 00–16).

OVA-treated asthma mouse model

Mice were immunized sc at 1-wk intervals with $1 \mu\text{g}$ OVA (Nakarai Pharmaceuticals, Tokyo, Japan) and 1.6 mg aluminum hydroxide (Nakarai Pharmaceuticals) in 0.4 ml of 0.9% saline. Control mice received the same volume of PBS in alum. One week later, all mice were challenged for 30 min with 5% OVA inhaled through a nebulizer a total of three times at 4-d intervals. For control animals, PBS was injected or administered through a nebulizer.

Measurement of ventilation by whole body plethysmography

We used double-chamber, whole-body plethysmography as described in our previous papers (20) with few modifications. This method was originally described by Jacky (21). The mouse was placed in one chamber (750 ml) that was used as a barometric chamber, and reference pressure was measured in the other chamber. The outlet ports of both chambers were connected by a long tube so that the time constant for gas leakage from the chamber was large, compared with the duration of the respiratory cycle. Such modification allowed for both respiratory frequency and tidal volume to be measured by the flow-through system (21). Plethysmographic signals were recorded as changes in the pressure difference between the two chambers by use of a differential pressure transducer (TP-602T; Nihon Kohden, Tokyo, Japan). Amplified (AR-601G and AB-621G; Nihon Kohden) signals were fed into a computer (sampling frequency of 100 Hz). The respiratory frequency and amplitude of the plethysmographic signals (representing tidal volume) were calculated using the Chart signal analysis software (AD Instruments Japan Inc., Nagoya, Japan). Ambient temperature and pressure and the chamber temperature were measured intermittently (approximately every 2 h) during the recording, and the average values were used for later calculation of tidal volume. Chamber temperature in the thermoneutral range was maintained between 22 and 25 °C by controlling the room temperature to minimize possible metabolic effects on respiration. Animal core temperature was not measured continuously.

Individual measurements of rectal temperature revealed a mean value ($\pm$ SE) of 35.8 ± 0.7 °C over a 24-h-based value. On the basis of these values, the tidal volume was calculated according to a formula used by Epstein *et al.* (22). Minute volume was defined as the product of inspiratory tidal volume and respiratory frequency. To show the daily change of minute volume, mean values for minute volume were calculated at 3-h intervals. Each chamber was continuously flushed with a gas mixture at a rate of 500 ml/min of room air. We already confirmed that the flushing rate in this study was sufficient to avoid both CO₂ accumulation and an O₂ decrease in the chamber while animals breathed room air (20). Airway responsiveness to acetylcholine (ACh) was measured in mice under light anesthetization with ketamine (45 mg/kg; Sigma-Aldrich, St. Louis, MO). Under ketamine anesthesia, respiratory pressure curves were recorded by double-chamber, whole-body plethysmography in response to inhaled ACh (Sigma-Aldrich) at concentrations of 10–50 mg/ml for 1–2 min. Respiration volume was recorded in unrestrained, conscious mice using the same double-chamber whole-

body plethysmography over the course of 1–2 d. Daily rhythm of minute respiratory volume was calculated using the mean values of minute volume taken during each 3-h recording epoch.

Drugs and treatment

DEXA (sodium salt; Sigma-Aldrich) and SAL (Sigma-Aldrich) were inhaled through a compressor-assisted nebulizer (NE-C16, clinical use type; Omron, Tokyo, Japan) at 0.02–0.5 and 1 mg/ml, respectively, for 15 min using a chamber (6.5 liters) with a flow rate of 5 l/min. For an inhalation speed of 3 ml per 15 min, 0.06–1.5 mg DEXA per total 75 liters exchange air was calculated. The minute respiration volume was approximately 30 ml/min for both control and OVA-treated mice; therefore, the total ventilation for 15 min equaled 0.45 liters per 15 min. Thus, each mouse was estimated to intake 0.36–9 μg per 0.45 liters. At an average mouse body weight of 40 g, a dose of DEXA was then 9–225 $\mu\text{g/kg}$ or 0.54–13.5 mg per 60 kg mouse. In the clinical setting, DEXA is prescribed at between 0.1 and 2 mg/person ($\sim$ 60 kg). Thus, doses of DEXA were comparable with clinical doses, and we actually selected a middle range dose (0.02 and 0.1 mg/ml; 0.54 and 2.7 mg per 60 kg mouse) for chronic application experiments. SAL (1 mg/ml and 13.5 mg per 60 kg) was administered at a dose 5 times higher than the clinical dose (1–2.5 mg/person) because single exposure at this dose caused an increase of *mPer1* gene expression similar to that of DEXA at 0.1 mg/ml.

Acute or chronic inhalation schedule of DEXA and SAL

Two hours after acute inhalation of DEXA (0.02, 0.1, 0.5 mg/ml) and SAL (1 mg/ml) at ZT 6 for 15 min or acute ip injection of DEXA (0.4, 2, 10 mg/kg), mice were killed and lung and liver tissues were dissected. To examine whether DEXA and SAL inhalation protected ACh-induced trachea constriction in the OVA-treated mouse, DEXA was given four times 1 h before OVA challenge, or SAL was given one time 1 h before ACh challenge. To assess whether daily chronic inhalation of DEXA and SAL caused a phase shift of clock gene and clock-controlled gene expression rhythm in the OVA-treated mouse liver and lungs, these drugs were administered through a nebulizer for 15 min, once daily at ZT0 for 6 continuous days. Drugs were not administered on d 7 and 8, and animals were killed at ZT19 and ZT1 on d 7 and ZT7 and ZT13 on d 8. In the experiment on bioluminescence rhythm of *mBmal1*-luciferase transgenic mice, mice were killed at ZT3 on d 7 after chronic drug exposure once daily at ZT0, 6, 12, or 18 for 6 continuous days. In some control experiments, PBS was administered as a drug vehicle, and mice were killed at the same time points. To assess whether chronic inhalation of DEXA or SAL changed the phase of daily rhythm of minute respiration, a mouse was moved from the drug exposure apparatus to the double chamber to measure whole-body plethysmography from d 7 to 8 under DEXA or SAL withdrawal conditions.

Measurement of *mBmal1*-luciferase activity in peripheral tissues

A real-time monitoring technique of gene expression was successfully introduced to the field of circadian rhythm research in mammals, which has enabled us to measure promoter activity of clock genes long-term with bioluminescence, not only *in vitro* but also *in vivo* (23–26). In this experiment, we used *mBmal1*-luciferase transgenic mice (19) for this purpose.

Peripheral tissues such as the liver and lungs were rapidly removed from the transgenic mice and placed in ice-cold Hanks' balanced salt solution (pH 7.2; Sigma-Aldrich). These tissues were cut into fragments and explanted in a 35-mm petri dish, sealed with parafilm (Sigma-Aldrich), and cultured with 1.3 ml DMEM (Invitrogen, Carlsbad, CA) supplemented with NaHCO₃ (2.7 mm), HEPES (10 mm), kanamycin (20 mg/liter; Sigma-Aldrich), insulin (5 $\mu\text{g/ml}$; Sigma-Aldrich), putrescine (100 μM ; Sigma-Aldrich), human transferrin (100 $\mu\text{g/ml}$; Sigma-Aldrich), progesterone (20 nm; Sigma-Aldrich), sodium selenite (30 nm; Sigma-Aldrich), and 0.1 mM D-luciferin Na salt (Invitrogen). The cultures were incubated at 37 °C, and bioluminescence was monitored for 1 min at 10-min intervals with a dish-type luminometer (LumiCycle; Actimetrics, Wilmette, IL).

Analysis of circadian rhythms in bioluminescence

The circadian rhythms for *mBmal1*-luciferase were smoothed by a five-point moving average method, and the peak phases were calculated after subtracting the trends of basal levels. A trend line was obtained by connecting two consecutive troughs in a circadian cycle. The trend line was shifted toward the peak, and the point of contact with the bioluminescence curve was defined as the peak phase of the cycle. Circadian period was determined by measuring the peak interval.

RNA isolation and real-time RT-PCR

Mice were deeply anesthetized with ether, and the lungs and livers were rapidly isolated and frozen in liquid nitrogen, then stored at -80°C until RNA isolation. Total RNA was extracted using ISOGEN reagent (Nippon Gene, Tokyo, Japan). Fifty nanograms of total RNA were reverse transcribed and amplified using the one-step SYBR RT-PCR kit (TaKaRa, Otsu, Japan) in the iCycler (Bio-Rad, Hercules, CA). Specific primer pairs were designed based on the following published data on *β -actin*, *mBmal1*, *mPer1*, *mPer2*, *mClca3*, and *mPai-1* genes in GenBank: *β -actin* (131 bp, GenBank AK075973, 1009–1139), 5'-TGACAGGATGCAGAAGGAGA-3' (forward) and 5'-GCTGGAAGGTGGACAGTGAG-3' (reverse); *mBmal1* (71 bp, GenBank AB014494, 2407–2477), 5'-CCACCTCAGAGCCATTGATACA-3' (forward) and 5'-GAGCAGGTTTAGTTCCACTTGTCT-3' (reverse); *mPer1* (151 bp, GenBank AF022992, 1951–2101), 5'-CGCCTCCTTGCTACAGGTACAT-3' (forward) and 5'-GGTAGGAACAGCCAGAGGTTTC-3' (reverse); *mPer2* (142 bp, GenBank AF036893, 5563–5704), 5'-TGTTGTCTTACACGGGTGCTCA-3' (forward) and 5'-ACGTTTGGTTTGCATGAA-3' (reverse); *mClca3* (146 bp, GenBank AB017156, 2258–2403), 5'-TGACCTCTTACCCTGTCA-3' (forward) and 5'-CGATACTGGTGCTCATTCGGA-3' (reverse); and *mPai-1* (137 bp, GenBank BC054091, 858–994), 5'-CCGATGGGCTCAGATATGA-3' (forward) and 5'-TTGTCTGATGAGTTACAGCATCCA-3' (reverse).

RT-PCR was executed under the following conditions: cDNA synthesis at 42°C for 15 min followed by 95°C for 2 min, PCR amplification for 40 cycles with denaturation at 95°C for 5 sec, and annealing and extension at 60°C for 20 sec. A melt curve analysis was performed after PCR amplification. The software determined only the number of templates present in the reactions; if the amounts of RNA and cDNA used in the RT-PCR were to be considered, the number of specific molecules of mRNA per microgram in the samples had to be calculated. Results are expressed as the absolute copy number per microgram of RNA or as a relative expression. The number of clock gene mRNAs calculated were normalized with the *β -actin* housekeeping gene, and then the normalized values were converted to relative expression. To facilitate a comparison among phases of daily clock gene expression under several different experimental conditions, the peak-time value of each daily rhythm was set at 100%.

Measurement of locomotor activity

To assess the locomotor activity, mice were housed individually in transparent plastic cages connected to a nebulizer for DEXA exposure. Locomotor activity rhythms under light-dark conditions were recorded through area sensors (FA-05 F5B; Omron, Tokyo, Japan) with a thermal radiation detector system attached to the top of the cage as described in our previous paper (27).

Statistics

Results are expressed as the mean $\pm$ SEM. The significance of differences between groups was determined by a two-way or one-way ANOVA followed by Dunnett's test or Student's *t* test.

Results

Asthma mouse model evaluated by *mClca3* gene expression and minute respiration

We initially examined the basal values of respiratory parameters in anesthetized mice before ACh application. There

were no significant differences between control and OVA-treated mice in respiratory frequency (n per second) (2.2 ± 0.3 , $n = 19$ for control; 2.2 ± 0.3 , $n = 19$ for OVA), tidal volume (milliliters) (0.140 ± 0.030 for control, 0.142 ± 0.032 for OVA), and respiratory minute volume (milliliters per minute) (31.5 ± 9.0 for control, 30 ± 4.5 for OVA). To assess whether the procedure of OVA treatment in the present experiments could cause asthma in mice, we examined the effect of ACh on respiratory frequency and respiration volume response in OVA-treated mice when ACh was applied 2 d after the last inhalation of OVA (Fig. 1, A and B). Using the 5-min recording control value as a baseline, ACh ($10\text{--}50$ mg/ml) was administered through a nebulizer for 1–2 min under ketamine anesthesia. For control mice, these doses of ACh did not cause any changes in respiratory frequency and respiration volume. In contrast, ACh at 25 and 50 mg/ml concentrations significantly increased the respiration volume and decreased the respiratory frequency ($P < 0.05$, Dunnett's test). Finally three of eight and four of four OVA-treated mice died with exposure at the 25 and 50 mg/ml concentrations, respectively, whereas none of the control mice died. Seven days after the last OVA administration, the ACh-induced impairment in respiratory response was still significant (data not shown).

Previous reports have demonstrated that OVA-treated asthmatic mice exhibit a high level of *mClca3* gene expression in the lungs (18). We therefore examined the daily expression pattern of *mClca3* in the lungs of control and OVA-treated mice 2 d after the last OVA administration. In control mice, the expression level was very low but had a daily rhythm ($P < 0.01$, by one-way ANOVA) with a peak at ZT7 (Fig. 1C). The OVA-treated mice had an expression level that was approximately 20 times higher than that of the control group, and there was also a robust daily rhythm ($P < 0.01$, by one-way ANOVA) with a peak at ZT7 (Fig. 1C). This high level of *mClca3* gene expression was maintained 7 d after the last administration of OVA but disappeared within 1 month after inhalation (data not shown).

mPer gene expression and minute respiration rhythms in control and asthmatic mice and effect of daily OVA inhalation on *mPer1* gene expression

The daily expression rhythm of *mPer1* and *mPer2* was examined in the lungs of control and OVA-treated mice 2 d after the last OVA administration (Fig. 2A). The expression pattern showed a clear nocturnal rhythm with a peak at ZT13 in both control ($P < 0.01$ for *mPer1*, $P < 0.01$ for *mPer2*, $P = 0.6$ for *β -actin* by one-way ANOVA) and asthmatic mice ($P < 0.01$ for *mPer1*, $P < 0.05$ for *mPer2*, $P = 0.1$ for *β -actin* by one-way ANOVA). No significant differences of daily rhythmic pattern of *mPer1* ($F_3, 23 = 0.4$, $P = 0.8$ by two-way ANOVA), *mPer2* ($F = 0.4$, $P = 0.8$), and *β -actin* ($F = 0.4$, $P = 0.8$) expression were observed between control and OVA-treated mice. In the next experiment, we calculated the daily rhythm of mean minute respiration at 3-h intervals in control and OVA-treated mice under free-moving conditions, 2 d after the last OVA administration (Fig. 2B). The mean values of minute volume showed a clear rhythm with a peak at ZT12–15 in both control and OVA-treated mice. The ampli-

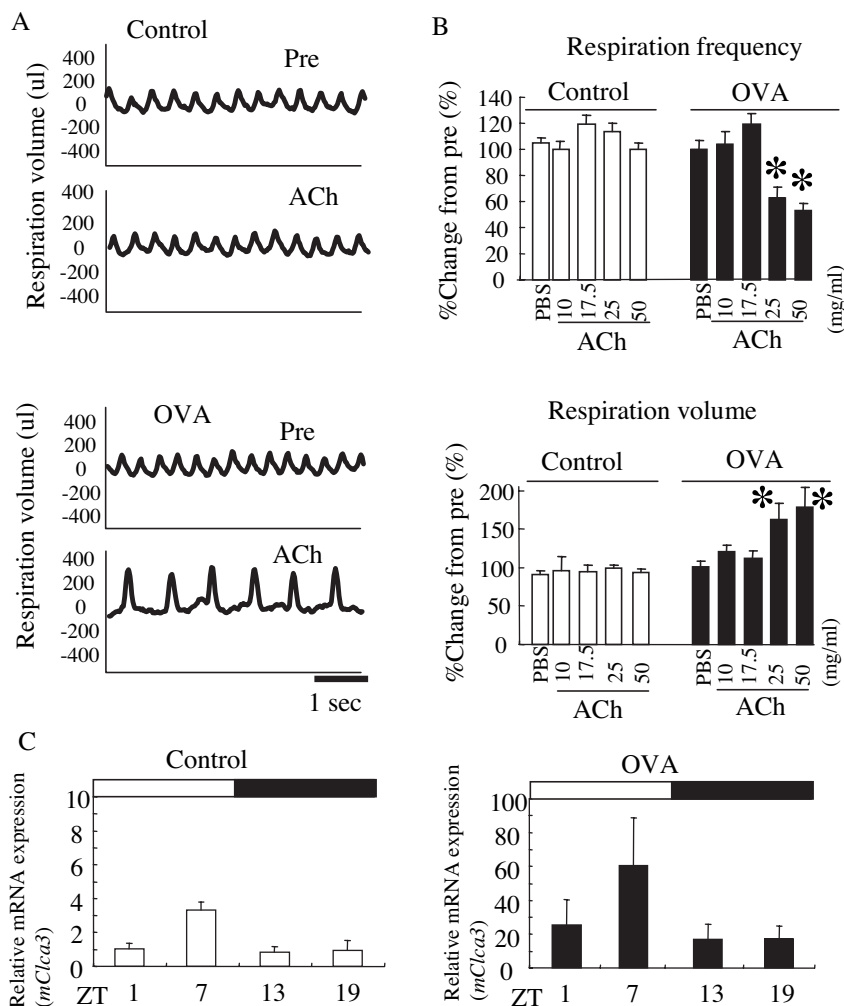


FIG. 1. ACh-induced respiration change and *mClca3* gene expression in the lungs of OVA-treated asthmatic mice. Under ketamine anesthesia, respiratory pressure curves were recorded by double-chamber, whole-body plethysmography in response to inhaled ACh. A, After recording of respiratory pressure curves for 5 min as a pre value, respiratory pressure curves were recorded again for 1–2 min under ACh application (50 mg/ml). Control, PBS-treated mice; OVA, OVA-treated asthmatic mice. B, Summarized data. Pre value was set as 100%. *, $P < 0.05$ vs. control (PBS) treatment (Dunnett's test). Number of animals ranged from three to six. C, Daily expression pattern of *mClca3* expression in the lungs of control and OVA-treated mice. One-way ANOVA revealed a significant daily rhythm in control ($F_{3,14} = 34$, $P < 0.01$) and OVA-treated ($F_{3,14} = 6.5$, $P < 0.01$) mice. The vertical values reflect the relative mRNA levels of *mClca3* as the ratio to β -actin mRNA levels. Number of animals ranged from three to six.

tude of the peak and trough was larger in OVA-treated mice, compared with control mice. Figure 2C illustrates an example of a trace of the respiratory response during the light period (ZT6) and dark period (ZT15). The respiratory frequency and tidal volume were increased during dark period rather than during light period. To determine whether an allergic reaction itself may be affecting the daily rhythm of clock gene expression in the lungs, we examined mice after the once-daily OVA exposure at ZT0 for 6 d. This procedure had no effect on the daily *mPer1* and *mPer2* gene expression rhythm (Fig. 2D). The expression profile was very similar to that of the control groups (Fig. 2A).

Acute effect of DEXA and SAL on clock gene expression in asthmatic mice

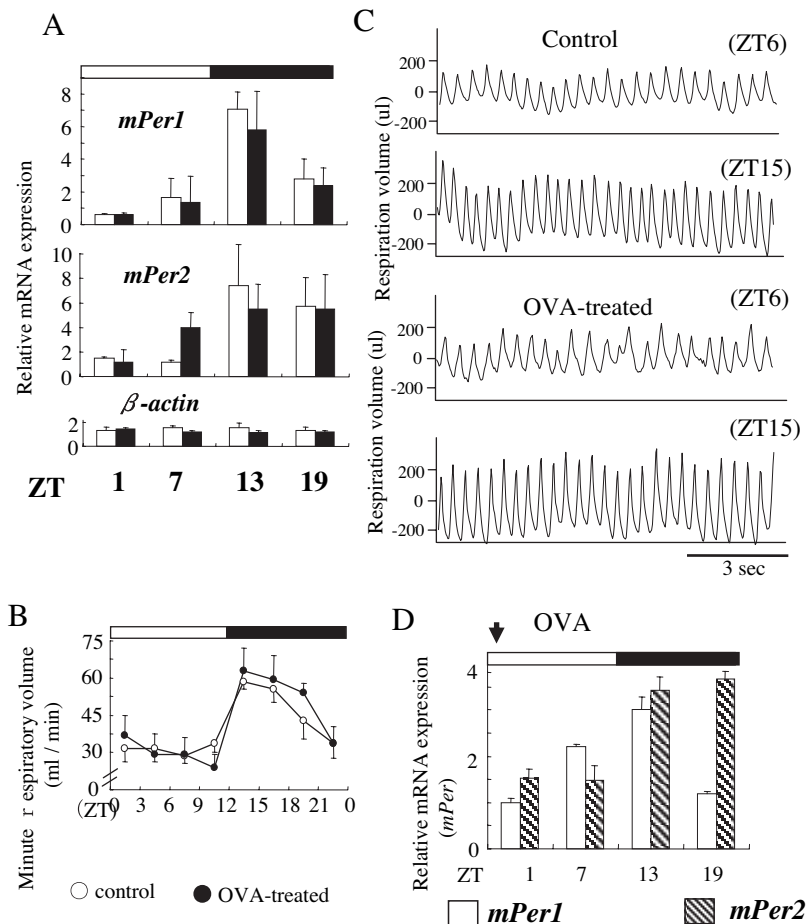
The concentration-dependent effect of acute inhalation of DEXA on *mPer* gene expression was examined in the lungs and liver of OVA-treated mice 2 d after the last OVA administration (Fig. 3A). DEXA significantly increased *mPer1* gene expression in the lungs at concentrations of 0.1 mg/ml ($P < 0.01$, Dunnett's test) and 0.5 mg/ml ($P < 0.05$) without affecting the liver ($P > 0.05$). Acute ip injection of DEXA strongly and significantly ($P < 0.01$, at any dose, Dunnett's test) enhanced *mPer1* gene expression in the liver. Although

ip injection of DEXA significantly increased *mPer1* gene expression in the lungs, the efficacy of DEXA in the lungs was weaker (approximately one third) than that of DEXA in the liver (Fig. 3A). DEXA (0.1 mg/ml). SAL (1.0 mg/ml) inhaled through a nebulizer caused a similar increase in the expression of the *mPer1* gene in the lungs of both control and OVA-treated mice (Fig. 3B). Although SAL slightly but not significantly ($P > 0.05$, Dunnett's test) affected *mPer2* gene expression in OVA-treated mice, DEXA and SAL had almost no effect on *mPer2* gene expression in the lungs of both control and OVA-treated mice.

Daily exposure of DEXA at ZT0 or ZT12 and clock gene expression rhythm

A previous study demonstrated that acute ip injection of DEXA at ZT0 strongly phase-advance circadian clock gene expression rhythm in the mouse liver (15). In this experiment, we administered DEXA or PBS through a nebulizer once daily to OVA-treated asthmatic mice at ZT0 for 6 consecutive days. The daily rhythm of *mPer1*, *mPer2*, and *mBmal1* gene expression was then examined on d 7 and 8. DEXA but not PBS inhalation at ZT0 clearly caused a phase advance of *mPer1* ($F_{3,16} = 37$, $P < 0.01$ by two-way ANOVA), *mPer2* ($F = 25$, $P < 0.01$), and *mBmal1* ($F = 5.1$, $P < 0.05$) but not β -actin

FIG. 2. Comparison of daily pattern of *mPer1* and *mPer2* gene expression in the lungs and of minute respiratory values in OVA-treated asthmatic mice and effect of daily OVA treatment on clock gene expression rhythm. A, Daily expression rhythm of *mPer1* and *mPer2* mRNA in the lungs of control (open bar) and OVA-treated mice (solid bar). Expression rhythm was examined 2 d after last OVA exposure. The vertical values reflect the relative mRNA levels of *mPer1*, *mPer2*, or β -actin mRNA levels. Minimum values of expression level are set at 1.0. Number of animals for each point ranged from three to six. One-way ANOVA revealed a significant daily rhythm of *mPer1* ($P < 0.01$ for control mice and OVA mice) and *mPer2* ($P < 0.01$ for control and $P = 0.05$ for OVA) but not β -actin ($P = 0.6$ for control and $P = 0.2$ for OVA) mRNA. B, Daily rhythm of minute respiratory volume. Each value shows the mean from every 3-h interval. Two-way ANOVA shows no significant differences between daily patterns of the two groups. C, Examples of respiratory pressure curves of control and OVA-treated mice during light period (ZT6) and dark period administration (ZT15). During dark period, respiratory pressure curves showed a high frequency with a high amplitude in both control and OVA-treated mice. D, Effect of daily inhalation of OVA at ZT0 for 6 d on *mPer1* and *mPer2* gene expression rhythm in the lungs of OVA-treated mice. One-way ANOVA revealed a significant rhythmicity of *mPer1* ($F_{3,8} = 8.3$, $P < 0.01$) and *mPer2* ($F_{3,8} = 8.3$, $P < 0.01$) mRNA expression in the lungs with a peak at ZT13 and ZT13–ZT19, respectively. The vertical values reflect the relative mRNA levels of *mPer1* and *mPer2* as the ratio to β -actin mRNA levels. Number of animals per point was three.



($F = 0.9$, $P = 0.5$) gene expression in the lungs of OVA-treated mice (Fig. 4B). A similar DEXA-induced phase advance of the *mPer1* ($F_{3,16} = 32$, $P < 0.01$ by two-way ANOVA), *mPer2* ($F = 13$, $P < 0.01$), and *mBmal1* ($F = 8.1$, $P < 0.01$) but not β -actin ($F = 2.0$, $P = 0.1$) expression rhythm was also observed in the lungs of nonasthmatic control mice (Fig. 4A). Because a previous study demonstrated that acute ip injection of DEXA at ZT12 has a weak effect on circadian clock gene expression rhythm in the mouse liver (15), DEXA was administered through a nebulizer once daily to OVA-treated asthmatic mice at ZT12 for 6 consecutive days in this study. DEXA inhalation at ZT12 did not cause a phase shift of *mPer1* ($F_{3,16} = 1.0$, $P = 0.2$ by two-way ANOVA) and *mPer2* ($F = 0.9$, $P = 0.5$) gene expression in the lungs of OVA-treated mice (Fig. 4C).

Effect of daily DEXA exposure at ZT0 on *mClca3* gene expression, ACh-induced impairment of tidal volume and respiratory frequency, and locomotor activity rhythm

To assess whether the procedure used for inhalant administration (exposure concentration and time) of DEXA was appropriate for relieving asthma symptoms, *mClca3* gene expression and ACh-induced impairment of respiratory response were examined in DEXA-treated asthmatic mice. Daily DEXA inhalation (0.1 mg/ml, for 15 min) at ZT0 for 6 d significantly reduced the *mClca3* gene expression level

($F_{3,21} = 7.8$, $P < 0.01$ by two-way ANOVA; $P < 0.05$ at ZT1, $P < 0.01$ at ZT7 by Student's *t* test) on d 7 and 8 after withdrawal of DEXA, compared with the PBS-treated group (Fig. 5A). Because the expression level was extremely different between PBS and DEXA groups, the maximum value was set to 100%, and the daily expression profile was recalculated. DEXA treatment provided a clear phase advance of the peak time, compared with the PBS-treated group (Fig. 5B). This treatment also phase advanced the expression rhythm of *mPai-1* in the lungs (Fig. 5C).

In the next experiment, for 4 d we administered DEXA (0.1 mg/ml) through a nebulizer for 15 min before each daily exposure to OVA. This procedure for administering DEXA also significantly attenuated the ACh-induced increase of tidal volume and decrease of respiratory frequency (Fig. 5D). Daily treatment with DEXA at ZT0 did not affect the shape of the minute respiration rhythm (Fig. 5E) or locomotor activity rhythm (Fig. 5F) in OVA-treated mice.

Effect of once-daily DEXA administration at ZT0, 6, 12, or 18 on bioluminescence rhythm in *mBmal1-luciferase* transgenic mice

A previous study demonstrated that DEXA injection at different ZTs advanced or delayed clock gene expression in the mouse liver (15). In this study, we administered DEXA through a nebulizer for 6 d at ZT0, ZT6, ZT12, or ZT18. The

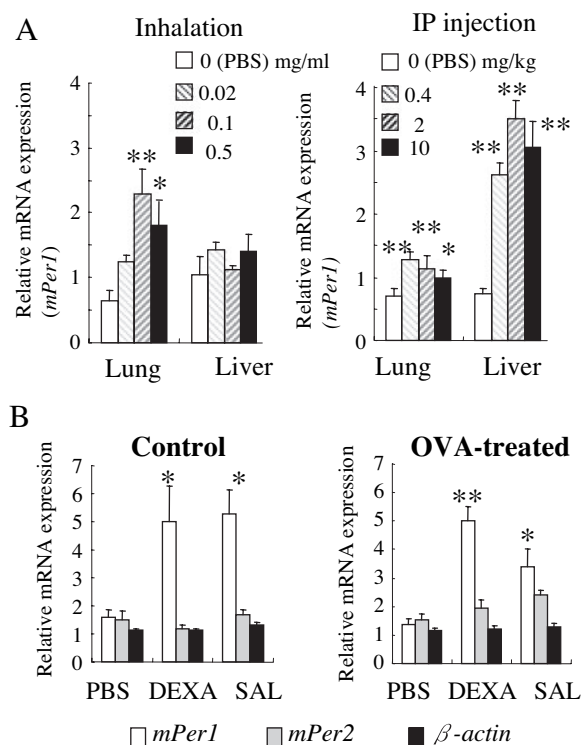


FIG. 3. Effect of acute ip or inhalant administration of DEXA or SAL on *mPer1* and *mPer2* gene expression in the lungs. A, DEXA was administered to OVA-treated mice through ip injection or inhalation, and then 2 h after administration, the lungs and liver were dissected. Expression level of *mPer1* was significantly increased by inhalant administration in the lungs but not in the liver. On the other hand, ip injection of DEXA strongly increased *mPer1* gene expression in the liver but only moderately in the lungs. The vertical values reflect the relative mRNA levels of *mPer1* as the ratio to β -actin mRNA levels. Each point represents three to four animals. *, $P < 0.05$; **, $P < 0.01$ vs. vehicle (PBS) treatment (0 mg/kg) (Dunnett's test). B, Effect of DEXA and SAL on *mPer1* and *mPer2* gene expression in the lungs of control and OVA-treated mice. DEXA and SAL significantly increased *mPer1* in the lungs of both control and OVA-treated mice, with the exception of a weak effect of SAL on OVA-treated mice. *mPer2* gene expression was slightly increased by DEXA and SAL in OVA-treated mice. However, these drugs had no effect on β -actin mRNA levels. The vertical values reflect the relative mRNA levels of *mPer1*, *mPer2*, or β -actin mRNA levels. Minimum values of the expression level are set at 1.0. Each point represents three to four animals. *, $P < 0.05$; **, $P < 0.01$ vs. vehicle treatment (PBS) (Dunnett's test). Each point represents three to four animals.

peak time of bioluminescence rhythm in the lungs of OVA-treated mice was advanced with daily DEXA treatment at ZT0, delayed with administration at ZT18, and unaffected by administration at ZT6 or ZT12 (Fig. 6, A and B). Intact control mice showed a similar phase advance or phase delay when DEXA was applied at ZT0 or ZT18, respectively (Fig. 6C). Vehicle exposure did not affect the bioluminescence rhythm in both intact and OVA-treated mice (Fig. 6C). Treatment with DEXA did not affect the period of the free-running rhythm of bioluminescence in the lungs (data not shown). On the other hand, daily DEXA treatment at any ZT did not affect peak time of the bioluminescence rhythm in the liver (Fig. 6B). Daily OVA inhalation at ZT0 for 6 d failed to change the peak of the bioluminescence rhythm in the lungs of OVA-treated mice (Fig. 6C).

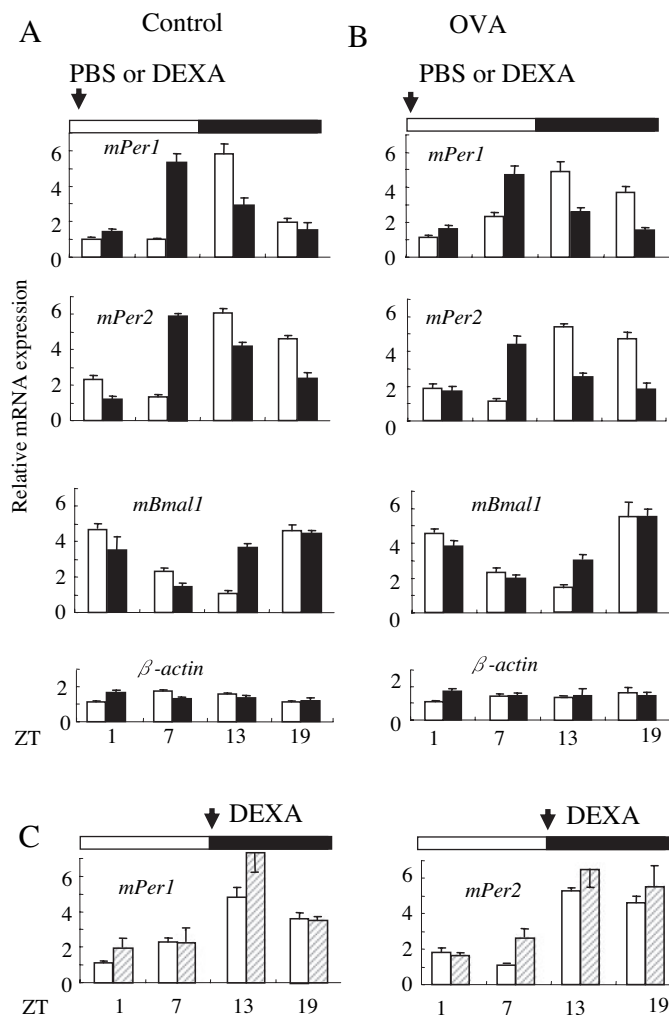


FIG. 4. Phase-shifting effect of DEXA inhalant administration at ZT0 or ZT12 on *mPer1*, *mPer2*, and *mBmal1* gene expression rhythm. PBS (open bar) or DEXA (0.1 mg/ml) (solid bar) was administered for 6 d at ZT0 to control (A) or OVA-treated (B) mice, and then the daily pattern of *mPer1* and *mBmal1* gene expression level was examined on d 7 and 8 under drug withdrawal conditions. Peak time of *mPer1* ($F_{3,16} = 32$, $P < 0.01$ for control mice; $F_{3,16} = 37$, $P < 0.01$ for OVA mice by two-way ANOVA), *mPer2* ($F = 12.5$, $P < 0.01$ for control; $F = 25$, $P < 0.01$ for OVA), and trough time of *mBmal1* ($F = 8.1$, $P < 0.01$ for control; $F = 5.1$, $P < 0.05$ for OVA) gene expression was significantly phase advanced by DEXA administration. C, DEXA (0.1 mg/ml) was administered for 6 d at ZT12 to OVA-treated mice, and then the daily pattern of *mPer1* and *mPer2* gene expression level was examined on d 7 and 8 under drug withdrawal conditions. The open bars refer to the data from Fig. 2B (open bar) and the hatched bars refer to daily DEXA inhalation at ZT12. Two-way ANOVA revealed no significant phase-shifts of the *mPer1* ($F_{3,16} = 1$, $P = 0.2$) and *mPer2* ($F_{3,16} = 0.9$, $P = 0.5$) mRNA expression rhythm by DEXA treatment. The vertical values reflect the relative mRNA levels of *mPer1*, *mPer2*, *mBmal1*, or β -actin mRNA levels. Minimum values of the expression level are set at 1.0. Each point represents three animals.

Effect of daily SAL exposure on the *mPer1* and *mPer2* gene expression rhythm and the bioluminescence rhythm in *mBmal1*-luciferase transgenic mice

Our previous paper (16) elucidated that daily adrenaline injection could cause an *mPer1* and *mPer2* gene expression rhythm in the liver of SCN-lesioned arrhythmic mice. This

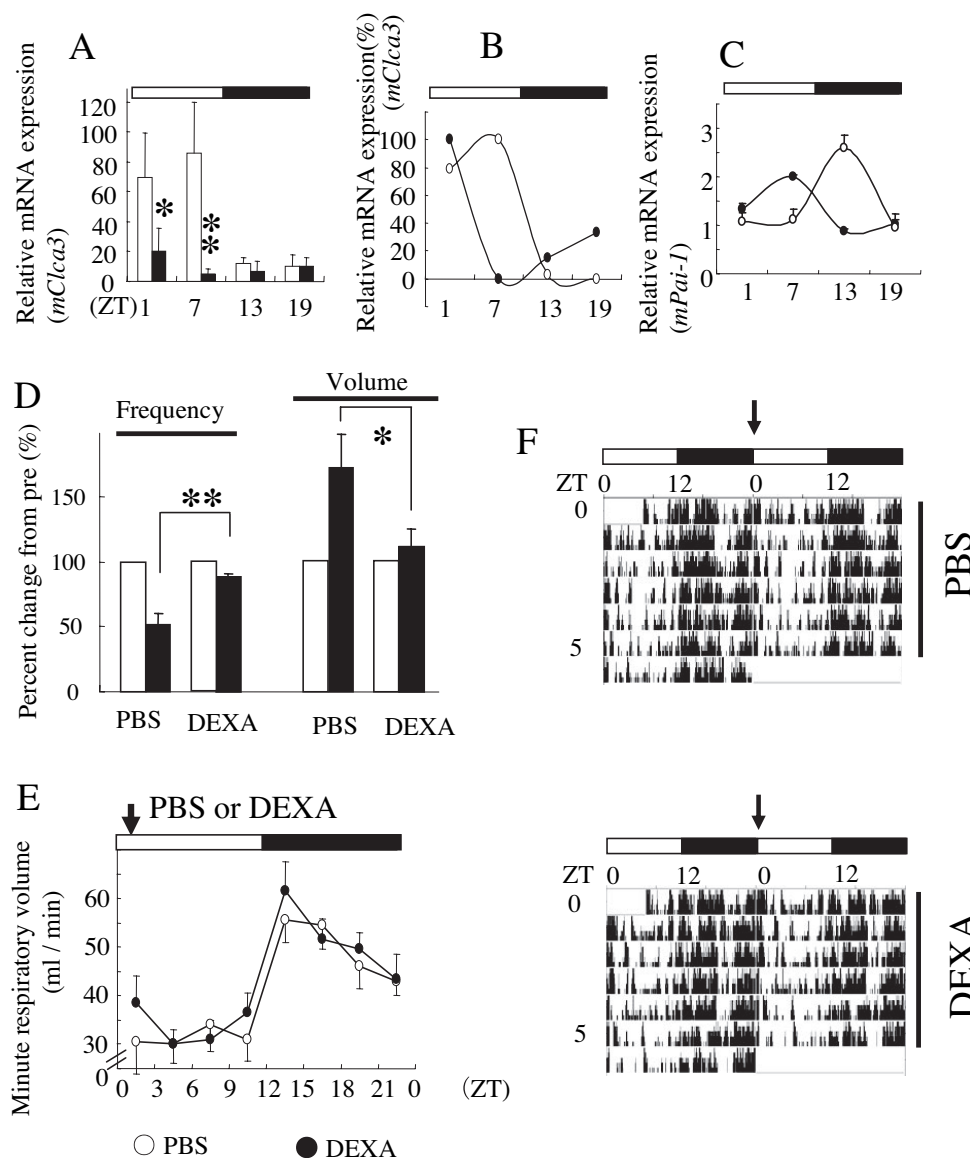


FIG. 5. Effect of DEXA inhalant administration for 6 d at ZT0 on *mClca3* and *mPai-1* gene expression rhythm, minute respiratory response rhythm, and locomotor activity rhythm in OVA-treated asthmatic mice. A, PBS (open bar) or DEXA (0.1 mg/ml) (solid bar) was administered for 6 d at ZT0 to OVA-treated mice, and then the daily pattern of *mClca3* gene expression level was examined on d 7 and 8 under drug withdrawal conditions. Expression level was significantly attenuated by DEXA treatment ($F_{3,21} = 7.8$, $P < 0.01$ by two-way ANOVA). The vertical values reflect the relative mRNA levels of *mClca3* as the ratio to β -actin mRNA levels. Each point represents three to six animals. *, $P < 0.05$; **, $P < 0.01$ vs. PBS treatment (Student's *t* test). B, To facilitate the phase differences between PBS and DEXA groups, peak value and trough values were set as 100 and 0, respectively. Peak time of *mClca3* gene expression was phase advanced by 6 h after DEXA administration. Open circle, PBS treatment; closed circle, DEXA treatment. C, Peak time of *mPai-1* gene expression was also phase advanced by 6 h after DEXA administration ($F_{3,21} = 23$, $P < 0.01$ by two-way ANOVA). Open circle, PBS treatment; closed circle, DEXA treatment. The vertical values reflect the relative mRNA levels of *mPai-1* as the ratio to β -actin mRNA levels. Each point represents three to six animals. D, Once-daily inhalation of DEXA through a nebulizer was conducted just before OVA inhalant administration for 4 d. Under ketamine anesthesia, respiratory pressure curves were recorded by double-chamber, whole-body plethysmography in response to inhalation of ACh. After recording of respiratory pressure curves for 5 min as a pre value, respiratory pressure curves were again recorded for 1–2 min under ACh application (50 mg/ml). Mean values of respiratory frequency (left) and volume (right) recordings for 5 min before ACh administration were set as 100%. *, $P < 0.05$; **, $P < 0.01$ vs. vehicle (PBS) treatment (Student's *t* test). Number of animals ranged from three to six. Open bars show pre (before ACh exposure), and closed bars during ACh treatment. E, After administration of DEXA or PBS to OVA-treated mice for 6 d at ZT0, the daily rhythm of minute respiratory volume was recorded. Each value shows the mean of every 3-h interval. Two-way ANOVA shows no significant differences between daily patterns of PBS and DEXA groups. F, Effect of daily inhalation of DEXA on locomotor activity rhythm of OVA-treated mice. Double-plotted actograms were obtained for the PBS or DEXA groups. White-black bars on the top of the graph show the light-dark cycle. ZT0 and ZT12 show lights-on and lights-off times, respectively. Vertical axis shows the day. Arrows show the inhalation time. Daily inhalant administration did not affect locomotor activity rhythm.

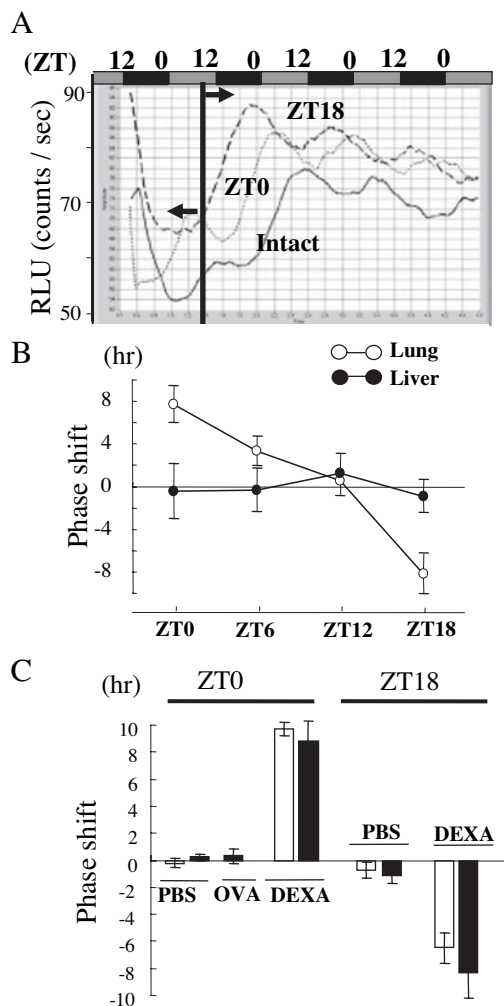


FIG. 6. Effect of daily inhalant exposure of DEXA on *mBmal1*-luciferase bioluminescence rhythm in the lungs of OVA-treated asthmatic mice. A, Solid line, intact nontreated mice; dashed line, DEXA administered OVA-treated mice at ZT0 or ZT18. Arrows show the direction of phase advance or phase delay, respectively. Once-daily dosing of DEXA (0.1 mg/ml) at ZT0 or ZT18 was carried out for 6 d, and then mice were killed at ZT3 on d 7. Bioluminescence in the lungs was recorded *in vitro* using LumiCycle. Vertical axis shows the RLU (counts per second), and horizontal axis shows the time course. White-black bars on the top of the graph show the light-dark cycle. ZT0 and ZT12 refer to lights-on and lights-off times, respectively. B, Phase-response curve to DEXA inhalation was obtained in the lungs (open circles) or liver (closed circles) of OVA-treated mice. Vertical axis shows the phase advance (positive value) or delay (negative value). Amplitude of the phase shift was calculated by phase differences between the phase of peak 1 of DEXA-exposed mice and the phase of peak 1 of nondrug administered mice (14.5 ± 0.9 o'clock, $n = 7$ for lung; 9.4 ± 0.5 o'clock, $n = 7$ for liver). C, Daily inhalant exposure of PBS at ZT0 or ZT18 did not change the peak of bioluminescence rhythm both in intact or OVA-treated mice. A similar phase-shifting effect of DEXA exposure at ZT0 or ZT18 was observed in both intact and OVA-treated mice. Daily inhalation of OVA to OVA-treated asthma mice did not cause a phase change of bioluminescence rhythm. Vertical axis shows the phase advance (positive value) or delay (negative value).

time we administered SAL through a nebulizer for 6 d at ZT0 and studied *mPer1* and *mPer2* gene expression in the lungs of OVA-treated mice. No phase shifts were observed with

SAL administration (Fig. 7A). SAL treatment either at ZT0 or ZT18 could not cause a phase shift of bioluminescence rhythm in *mBmal1*-luciferase OVA-treated transgenic mice (Fig. 7B). Exposure to SAL (1 mg/ml) once for 15 min before ACh significantly attenuated the ACh (25 mg/ml)-induced decrease of respiratory frequency and increase of tidal volume (Fig. 7C). The dose of SAL used in this experiment was

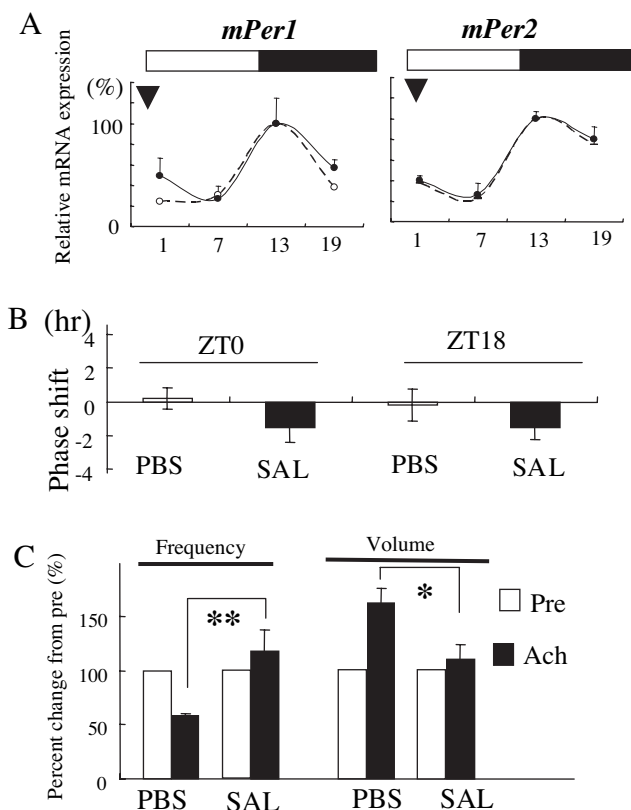


FIG. 7. Effect of daily inhalant exposure of SAL on daily rhythm of *mPer1* and *mPer2* gene expression and *mBmal1*-luciferase bioluminescence rhythm in the lungs of OVA-treated mice. A, Effect of once-daily inhalation of PBS or SAL (1 mg/ml) at ZT0 for 6 d on *mPer1* (left panel) and *mPer2* (right panel) gene expression rhythm in the lungs of OVA-treated mice. Open circle with dashed line, PBS-administered, OVA-treated mouse; closed circle with solid line, SAL-administered, OVA-treated mouse. Arrowheads show administration time point (ZT0) for 6 d. Daily pattern of *mPer* gene expression level was examined on d 7 and 8 under drug withdrawal conditions. Relative mRNA levels of *mPer1* and *mPer2* as the ratio to β -actin mRNA levels were calculated. To compare the phase angle of *mPer* gene expression rhythm in between PBS-administered OVA-treated mice and SAL-administered OVA-treated mice, the maximum value for each daily profile was set at 100% (vertical values). Number of animals for each point was three. B, Once-daily inhalation of SAL (1 mg/ml) at ZT0 or ZT18 for 6 d to OVA-treated, *mBmal1*-luciferase mice. Bioluminescence rhythm was recorded. Vertical axis shows the phase advance (positive value) or delay (negative value). Number of animals was four for each group. C, Acute inhalant exposure of SAL was applied for 15 min, and then under ketamine anesthesia, respiratory pressure curves were recorded by double-chamber, whole-body plethysmography in response to inhaled ACh. After recording of respiratory pressure curves for 5 min as a pre value, respiratory pressure curves were again recorded for 1–2 min under ACh application (50 mg/ml). Mean values of respiratory frequency (left) and volume (right) recordings for 5 min before ACh administration were set at 100%. *, $P < 0.05$; **, $P < 0.01$ vs. vehicle (PBS) treatment (Student's *t* test). Number of animals ranged from three to six.

considered to be enough to reduce asthmatic symptoms in mice.

Discussion

In the present study, once-daily inhalation of DEXA through a nebulizer for 6 d at ZT0 but not ZT12 could cause a 6-h phase advance of the *mPer1*, *mPer2*, and *mClca3* gene expression rhythm in the lungs of control and OVA-treated mice. Daily inhalation of DEXA at ZT0 or ZT18 caused a big phase advance or phase delay of bioluminescence rhythm in the lungs of OVA-treated *mBmal1*-luciferase transgenic mice, whereas DEXA inhalation at ZT6 or ZT12 failed to cause phase shifts.

Once-daily inhalant administration of glucocorticoids such as DEXA is a widely recommended drug formulation for asthma patients to reduce inflammation response (5). It is well known that nocturnal symptoms of cough and dyspnea are accompanied by circadian variations in airway inflammation and physiological variables such as airflow limitation and airway hyperresponsiveness (1). Although treatment dosage and times depend on the severity of asthma symptoms, once-daily, twice-daily, or four times/day dosing schedules have been implemented (5–7). Based on chronotherapeutic ideas, Pincus *et al.* (7, 10) compared the efficacy of triamcinolone inhaled from once-daily at 1500 h to up to four times a day or between once-daily inhalation at either 0800 or 1730 h to up to four times a day. They showed that early evening (1730 h) inhalation was preferable to morning (0800 h) inhalation, which did not produce effects comparable to the four times a day inhalation with regard to morning or evening peak flow. Beam *et al.* (28) studied chronotherapeutic treatment using oral corticosteroids according to a crossover design to evaluate the effect of a variably timed 50-mg oral dose of prednisone given at 0800, 1500, or 2000 h. Based on the results of overnight spirometry, blood eosinophil counts, and bronchial lavage cytology, a single prednisone dose at 1500 h resulted in improved asthma symptoms compared with the 0800 or 2000 h groups. Thus, clinical efficacy has been observed with glucocorticoid administration in the early evening at times like 1500 or 1730 h. Based on clinical evidence taken together finding from the present mouse experiments, we suggest that phase delay of clock function in the human lungs may occur when glucocorticoid is administered at 1500 or 1730 h (around ZT18 for mice). In other words, the present results strongly suggest that DEXA exposure at ZT6 or ZT12 (midnight to early morning for humans) may minimize a phase-shifting effect in the lungs.

Based on the clinical dosing for DEXA inhalation, we selected a DEXA concentration of 0.02 and 0.1 mg/ml, an estimated 0.009 and 0.045 mg/kg for the present mouse experiment. Although a previous ip injection experiment (2 mg/kg) (15) used approximately 50–250 times higher doses than our 0.54 and 2.7 mg, our doses were comparable with those used in the clinic (0.1–2 mg). It was interesting to find that our doses of DEXA caused a phase shift in clock gene expression. In the present experiment, inhalation of DEXA caused the elevation of *mPer1* mRNA expression in the lungs but not the liver. Also DEXA inhalation at any ZT time did

not cause phase shifts of bioluminescence rhythm in the liver of *mBmal1*-luciferase transgenic mice. From the viewpoints of pharmacokinetics, absorption of DEXA through the lungs and into the blood might be too insignificant to cause circadian change with inhalation. The higher elevation of *mPer1* gene expression in the liver *vs.* the lungs with ip injection of DEXA may refer to organ sensitivity differences.

Balsalobre *et al.* (15) first demonstrated that an ip injection of DEXA to mice induces a phase-shift response curve for the liver clock *in vivo*. In this study, the phase response curve of *mBmal1*-luciferase bioluminescence rhythm was comparable with their data in the lungs but not the liver. Koyanagi *et al.* (29) demonstrated that whereas prednisolone (5 mg/kg, sc) injection to mice at ZT0 for 7 d causes an advance of *mPer1* gene expression with reduced daily rhythms of *mPer2*, *mRev-erb α* , and *mBmal1* gene expression in the liver, injection at ZT12 has no effect on clock gene expression rhythm, and chronic application of prednisolone through an osmotic minipump causes attenuation of the daily clock gene rhythm in the liver. Although gene expression rhythm in this study was strongly affected in the liver by glucocorticoids, we could not determine whether glucocorticoids directly or indirectly affected liver clock gene expression. Compared with ip injection, sc injection, or an osmotic minipump, inhalation of the drug through a nebulizer affected clock gene expression only in the lungs. Thus, this is the first report of a successful protocol that could obtain a phase-shift response curve of clock gene expression rhythm in isolated peripheral organs *in vivo*. The procedure for DEXA administration used in the present experiment may be advantageous in that it provides some molecular understanding of peripheral clock function *in vivo*.

In the present experiment, we found extremely high expression of the *mClca3* gene together with the daily change of mRNA level in the lungs of OVA-treated mice *vs.* normal control mice. To elucidate the possibility of clock-controlled *mClca3*, we used the database to determine the clock-controlled gene-specific motif analysis of the promoter region (10 kb upstream from transcription initiation point) of the *mClca3* gene. We found one E-box element, two albumin D-site binding protein (DBP)/adenovirus E4 promoter-binding protein (E4BP4) binding elements, and eight Rev-erb- α /retinoid-related orphan receptor (ROR) binding elements. At least one of these DNA binding elements is necessary for clock-controlled genes (30–33). *mClca3* has been identified as a key molecule in the induction of murine asthma through mucus overproduction (18). Although *mClca3* mRNA expression in OVA-treated mice is about 20 times higher than that in control mice, we do not know any differences in the regulatory mechanism underlying the rhythmic expression of *mClca3* mRNA between the two groups. During a previous experiment, we found a similar rhythmic and high expression (about 10–20 times) of *mPai-1* mRNA in the liver of mice fed a cholesterol diet *vs.* a normal diet (34). Thus, expression of some of the clock-controlled genes may be highly up-regulated by abnormal environmental conditions. Chronic exposure of TNF- α is reported to increase the airway *mClca3* gene expression in mice (35). TNF- α is recognized as an important proinflammatory cytokine with the ability to induce airway hyperresponsiveness

(36). DEXA can suppress the transcriptional up-regulation of bradykinin receptors induced by TNF- α in the murine *in vitro* model of chronic airway inflammation (37). Thus, in the present experiment, DEXA may suppress *mClca3* gene expression in the lungs of OVA-treated asthmatic mice. DEXA treatment at ZT0 dramatically reduced the *mClca3* expression level and caused a phase advance of its expression rhythm. Consequently, DEXA seemed to move the phase of not only clock genes such as *mPer1* and *mPer2* but also clock-controlled genes such as *mClca3* and *mPai-1* (34).

The mean value of minute respiration calculated at 3-h intervals showed a robust daily rhythm with a peak at ZT12–15. There was a significant difference in the expression of *mClca3* mRNA and the responsiveness to ACh but not in the daily rhythm of minute respiratory function between OVA-treated mice and control mice. Although there were no significant differences, amplitude of the peak and trough of minute respiratory volume tended to be high in OVA-treated mice *vs.* control mice (Fig. 2B). To clearly define the characteristics of the asthma mouse model, further experiments on the measurement of respiratory function in mice under stress and/or antigen exposure are required.

Surprisingly, the rhythm pattern of mean minute respiration was never affected by once-daily inhalation of DEXA at ZT0. To confirm this unexpected data, we examined the locomotor activity rhythm after once-daily DEXA exposure at ZT0. Similar to respiration data, locomotor activity rhythm was unaffected by DEXA treatment. Thus, we found a clear dissociation between phase advance of the clock gene expression rhythm and the same phase of minute respiration rhythm after DEXA treatment at ZT0. This may mean that the SCN, a main oscillator, controls the minute respiration rhythm independent of DEXA-induced phase changes in the clock and clock-controlled gene expression rhythm in the lungs. Balsalobre *et al.* (15) and Koyanagi *et al.* (29) already demonstrated that DEXA or prednisolone injection to mice does not cause a phase change in the SCN circadian rhythm. Thus, glucocorticoid hormones could be the *Zeitgeber* for peripheral tissues and cells but not the SCN.

SAL is also widely used for its bronchodilator effect in the presence of low bronchomotor tone among asthma patients, and the combination of a corticosteroid plus a long-acting β -agonist are recommended for patients with moderate to severe and persistent asthma (6, 38). Once-daily or twice-daily inhalation of a long-acting β -agonist has become a common dosing schedule. Very different from DEXA, once-daily SAL inhalation at ZT0 or ZT18 did not cause any phase shifts of *mBmal1*-luciferase bioluminescence rhythm in the lungs of OVA-treated mice. A previous study of ours (16) clearly demonstrated that *mPer1* gene expression is induced by phenylephrine, an α -adrenoceptor agonist, as well as isoproterenol, a β -adrenoceptor agonist, and that daily adrenaline injection provides a phase-resetting oscillation in the lungs of SCN-lesioned mice. In consideration of our current findings taken together with the previously mentioned findings, we believe that the adrenergic receptor-operated reset of clock gene expression is organ dependent; the liver is a sensitive organ and the lungs are insensitive.

In this experiment, the daily rhythm of clock gene expression was unaffected by once-daily OVA inhalation. In ad-

dition, both DEXA-induced phase-advances and acute *mPer1* induction were comparable between OVA-treated asthmatic and control mice. Allergic responses in the lung itself may not interact with clock functions. Therefore, the DEXA-induced phase-resetting effect may be dissociated from DEXA-induced antiallergic action. It is important to clearly clarify the dosing time-dependent pharmacological effect of DEXA on lung function. Unfortunately, in the present experiment, we could not find treatment differences related to ZT (at ZT0 or ZT12) in ACh-induced impairment of tidal volume and respiratory frequency in DEXA-treated mice (data not shown). Further experiment is required to elucidate the dosing time-dependent pharmacological effect of DEXA on *mClca3* mRNA expression and bronchial lavage cytology.

In summary, the present results denote that nebulizer administration of DEXA at ZT6 or ZT12 rather than ZT0 or ZT18 minimized the phase-shifting effect of clock gene expression in the lungs of asthmatic mice.

Acknowledgments

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Address all correspondence and requests for reprints to: Shigenobu Shibata, Laboratory of Physiology and Pharmacology, School of Science and Engineering, Waseda University, Higashifushimi 2-7-5, Nishitokyo-Shi 202-0021, Japan. E-mail: shibatasa@waseda.jp.

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