Advanced Drug Delivery Reviews 62 (2010) 946–955

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Asthma: Chronopharmacotherapy and the molecular clock ☆

Naoto Burioka [a,e,*], Yasushi Fukuoka [a], Satoru Koyanagi [b], Masanori Miyata [c], Miyako Takata [a], Hiroki Chikumi [a], Hiroshi Takane [d], Masanari Watanabe [a], Masahiro Endo [a], Takanori Sako [c], Hisashi Suyama [a], Shigehiro Ohdo [b], Eiji Shimizu [a]

[a] Division of Medical Oncology and Molecular Respirology, Faculty of Medicine, Tottori University, 36-1 Nishimachi, Yonago 683-8504, Japan
[b] Division of Clinical Pharmacy, Department of Medico-Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan
[c] Department of Internal Medicine, Obase Hospital, 1598 Shintsu, 800-0344, Japan
[d] Department of Pharmacology, Faculty of Medicine, Tottori University, 36-1 Nishimachi, Yonago 683-8504, Japan
[e] Division of School of Health Science, Department of Pathobiological Science and Technology, Faculty of Medicine, Tottori University. 36-1 Nishimachi, Yonago 683-8503, Japan

ARTICLE INFO

Article history:
Received 11 November 2009
Accepted 23 March 2010
Available online 30 March 2010

Keywords:
β_2-adrenoceptor agonist
Bronchial asthma
Chronopharmacology
Chronotherapy
Circadian rhythm
Clock gene
Glucocorticoid
Hypoxia
Theophylline

ABSTRACT

Bronchial asthma is characterized by chronic airways inflammation and reversible airflow limitation. In patients with asthma, symptoms generally worsen during the early hours of the morning, and pulmonary function often deteriorates at the same time, suggesting a role for chronopharmacotherapy. Several drugs for asthma have been developed based on chronopharmacology. Most medications employed for the chronotherapy of asthma are administered once at night with the goal of preventing chronic airway inflammation or development of airflow limitation. In addition to bronchodilators, the inhaled glucocorticosteroid ciclesonide is now available with once-daily dosing, which also improves patients' compliance. Numerous investigations have demonstrated the usefulness of chronotherapy for asthma, especially for patients with nocturnal asthma. This review focuses on chronotherapy of asthma, and also provides a molecular biological explanation for the influence of asthma medications on the clock genes.

Contents

☆ This review is part of the *Advanced Drug Delivery Reviews* theme issue on Chrono-Drug-Delivery Focused on Biological Clock: Intra- and Inter-Individual Variability of Molecular Clock.

* Corresponding author. Division of School of Health Science, Department of Pathobiological Science and Technology, Faculty of Medicine, Tottori University. 36-1 Nishimachi, Yonago 683-8503, Japan. Tel.: +81 859 38 6537; fax: +81 859 38 6539.
E-mail address: burioka@grape.med.tottori-u.ac.jp (N. Burioka).

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doi:10.1016/j.addr.2010.03.012

1. Introduction

Bronchial asthma is characterized by chronic inflammation of the airways and reversible limitation of airflow. When treatment is inadequate, however, airway remodeling can occur and reversibility of obstruction becomes incomplete. Chronic inflammation of the airways is associated with infiltration of eosinophils, T lymphocytes, mast cells and other inflammatory cells, as well as with production of various humoral factors. Persistent inflammation may lead to airway obstruction or airway hyperreactivity. The symptoms of asthma include paroxysmal dyspnea, chest tightness, wheezing, cough, and hypoxia during acute attacks [1].

Symptoms of asthma frequently show exacerbation in the early hours of the morning [2]. Nocturnal symptoms of asthma are associated with increased morbidity and a lower quality of life. Inhaled glucocorticosteroid (ICS) has long been used to control airway inflammation in asthma patients. In addition, β_2-adrenoceptor agonists are employed to improve symptoms and airway obstruction because β_2-adrenergic receptors are widely distributed throughout the respiratory tract and airway smooth muscle [3,4]. Newer drugs developed on the basis of chronopharmacology and those using specific drug delivery systems are also being used clinically in the treatment of asthma [2]. Since pulmonary function frequently deteriorates after midnight during the early morning hours, chronotherapy has been used to alleviate such dysfunction.

It was recently reported that multiple signaling pathways can elicit the expression of clock genes [5,6], and that several drugs alter the functioning of these genes [7]. *Period1* (*Per1*) is one of the important circadian clock genes. Since *Per1* has both glucocorticoid response element (GRE) [8,9] and cAMP response element (CRE) in its 5′-upstream sequence [10–12], administration of glucocorticoids or β-adrenoceptor agonists may induce *Per1* mRNA expression. Although asthma medications may theoretically influence clock gene function, the actual physiologic impact on clock genes in asthma patients remains unclear. It is also unclear whether hypoxia related to exacerbation of asthma has any influence on the expression of clock genes.

This article reviews chronotherapy for asthma and the chronopharmacology of drugs developed for administration by new drug delivery systems. The present review also summarizes recent findings concerning the influence of glucocorticoids, β_2-adrenoceptor agonists, and hypoxia on clock genes.

2. Objective evaluation of asthma

Objective evaluation of the severity of asthma is important for proper management of this disease. The Global Initiative for Asthma (GINA) Report recommends the monitoring of peak expiratory flow (PEF) with a peak flow meter for evaluation of the severity of asthma [1]. PEF is an index of respiratory function. Fingertip pulse oximetry is an easy and objective method for measuring arterial oxygen saturation as an indicator of the partial pressure of oxygen in arterial blood. Both PEF and arterial oxygen saturation are important indices, especially since subjective assessment of dyspnea is impaired in asthma patients and nocturnal symptoms may be underestimated [13]. Because a peak flow meter can be used at home, each patient can measure and record his/her PEF data for evaluation of the response to treatment. More recently, measurement of exhaled nitric oxide (NO) has become available as an objective tool for management of asthma [14], and the induced sputum eosinophil count also serves as a marker of the efficacy of treatment [15].

3. Chronotherapy for asthma

Chronotherapy provides a more rational approach to treatment as a means of optimizing its efficacy and reducing adverse reactions by delivering medications at the appropriate time of day to match the specific needs of patients [2]. To perform chronotherapy for asthma with adequate efficacy, it is important to analyze the blood concentration profile of a drug over time and ensure that the drug level is adequate when worsening of symptoms is predicted. Bronchodilators such as β_2-agonists and sustained-release theophylline have been used for chronotherapy. Improvement of chronic airway inflammation is another important goal of chronotherapy. The GINA Report recommends the use of ICS [1]. Ciclesonide is a novel ICS that became available recently, and it can be used for chronotherapy of asthma with a single nocturnal dose [16–18].

Since the PEF decreases along with increasing severity of asthma, circadian and day-to-day variations of the PEF should be measured to determine the best time for administration of a drug and to evaluate its efficacy. Because chronopharmacological data, such as the profile of bronchodilator concentration in the blood over time, are now available, circadian variation of the PEF can be studied for the application of chronotherapy. Self-measured PEF data obtained by patients can be used for objective evaluation of the severity of asthma and for chronotherapy. In other words, circadian variation of the PEF as an index of each patient's biorhythms can be employed to individualize the treatment of asthma.

3.1. Chronobiological aspect in bronchial asthma

It is well known that one of the main chronobiological features of asthma is the worsening of symptoms between midnight and early morning, which is referred to as the morning dip [19] (Fig. 1). Many factors are associated with nocturnal exacerbation of asthma (Fig. 2) [20]. When there is poor management of nocturnal asthma, the morning PEF is markedly lower than the evening PEF. Daily PEF variation of over 20% also indicates poor management of asthma [1].

Drugs that were developed on the basis of chronopharmacology data are now available for adequate management of the nocturnal exacerbation of asthma. For the purpose of enhancing the efficacy as well as reducing adverse effects, the administration time and drug delivery system are arranged considering the status and symptoms of the diseases [2]. Most of the drugs currently used for chronotherapy of asthma are administered once at night with the goal of preventing chronic airway inflammation or the onset of airflow limitation. A single dose at night contributes to improve patients' adherence and better self-management of asthma.

3.2. Chronotherapy with synthetic glucocorticoids

Glucocorticoids can suppress both systemic and local inflammation. Since airway inflammation is a primary problem underlying asthma, ICSs are recommended as first-line treatment [1].

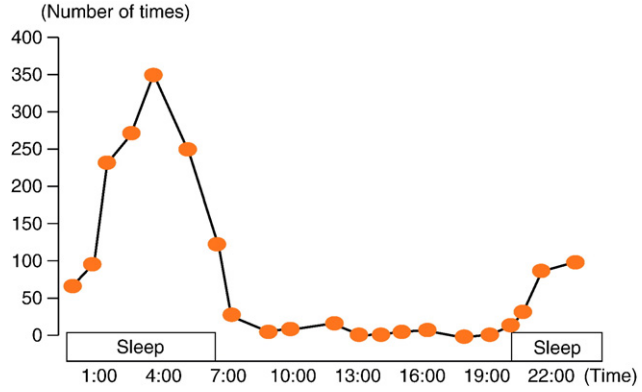

Fig. 1. Early morning exacerbation of symptoms in patients with asthma. A total of 3129 asthma patients recorded the times when they experienced dyspnea in their diaries, revealing that symptoms became worse during the early morning hours (redrawn from the data of Dethlefsen and Repges [19]).

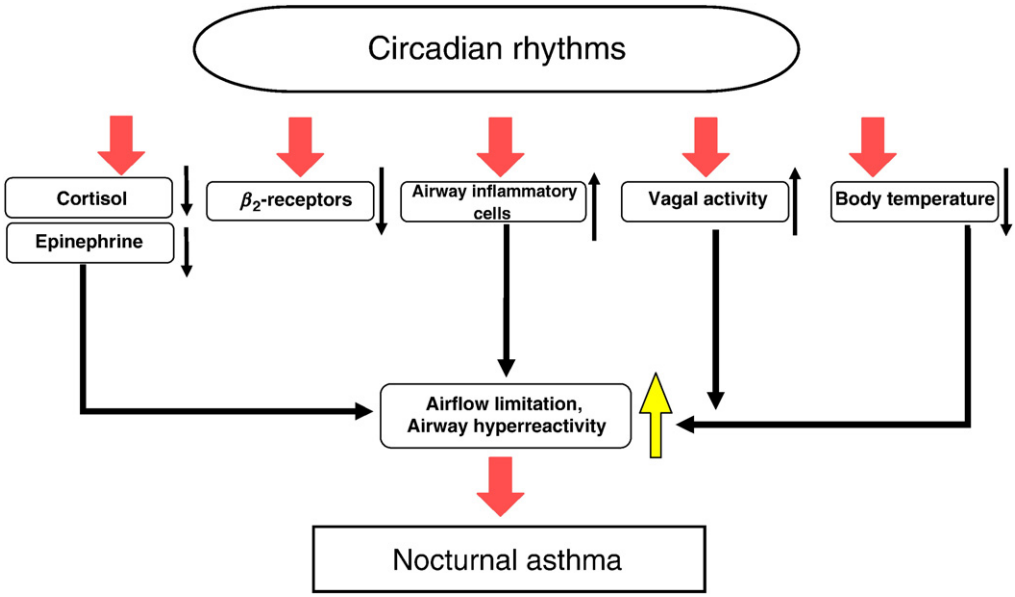

Fig. 2. Mechanisms underlying the nocturnal exacerbation of asthma. Airflow limitation and airway hyperreactivity are caused by chronic airway inflammation, and are associated with changes of the parasympathetic nervous system and endocrine system at night. Because of the circadian rhythm of these factors, exacerbation of asthma often occurs in the early morning (redrawn from the data of Martin [20]).

Glucocorticoids are systemically only used for the treatment of acute attacks or for severe chronic asthma because of the severe side effects.

It has been reported that the efficacy of glucocorticoid therapy depends on the timing of administration. Prednisone was administered to asthma patients as a dose of 50 mg at 08:00, 15:00, or 20:00 h, with measurement of the forced expiratory volume in one second (FEV_1) and investigation of the bronchoalveolar lavage fluid (BALF). In the patients given the drug at 15:00 h, there was a significant increase of FEV_1 and a decrease of neutrophils, eosinophils, lymphocytes, and macrophages, in the BALF compared with the findings in the placebo group [21]. Reinberg et al. administered methylprednisolone at a dose of 40 mg to patients with asthma at 03:00, 07:00, 15:00 or 19:00 h, and reported that administration at 15:00 h led to the greatest increase of PEF [22].

ICSs have a strong local anti-inflammatory action on the airways without systemic adverse effects, and these drugs are currently first-line treatment for asthma. Patients with asthma ranging from mild to severe are considered to be candidates for ICS, in order to prevent chronic airway inflammation [1]. Several studies have investigated the usefulness of chronotherapy for asthma with ICS. The safety and efficacy of inhaled beclomethasone dipropionate (BDP) for patients with moderate asthma were investigated by comparing once-daily administration (at 17:00 or 22:00 h) and twice-daily administration (at 08:00 and 22:00 h), with the results revealing that once-daily administration was effective for controlling asthma [23]. Another study revealed no significant difference in efficacy between once daily (at 15:00 h) and four times daily administration of triamcinolone at a dose of 800 μg [24]. Furthermore, flunisolide was administered at a dose of 1000 μg either once-daily (morning or evening) or twice-daily (morning and evening), with the result that there were no significant differences of efficacy among the three treatment groups [25]. Although these studies showed that the differences were not significant, administration of ICS in the afternoon tended to be more effective. Because of sustained retention in the lungs due to esterification, budesonide is effective when inhaled once-daily [26,27] and there are no significant differences between once-daily and twice-daily administration [28]. Several studies have demonstrated that once-daily inhalation of mometasone furoate is effective for asthma [29–33]. Noonan et al. evaluated the influence of treatment time and dose on the airways when mometasone furoate was administered to 286 patients with mild to moderate persistent asthma. Once-daily dosing with 400 μg in the morning was equivalent in efficacy to 200 μg twice daily, and was significantly better than placebo. Once-daily administration of a low dose (200 μg) in the evening was also as effective as 200 μg twice daily to maintain airway caliber [34].

Ciclesonide is a novel ICS that is administered once-daily at night. It is a pro-drug that is hydrolyzed by esterases to form the active metabolite. Although ciclesonide itself has a low affinity for the glucocorticoid receptor, its metabolite desibobutyryl-ciclesonide exhibits approximately 100-fold greater affinity (similar to that of fluticasone) for this receptor compared with the parent compound [16,17]. The commercially available formulation of ciclesonide (Alvesco®, Teijin Pharma Ltd., Japan) is presented in a pressurized metered dose inhaler with hydrofluoroalkane, a chlorofluorocarbon substitute, as the propellant. It is highly lipophilic and completely soluble with reversible formation of fatty acid conjugates of the active metabolite in the lung, allowing the generation of a very fine aerosol (particles with a diameter of 1 μm) and achieving a 52% pulmonary deposition rate [16,17]. Ciclesonide is usually administered as a single dose at night without a spacer. When patients with mild to moderate asthma were given administered fluticasone propionate twice-daily or ciclesonide once-daily, similar improvement of FEV_1 was observed in both groups [17]. In another study, inhaled ciclesonide showed greater clinical benefit than inhaled BDP with a chlorofluorocarbon propellant [18]. Ciclesonide also has fewer systemic side effects, because the oral absorption rate is less than 1% and the drug is inactivated by the hepatic first-pass effect. Moreover, ciclesonide treatment is associated with an extremely low incidence of oral candidiasis, dysphonia, hoarseness, or sore throat due to a low rate of transformation to the active metabolite in the mouth [35]. We used inhaled ciclesonide (Alvesco®) to treat asthma patients who had hoarseness due to other ICSs, and improvement of their hoarseness was observed in almost all cases (unpublished data, Fig. 3). Adequate control of asthma symptoms was also achieved, and exacerbation did not occur in any of the patients.

3.3. Chronotherapy with theophylline

Theophylline is a bronchodilator, and it has also been reported to suppress airway inflammation [36]. Sustained-release theophylline is

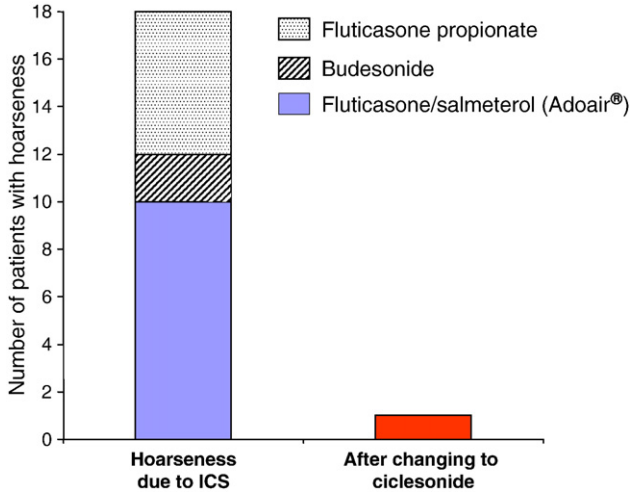

Fig. 3. Hoarseness in asthma patients receiving inhaled glucocorticosteroid (ICS). Hoarseness was found in 18 asthma patients receiving ICS such as fluticasone propionate, budesonide, or fluticasone/salmeterol (Adoair®, Glaxo Smith Klein, K.K., Japan). The symptom of hoarseness resolved in 17/18 patients after ICSs were switched to ciclesonide (Alvesco®).

administered orally. Round-the-clock therapy with theophylline is the common conventional method, in which the drug is administered to obtain a stable serum theophylline concentration throughout a day. On the other hand, since many patients with asthma have more difficulty in breathing and worse symptoms at night [19], once-daily dosing in the evening with a sustained release theophylline can increase the nocturnal serum theophylline concentration and contribute to prevent airflow limitation during sleep. Numerous investigations have demonstrated the usefulness of chronotherapy with sustained-release theophylline for asthma [37–45].

When once-daily dosing in the evening (chronotherapy) and twice-daily dosing (round-the-clock therapy) with a sustained release theophylline formulation were compared in asthma patients showing a decrease of PEF between 02:00 and 04:00 h, once-daily evening administration was more effective for increasing the serum theophylline concentration at the time when lung function was worse, and this regimen improved both symptoms and PEF [46]. Although oral theophylline is widely used for chronotherapy of asthma, it is important to examine the time when the maximum concentration of the drug occurs (Tmax), because this differs among products. Uniphyl® (Purdue Frederik, USA; Mundipharma, Germany; Otsuka Pharmaceutical, Ltd., Japan) is a sustained-release theophylline preparation that was designed for chronotherapy of asthma. A single dose of Uniphyl® in the evening leads to controlled release of theophylline that produces the highest serum theophylline concentration in the early morning.

Changes in the circadian rhythm of PEF over 24 h were examined before and after administration of Uniphyl® to the patients with nocturnal asthma. During the first part of the study, subjects were investigated when they were symptom-free. During the second part, the subjects were studied while their asthma was unstable and when they had nocturnal symptoms and asthma attacks. During the third part, the subjects were studied on the 6th day of once-daily (18:00–19:00 h) chronotherapy with theophylline. Patients recorded their PEF every 4 h from 07:00 to 23:00 h for one day during each part of the study. When asthma was unstable, PEF was lowest at 07:00 h, with a significant morning dip, compared to the PEF during the period without symptoms. To identify circadian variation, the data were analyzed by the group mean cosinor method [47], which showed that the mean daily PEF increased and circadian variation disappeared. The group-mean cosinor analysis is based on the results of the single cosinor test that is the approximation of single cosine curve to the time series data by the method of least squares. The statistical significance of the approximation, i.e., circadian rhythm detection, was tested by the zero-amplitude test, an *F*-test of the amplitude being non-zero in value [47]. When Uniphyl® is administered to patients with a decrease of PEF, the morning PEF increases, the mean daily PEF increases, and the circadian variation of PEF disappears, along with resolution of the symptoms of nocturnal asthma [48]. Generally, the serum theophylline concentration should be measured in each patient to avoid side effects.

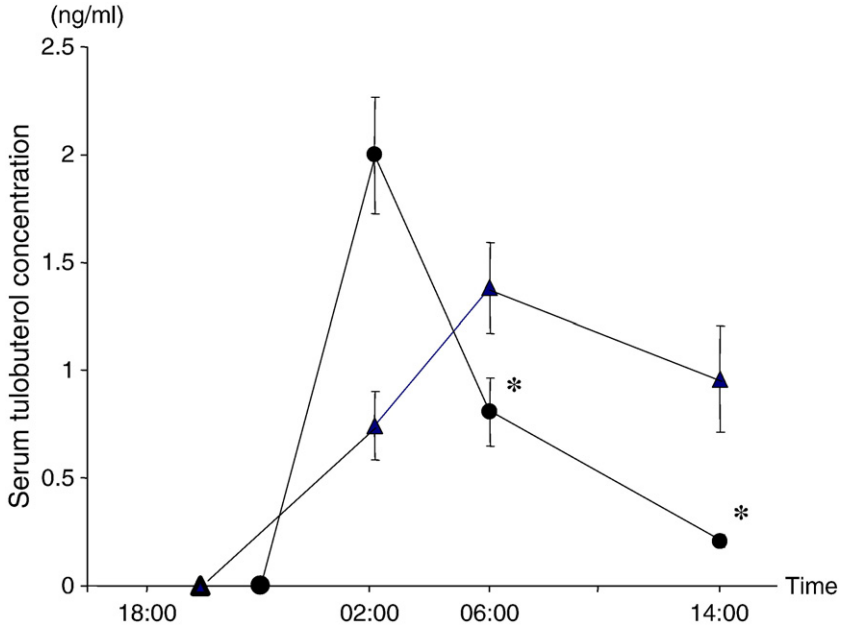

Fig. 4. Serum tulobuterol concentration after a single dose (2 mg) delivered orally or transdermally. Oral tulobuterol (closed circles) was administered to each subject at 22:00 h, while the tulobuterol patch (closed triangles) was applied at 20:00 h. Data represent the mean ± SEM ($n = 5$). Serum tulobuterol concentrations were significantly lower with the tablet than with the patch at 06:00 and 14:00 h (Wilcoxon ranked test, $^*p < 0.05$) (redrawn from the data of Burioka et al., [69]).

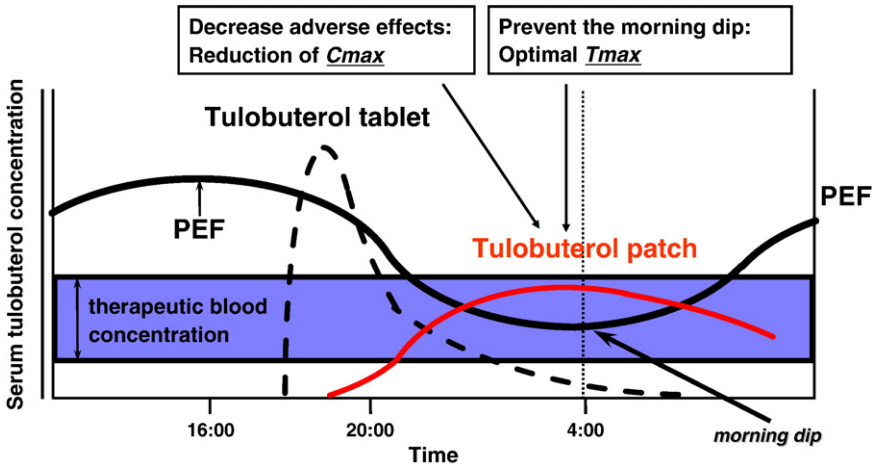

Fig. 5. Chronopharmacology of the tulobuterol patch. When a tablet is administered at night, the serum tulobuterol concentration increases rapidly and the drug is not so effective for preventing the worsening of respiratory function during sleep. Also, a rapid increase of tulobuterol in the blood may be associated with adverse effects such as palpitations and finger tremor. In contrast, the tulobuterol patch employs a crystal reservoir system for slow release, so the serum tulobuterol concentration increases gradually and remains within the therapeutic range for 24 h. The patch is designed to avoid a rapid increase of the serum tulobuterol concentration and improves PEF in the early morning.

3.4. Chronotherapy with β_2-adrenoceptor agonists

Since β_2-adrenergic receptors are widely distributed in the lungs, β_2-agonists are effective as bronchodilators in the treatment of asthma. Plasma epinephrine shows a circadian rhythm with the lowest level at 04:00 h and the highest at 16:00 h in both healthy subjects and patients with asthma [49]. Administration of sustained-release β_2-agonists directly improves bronchoconstriction in the early morning. There have been many reports about chronotherapy with β_2-agonists for patients with asthma [50–68].

A transdermal β_2-agonist preparation has been developed in Japan (Hokunalin® tape, Abbott Japan Co., Ltd.) to provide slow release of the drug for chronotherapy of nocturnal asthma when applied at night. Fig. 4 shows the serum concentration profiles of tulobuterol after oral administration as a tablet and transdermal administration as a patch [69]. The transdermal preparation of this β_2-agonist is designed to avoid a rapid increase of the serum concentration [70], and the increase of the serum transdermal level is slower with the patch than after oral administration. In most patients with nocturnal asthma, PEF is the lowest at around 04:00 h. When conventional tulobuterol tablets are administered after dinner, the serum concentration of the drug increases rapidly and it is not so effective for preventing the deterioration of respiratory function during sleep. Moreover, a rapid increase of the tulobuterol concentration may be associated with adverse effects such as palpitations and finger tremor. On the other hand, Hokunarin® tape provides slow transdermal absorption by employing a crystal reservoir system so that Tmax is longer and adverse effects are reduced, while the serum tulobuterol concentration is increased at the appropriate time of night (Fig. 5). There have been several reports about the efficacy of chronotherapy with the transdermal tulobuterol patch for nocturnal asthma [71–75]. Tamura et al. found that use of the tulobuterol patch led to better compliance with treatment [71]. It was also reported that addition of the tulobuterol patch to ICS therapy was beneficial for elimination of the morning dip as well as for improvement of bronchial hyperresponsiveness and allergic airway inflammation [75].

In another study, the efficacy of transdermal tulobuterol for chronotherapy was investigated from the change in the circadian rhythm of PEF [73]. That study found alterations in the circadian rhythm of PEF in patients with nocturnal asthma when a 2-mg tulobuterol patch was applied between 19:00 and 21:00 h. The circadian rhythm of PEF was analyzed by the group mean cosinor method [47] before and during transdermal tulobuterol treatment. When the tulobuterol patch was applied for 6 days, the 24-h mean PEF increased, and the circadian amplitude of PEF decreased significantly. These changes of the circadian rhythm indicated the increased stability of PEF and improvement of asthma by use of the tulobuterol patch. All of the subjects showed improvement of nocturnal symptoms following evening application of the tulobuterol patch for 6 days, and the effectiveness of this patch for chronotherapy was confirmed by cosinor analysis of the PEF data [73]. Thus, patients with nocturnal asthma should be instructed to apply a transdermal tulobuterol patch at night. Since the Tmax of tulobuterol is approximately 9–12 h with the patch preparation, it is more effective when applied before 20:00 h (Fig. 5). Low-dose (0.5 and 1 mg) tulobuterol patches are also available for children.

3.5. Chronotherapy with leukotriene receptor antagonists

Cysteinyl leukotrienes are derivatives of the 5-lipoxygenase pathway for arachidonic acid metabolism, and are important mediators of airway inflammation. These leukotrienes are the potent constrictors of human airway smooth muscle, and also cause tissue edema and eosinophil migration, which can stimulate the production of airway secretions and airway smooth muscle proliferation [76]. Leukotriene receptor antagonists are used as an on-add therapy to improve the symptoms of asthma patients [1]. Montelukast is one of the leukotriene receptor antagonists. The similarity of response between once-daily and twice-daily administration and the persistent effect throughout the once-daily dosing regimen were reported [77,78].

4. Influence of asthma medications on clock genes

In mammals, a heterodimer of CLOCK and BMAL1 binds to E-box sequences in the promoters, activating transcription of the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. Once PER and CRY proteins have reached a critical concentration, they suppress CLOCK/BMAL1-mediated transactivation, thus generating circadian oscillation of their own transcription. Expression of clock genes is also driven by another loop composed of two orphan nuclear receptors, Rev-erbα and retinoid-related orphan receptor-α (RORα), which generate circadian changes in the transcription *Bmal1*. Several cis-elements including the E-box and the ROR/Rev-erbα binding element (RORE) are known to be involved in the circadian regulation of clock gene expression. The CLOCK/BMAL1 heterodimer binds to the E-box in the promoter regions of the *Rev-erbα* and *RORα* genes. RORα protein binds to ROR/Rev-erbα-binding element in the promoter of *Bmal1*, and activates the transcription of *Bmal1*. On the other hand, Rev-erbα protein competes with RORα for the binding site

on ROR/Rev-erbα binding element and thus inhibits transcription of *Bmal1* [9].

The clock genes regulate the circadian clock in suprachiasmatic nucleus (SCN) and slave clocks in peripheral organs. Many mammalian biorhythms are generated by clock genes, which have been found in the SCN, the peripheral tissues, and cultured cells [79]. Circadian rhythms of peripheral cells are not directly controlled by light, but these cells are entrained by various nonphotic cues [80]. Dysfunction of the central clock genes in the SCN can cause sleep disorders and psychiatric diseases, while dysfunction of peripheral clock genes is related to disorders such as tumorigenesis, abnormal lipid metabolism, and cardiovascular disease [81–83]. Although the relationship between asthma and clock gene function remain unclear, some asthma medications like glucocorticoids or β_2-adrenoceptor agonists may influence clock genes *in vivo* [12].

Recently, human peripheral blood mononuclear cells (PBMCs) have been used to assess the expression of mRNAs for peripheral clock genes [84–88]. In humans, the maximum level of human *Period1* (h*Per1*) transcription is observed at the time of subjective morning [85,87]. Since peripheral blood samples can be easily collected, PBMCs may be useful to evaluate changes in the expression of peripheral clock genes and to examine the effects of various medications.

4.1. Influence of glucocorticoids on clock genes

Glucocorticoids have various physiological effects and their levels show marked daily oscillation, which is thought to be driven by the master circadian clock in SCN of the hypothalamus via the hypothalamo–pituitary–adrenal axis. Glucocorticoids bind to glucocorticoid receptors in the cytoplasm of target cells and then are transported to the nucleus to act as transcription factors. It was reported that the glucocorticoid analogue dexamethasone did not induce h*Per1* mRNA expression in the SCN because there were no glucocorticoid receptors in this brain region [89]. However, glucocorticoids are particularly potent at eliciting the rhythmic expression of the mRNAs for peripheral clock genes. Dexamethasone has been shown to strongly induce *Per1* mRNA expression in cultured rat-1 fibroblasts [79,89] due to signaling via the GRE consensus sequence in *Per1* [8,9,90,91].

In mice, ablation of the adrenal clock alters behavioral rhythms and also attenuates mouse *Per1* (m*Per1*), but not mouse *Period2* (m*Per2*) rhythmicity in several organs including the adrenal gland, liver, kidney, and pancreas [92]. The expression of clock genes is differentially regulated by glucocorticoid signaling because *Per1* expression can be directly modulated via a distal glucocorticoid-responsive promoter element [9,93]. In a previous study of nocturnally active rodents, administration of prednisolone at Zeitgeber time (ZT) 0 for 7 days altered the rhythm of hepatic expression of mRNAs for m*Per1*, m*Per2*, m*Rev-erbα*, and m*Bmal1*, while administration at ZT12 had no significant effect on the expression of these clock genes [9]. The times of ZT0 and ZT12 were the lights-on and lights-off times, respectively. Since secretion of endogenous glucocorticoids by rodents peaks from the late light to early dark phases, administration of prednisolone at ZT0 would be likely to disturb the endogenous glucocorticoid rhythm and thus alter hepatic expression of clock genes [9]. In diurnally active humans, endogenous glucocorticoid secretion peaks early in the morning, and morning administration of glucocorticoids is less likely to influence clock genes. When it was investigated whether ICS therapy could affect clock genes in mice, once-daily inhalation of dexamethasone via a nebulizer for 6-days at ZT0, but not at ZT12, led to a 6-h phase advance in the rhythm of m*Per1* and m*Per2* mRNA expression in the lungs [94].

It has been reported that glucocorticoids influence human peripheral clock genes both *in vitro* and *in vivo*. When human monocytes and lymphocytes were separated from PBMCs and stimulated *in vitro* by prednisolone, strong induction of h*Per1* mRNA expression was found in both monocytes and lymphocytes according to reverse-transcription

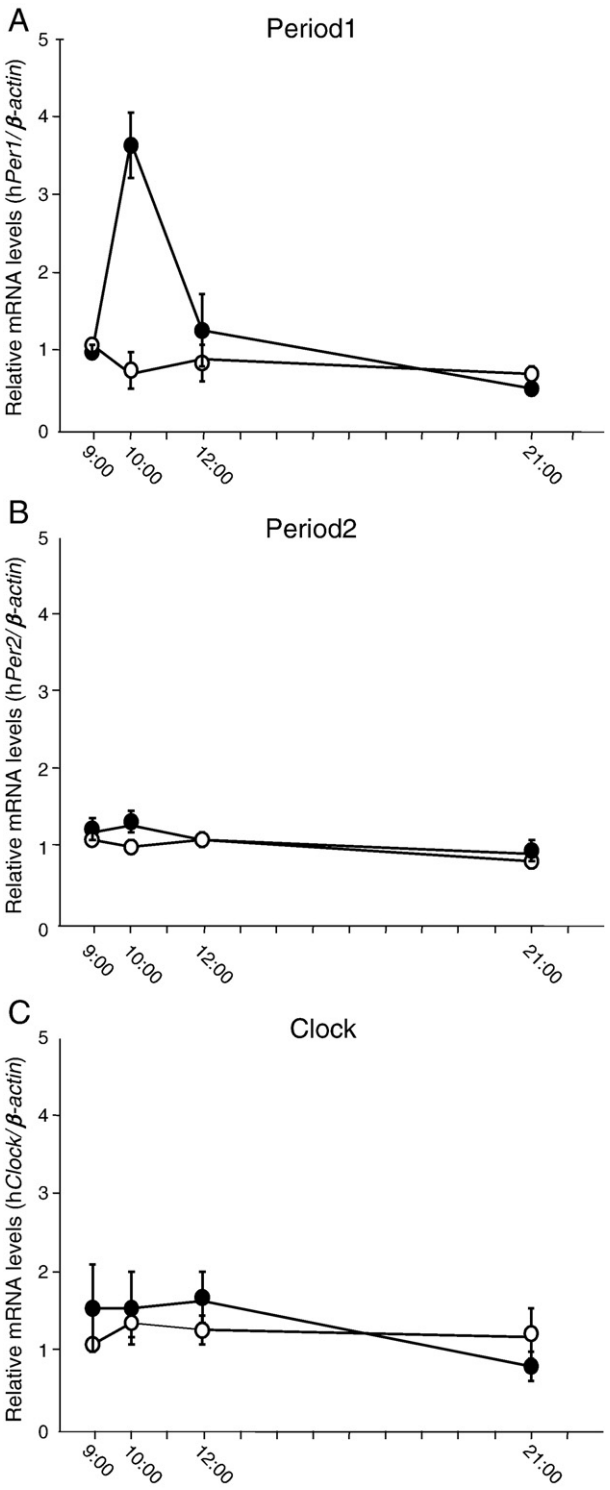

Fig. 6. Induction of h*Per1*, h*Per2* and h*Clock* mRNA by prednisolone in healthy subjects. In three healthy volunteers, clock gene (h*Per1*, h*Per2* and h*Clock*) mRNA expression was assessed at 4 times (09:00, 10:00, 12:00, and 21:00 h) on 2 consecutive days. Prednisolone was injected at 09:00 h on the second day. The mRNA level at 09:00 h on day 1 was defined as 1.0 and relative values obtained at the other times were plotted after normalization for *β-actin* mRNA expression (mean ± SD). Open circles indicate the result of first day without prednisolone, and closed circles indicate that of second day (injection of prednisolone at 09:00 h). The expression of h*Per1* mRNA showed a rapid increase after prednisolone injection, but its expression almost returned to baseline by 21:00 h (A). In contrast, expression of h*Per2* (B) and h*Clock* (C) mRNA was unchanged after prednisolone injection. (Redrawn from the data of Fukuoka et al., [95]).

(RT)-PCR and real-time PCR method. However, transcription of other clock genes, h*Per2* and h*Clock*, was not detectably induced mRNA by prednisolone. [95]. Fig. 6 presents h*Per1,* h*Per2,* and h*Clock* mRNA expression in peripheral blood mononuclear cells obtained from 3 healthy volunteers over 2 consecutive days [95]. Venous blood in each subject was sampled at 09:00, 10:00, 12:00 and 21:00 h. PBMCs were isolated immediately to determine baseline expression in the first day. On the second day, the volunteers were injected with 20-mg prednisolone at 09:00 h, and separated PBMCs were obtained at the same four times. Administration of prednisolone strongly induced h*Per1* mRNA expression, but h*Per2* and h*Clock* mRNA expression were not induced [95]. Expression of h*Per1* mRNA then rapidly decreased and returned to baseline by 12 h. Thus, the influence of glucocorticoids on the clock genes of blood cells was confirmed *in vivo*. Accordingly, glucocorticoid therapy rapidly increases the expression of h*Per1* mRNA, but it soon decreases again.

These results suggest that peripheral h*Per1* that is one of the important clock genes can be influenced by treatment of glucocorticoid [96], although administration of glucocorticoid does not affect the central clock in the SCN [89]. Moreover, this influence may be temporary *in vivo* depending on the time of administration [9,94,95]. However, it was also reported that a positive GRE causes the induction of *Per1* by glucocorticoids, whereas the promoter of *Rev-erbα* has been proposed to contain a negative GRE that mediates glucocorticoid-induced repression of *Bmal1* transcription [96,97]. Thus, further studies are needed to examine the influence of glucocorticoids on each clock gene.

4.2. Influence of β_2-adrenoceptor agonists on clock genes

The human β-adrenoceptor is a member of seven transmembrane family of receptors, and is classified into β_1, β_2, and β_3 subgroups. Among these, β_2-adrenoceptors are widely distributed in the respiratory tract and airway smooth muscle. Intracellular signaling after β_2-adrenoceptor activation involves Gs-protein coupling to adenylate cyclase, and cAMP induces airway dilation through phosphorylation of muscle regulatory proteins and the cAMP response element binding protein (CREB) via protein kinase A (PKA), as well as by reducing the intracellular Ca^{2+} concentration [4]. Thus, β_2-adrenoceptor agonists can effectively improve airway obstruction in patients with nocturnal symptoms or acute asthma attacks.

The m*Per1* gene expression is reportedly induced by the α-adrenoceptor agonist phenylephrine and the β-adrenoceptor agonist isoproterenol, and is decreased by MAPK (mitogen-associated protein kinase) kinase and PKA inhibitors, respectively [98]. Induction of *Per* gene expression in cultured cells is related to phosphorylation of CREB through the activation of MAPK and PKA [98–101]. Adrenoceptor agonists induce *Per1* gene expression *in vitro* via signaling pathways that involve cAMP-PKA-CREB or MAPK-CREB because *Per1* has CRE sequences in its 5′-upstream region to which phosphorylated CREB binds and induces transcription [10–12,98–101].

It has been reported that two β_2-adrenoceptor agonists, procaterol and fenoterol, strongly induce h*Per1* mRNA expression in human bronchial epithelial cells *in vitro* [11]. Moreover, administration of tulobuterol has been shown to induce h*Per1* mRNA expression in human peripheral blood cells according to real-time PCR analysis [69]. That study also revealed that the induction of *Per1* mRNA was weakly correlated with the serum tulobuterol concentration in healthy subjects. When a tulobuterol patch is applied in the evening, the peak blood concentration occurs after 9 to 12 h (i.e., the early morning) [70], so evening application of a tulobuterol patch may be useful to avoid an excessive influence of the medication on clock genes during sleep.

Noradrenaline is another adrenoceptor agonist, and it was reported to have no effect on the central clock genes in the SCN [102]. Noradrenaline induced m*Per1* mRNA expression in a concentration-dependent manner by cultured NIH3T3 cells. However, when noradrenaline was administered to 3 mice via an osmotic pump for 6 days, it was found that m*Per1* mRNA was not induced in the SCN during the light or dark periods, but m*Per1* mRNA expression was significantly induced in the cerebral cortex during the light period when the mice were inactive [102]. Although further studies are needed, it seems that adrenoceptor agonists influence peripheral clock genes, but not central clock genes in the SCN.

4.3. Influence of anticholinergic agents on clock genes

The cholinergic tone from vagal nerves in parasympathetic system increases at night, and may contribute to the worsening of asthmatic symptoms [2]. The electrical stimulation of vagal nerves causes bronchoconstriction and mucus secretions in mammals, which are inhibited by muscarinic antagonists [103]. The vagal nerves are implicated in the pathophysiology of airflow obstruction [1]. Inhaled anticholinergic agents are of noteworthy value to prevent the bronchoconstriction from parasympathetic cholinergic pathways. For asthmatic patients whose conditions are not sufficiently controlled by ICS, combining medications of different classes, an inhaled anticholinergic agent with a β_2-adrenoceptor agonist is clinically used [103].

Bando et al. reported that expressions of clock genes in respiratory tract were influenced by the parasympathetic regulation in mice [104]. They found that m*Per1*, m*Per2*, m*Bmal1* and m*Clock* mRNA levels showed the robust rhythms in cells of the respiratory tract including larynx, trachea, bronchus, and lung. The oscillations were abolished in arrhythmic $Cry1^{-/-}Cry2^{-/-}$knockout mice and after lesioning of the master clock in SCN in wild-type animals. These findings indicated that respiratory system cells contained a functional peripheral oscillator that was controlled by the SCN. It was also found that the muscarinic acetylcholine receptor genes *Chm2, Chm3, and Chm4* were expressed in a circadian manner. The vagal nerve mainly transmitted signals from the SCN because unilateral vagotomy completely abolished rhythms in mucin and PER2 protein levels in the operated ipsilateral side of the submucosal glands, but not in the intact contralateral side. It was suggested that the vagal nerve was one of the dominant pathways for conveying circadian signals from the SCN master clock to the peripheral clock in the respiratory tract [104]. Anticholinergic drugs may influence clock gene function *in vivo*. However, actual influence of inhaled anticholinergic agents on clock genes is still unclear.

5. Influence of asthma attack on clock genes

An acute asthma attack features symptoms such as paroxysmal dyspnea, chest tightness, wheezing, cough, and hypoxia [1], but the influence of transient hypoxia on clock genes has not been clarified despite several studies on hypoxia and the expression of clock genes. It was reported that exposure of mice to sustained hypoxia increased PER1 protein levels in the brain [105], and stabilization of PER1 protein through interaction with hypoxia inducible factor-1α (HIF-1α) was suggested to be involved. On the other hand, another study showed that either intermittent hypoxia or sustained hypoxia had little effect on the transcription of circadian clock genes [106].

The transcription factor HIF-1 is activated by sustained hypoxia, resulting in the increased expression of a number of genes that encode various proteins, including vascular endothelial growth factor (VEGF) [107]. On the other hand, intermittent hypoxia activates nuclear factor-κB (NF-κB) and induces various proinflammatory cytokines, including IL-6 and TNF-α [106,108–110]. Fig. 7A shows that NF-κB signaling is activated by increasing the number of cycles of intermittent hypoxia. Significant activation of NF-κB signaling was observed after exposing cells to 8 or 10 cycles, whereas there was no significant activation of NF-κB signaling when cells were exposed to sustained hypoxia for 3 or 24 h. On the other hand, intermittent hypoxia had little effect on hypoxia response element (HRE)-mediated signal transduction although exposure of cells to sustained hypoxia significantly activated the HIF-1 signaling pathway (Fig. 7B). As shown in Fig. 7C, all three types of clock

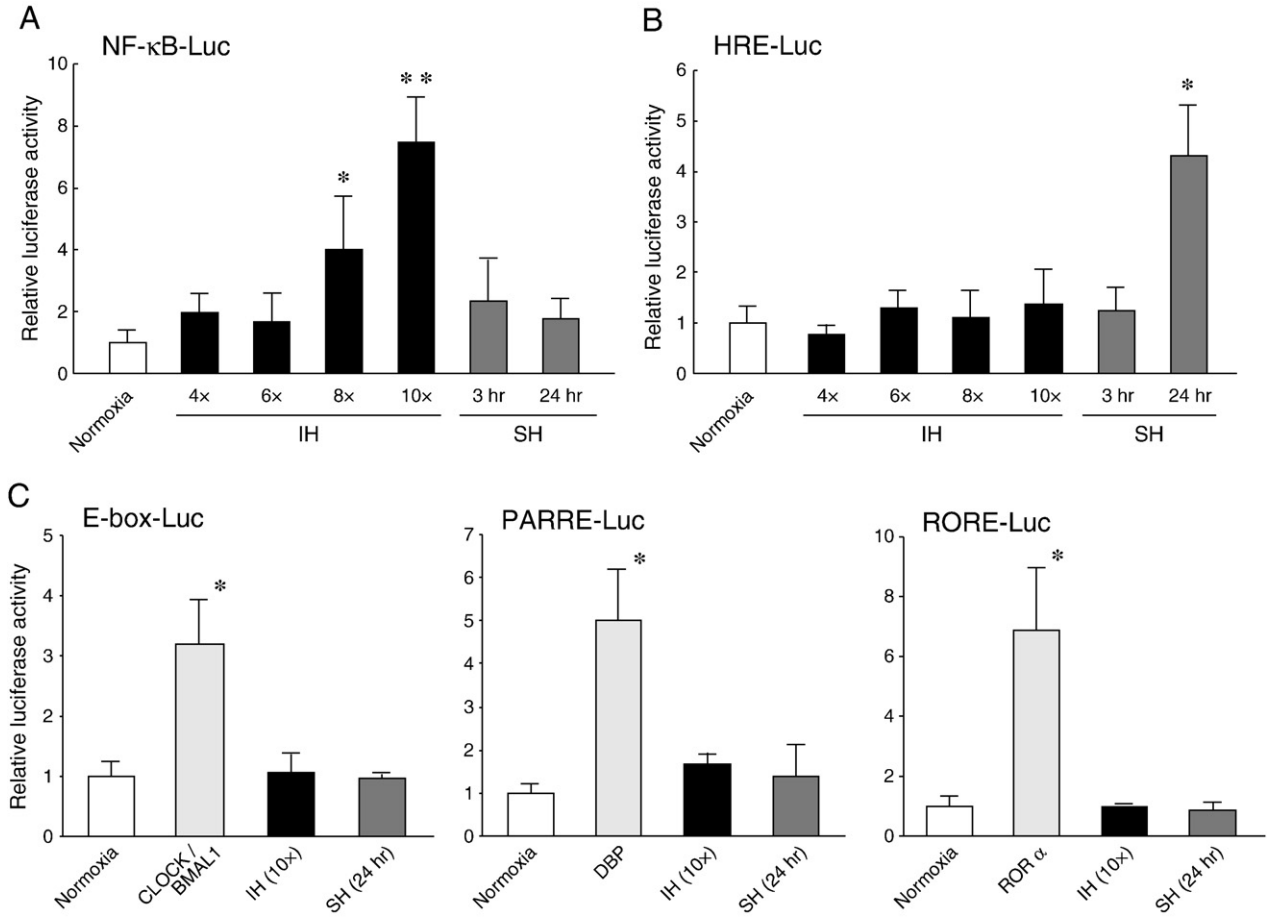

Fig. 7. Activation of transcriptional factors in cultured A549 cells by intermittent hypoxia (IH) or sustained hypoxia (SH). Cells were transfected with luciferase reporter constructs and then exposed to intermittent hypoxia (20% O_2/2% O_2) for the indicated number of cycles. Transfected cells were also exposed to SH (2% O_2) for 3 or 24 h. The mean levels obtained in cells cultured under normoxic conditions were set as 1.0 and data represent the mean ± S.D. of 3 independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ compared with control cells exposure to 20% O_2 (normoxia). (A) Influence of IH or SH on NF-κB-driven transactivation. (B) Influence of IH or SH on HRE-driven transactivation. (C) Influence of IH or SH on clock gene response element-driven transcription. Each luciferase reporter construct (E-box-Luc, PARRE-Luc, or RORE-Luc) was also cotransfected with expression constructs encoding CLOCK/BMAL1, DBP, and RORα, respectively (redrawn from the data of Burioka, Koyanagi et al., [106]).

gene response element (E-box, PARRE, and RORE) respond to CLOCK/BMAL1, DBP, and RORα, respectively, but both intermittent and sustained hypoxia had no significant effect on clock gene response element-driven transcriptions (Fig. 7C). Moreover, the levels of mRNAs for various clock genes (*Per1*, *Per2*, *Cry1*, *Rev-erbα*, *Clock*, and *Bmal1)* were not significantly altered by exposure of cells to intermittent hypoxia [106]. These results suggest that neither intermittent nor sustained hypoxia has much effect on the transcription of circadian clock genes, so that transient hypoxia during asthma attacks may not alter clock gene function.

6. Conclusion

Chronotherapy is effective for asthma because worsening of the asthma symptoms characteristically occurs between midnight and early morning. Several drugs developed on the basis of chronopharmacology are clinically used to treat bronchial asthma. Most drugs used for the chronotherapy of asthma are administered only once at night with the goal of preventing the chronic airway inflammation or the development of airflow limitation. These drugs allow nocturnal asthma to be easily treated at home and once-daily dosing at night often improves patients' adherence. ICS therapy is important for chronic airway inflammation in patients with asthma, since these medications have a strong anti-inflammatory action on the airways without causing systemic adverse effects. Glucocorticoids bind to the glucocorticoid receptors in airway cells and then enter the nucleus to act as transcription factors that increase β_2-receptor gene expression and the number of β_2-receptors, or repress the expression of genes related to inflammation [111,112]. Combined ICS and β_2-adrenoceptor agonist therapy is more effective for asthma than monotherapy. It is expected that new drugs with both optimal combination and new drug-delivery system will be developed based on chronopharmacology.

Glucocorticoids and β_2-adrenoceptor agonists have been shown to alter the function of clock genes *in vitro,* and these agents may influence the transcription of peripheral clock genes *in vivo*. Although it has been suggested that these drugs do not directly influence the function of central clock genes in the SCN, the actual impact on clock genes of medications for asthma remains a matter for future study.

Acknowledgements

This research was partially supported by a Grant-in-Aid for Scientific Research (No. 20590923 to N.B.) and a Grant-in-Aid for Young Scientists for Scientific Research (No. 21790771 to Y.F.) from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

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