Forum Minireview

Circadian Rhythms in the CNS and Peripheral Clock Disorders: Function of Clock Genes: Influence of Medication for Bronchial Asthma on Circadian Gene

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Abstract. Bronchial asthma is a chronic inflammatory disorder of the airways, in which inflammation causes bronchial hyper-responsiveness and flow limitation in the presence of various stimuli. Pulmonary function in asthmatic patients frequently deteriorates between midnight and early morning, which has suggested a role for chronotherapy. Although relationships between bronchial asthma and the function of clock genes remain unclear, some medications given for asthma such as glucocorticoids or β_2 -adrenoceptor agonists may influence clock genes in vivo. In our studies of clock gene mRNA expressions in human bronchial epithelial cells in vitro and peripheral blood cells in vivo, we demonstrated that glucocorticoid or β_2 -adrenoceptor agonist treatment strongly induced human *Per1* mRNA expression both in vitro and in vivo. Human peripheral blood cells provide a useful indication of peripheral clock gene mRNA expression in vivo.

Keywords: β_2 -adrenoceptor agonist, cAMP, clock gene, glucocorticoid, *Per1*

Introduction

Clock genes in the suprachiasmatic nucleus (SCN) as well as in peripheral organs regulate important mammalian circadian rhythms (1, 2). Dysfunction of clock genes can contribute to sleep disorders, psychiatric diseases, tumor genesis, dyslipidemia, and cardiovascular disease (2–5). Although molecular mechanisms involving the circadian oscillator in nocturnally active rodents have been studied in detail, few investigations have focused on diurnally active humans.

In bronchial asthma, chronic airway inflammation causes bronchial hyper-responsiveness and flow limitation in response to various external and internal stimuli.

Since pulmonary function in asthma frequently is worst between midnight and the early morning (6, 7), chronotherapy has been used to alleviate this dysfunction. Glucocorticoids have been used clinically to control inflammation in asthma. Moreover, β_2 -adrenergic receptors are widely distributed in the respiratory tract and airway smooth muscle (8), and β_2 -adrenoceptor agonists have been used to improve symptoms and airway obstruction in bronchial asthma (7, 8).

Multiple signaling pathways can elicit expression of clock genes (1, 2), and several drugs can alter the molecular functions of these genes. In particular, *Period1* (*Per1*) is an important circadian clock gene. Since *Per1* has both a glucocorticoid response element (GRE) (9–11) and cAMP response element (CRE) in its 5'-upstream sequence (12, 13), administration of glucocorticoid or β -adrenoceptor agonist may induce *Per1* mRNA expression in vivo. Although asthma

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medications may influence clock genes, this has been unclear in humans. This minireview summarizes recent findings from our laboratory and others concerning influences of glucocorticoid and β_2 -adrenoceptor agonist treatments on clock genes in humans.

Molecular signals of the mammalian circadian clock gene *Period1*

Circadian clock genes are expressed not only in the central pacemaker of the SCN but also in peripheral tissues such as kidney, liver, heart, skin, and peripheral blood mononuclear cells (PBMCs) (14–18). In mammals, three clock genes, *Per1*, *Per2*, and *Per3*, are expressed rhythmically in the SCN, and *Per1* and *Per2* are induced in response to light (19). Generally, negative feedback determines oscillation of circadian rhythm in both the SCN and peripheral tissues. BMAL1-CLOCK protein heterodimers are positive regulators that act through an E-box enhancer to activate transcription of *Period*, vasopressin, and *DBP* mRNA. The proteins PER1–3, CRY1–2, CK1 ϵ , and DEC1–2 are negative regulators that inhibit activation of BMAL1-CLOCK heterodimers. (20–22).

In addition, previous reports have shown that multiple signaling pathways, which elicit circadian expression of *Per* in cultured cells, are affected by retinoic acids, epidermal growth factor, glucocorticoids, equine serum in high concentration, forskolin, phorbol ester and sympathetic neuron-related factors (1, 23–28).

Glucocorticoid induces human *Period1* mRNA

Glucocorticoids are a particularly potent signal eliciting rhythmic mRNA expression in peripheral clock genes. Rat *Per1* mRNA expression in cultured rat-1 fibroblasts was strongly induced by a glucocorticoid hormone analogue, dexamethasone (1, 23). This in-

crease of *Per1* mRNA accumulation is caused by glucocorticoid signaling via GRE consensus sequences in *Per1* (9–11). Figure 1 shows the signaling pathway resulting in *Per1* mRNA expression via interaction with GRE.

Mechanisms of clock genes have been studied largely in nocturnally active rodents (14, 15, 27, 29). Much investigation remains to be carried out in diurnally active humans. Bjarnason et al. reported that clock genes such as *hClock*, *hTim*, *hPer1*, *hCry1*, and *hBmal1* are expressed in human oral mucosa and skin (30). A rhythmic expression profile was found for human *Per1*, *Cry1*, and *Bmal1* (30). However, biopsies to obtain samples of oral mucosa or skin from human subjects are limited by the need for local anesthesia when performing this procedure. Peripheral blood cells provide an easier way to evaluate changes in expression of peripheral clock genes. Recently, human PBMCs have been used as a surrogate to estimate likely mRNA expression of clock genes in other peripheral tissues (16–18). The greatest *hPer1* transcription was observed in human subjects during the morning hours (17, 18, 31).

We examined how stimulation with a glucocorticoid in vitro and in vivo affected expression of a clock gene, *hPer1*, in PBMCs. Exposure to dexamethasone strongly induced *hPer1* mRNA in cultured human bronchial epithelial cells, BEAS-2B; in contrast, *hClock* mRNA remained essentially unchanged after stimulation with dexamethasone (28). On average, relative *hPer1* mRNA expression according to the real-time polymerase chain reaction (PCR) was increased about 11-fold by stimulation with dexamethasone (28). This result was consistent with the previous report (23). Furthermore, when human monocytes and lymphocytes were separated from PBMCs, and stimulated in vitro by prednisolone, both monocytes and lymphocytes showed induction of *hPer1* mRNA expression according to reverse-transcription (RT)-PCR (Fig. 2). Transcription of other clock genes, *hPer2* and *hClock*, was not detectably induced by prednisolone (data not shown). Monocytes represent a relatively small fraction of PBMCs, so expression in monocytes and lymphocytes was assessed by the more sensitive real-time PCR method. Again, prednisolone at 10^{-7} M induced expression of *hPer1* mRNA in both monocytes and lymphocytes (Fig. 3), but not *hPer2* or *hClock* mRNA expression.

Figure 4 presents *hPer1* mRNA expression in PBMCs from three healthy volunteers for 2 consecutive days (32). We first collected 10-ml venous blood samples at 9:00 and 10:00 AM and 12:00 and 9:00 PM. PBMCs were isolated immediately to determine baseline expression. On the second day, the volunteers were injected with 20 mg of prednisolone at 9:00 AM, and separated

Fig. 1. Signaling pathway leading to *Per1* mRNA expression via the glucocorticoid response element (GRE).

Fig. 2. RT-PCR analysis for mRNA expression of a human clock gene, *hPer1*. In both monocytes and lymphocytes, the *hPer1* mRNA expression was intensified by exposure in vitro to prednisolone (PSL, 10^{-7} M).

Fig. 3. Effect of prednisolone stimulation in vitro on human clock gene mRNA expression in monocytes and lymphocytes according to real-time PCR analysis. Monocyte and lymphocyte samples were exposed to vehicle alone (ethanol) or to 10^{-7} M prednisolone (PSL) in vehicle for 1 h ($n = 6$; mean $\pm$ S.E.M.). Lymphocytes showed an increase similar to that in monocytes, but only about half as intense. * $P < 0.04$, compared to the relative values with vehicle alone (Wilcoxon test).

Fig. 4. Induction of *hPer1* mRNA by prednisolone injection in three healthy subjects according to real-time PCR analysis. Expression of *hPer1* mRNA in PBMCs was assessed at four time points on each of 2 days in three healthy subjects (mean $\pm$ S.D.). Expression at 9:00 AM on day 1 was defined as 1 (baseline). Open symbols indicate day 1 (no prednisolone), while closed symbols indicate day 2 (prednisolone given at 9:00 AM).

PBMCs were obtained at the same four times. We found that *hPer1* mRNA expression in PBMCs was strongly induced by prednisolone in vivo, while *hPer2* and *hClock* mRNA expressions were not induced (32). Expression of *hPer1* mRNA in PBMCs then rapidly decreased, returning to baseline by 12 h. Thus, the influence of glucocorticoids on clock genes was confirmed in PBMCs exposed to them in vivo. In a previous study of nocturnally active rodents, administration of prednisolone at ZT0 for 7 days altered hepatic mRNA expression rhythms of *mPer1*, *mPer2*, *mRev-erba*, and *mBmal1*, but administration of ZT12 had no significant effect on expression rhythms of clock genes (11). Since secretion of endogenous glucocorticoid hormones in rodent peaks from the late light phase to the early dark phase, administration of prednisolone at ZT0 would be likely to disturb the endogenous glucocorticoid rhythm and thus alter expression of clock genes in the liver (11). In diurnally active humans, the secretion of endogenous glucocorticoid hormones peaks early in the morning, so morning administration of glucocorticoid would be less

likely to influence clock genes. Although responses to glucocorticoid stimulation may differ between organs, human PBMCs are readily available to examine changes in mRNA transcription from clock genes.

β_2 -Adrenoceptor agonist induces human *Period1* mRNA

Factors related to sympathetic neurons recently have been reported to reset the clock genes (26). Many sympathetic nervous system functions are mediated by adrenoceptor signaling. Expressions of the mouse *Per1* gene were induced by an α -adrenoceptor agonist or a β -adrenoceptor agonist, but attenuated by a mitogen-activated protein kinase (MAPK) kinase inhibitor or protein kinase A (PKA) inhibitor (26). Induction of *Per1* gene expression in cultured cells is related to cAMP response element binding protein (CREB) phosphorylation via activation of MAPK and PKA (12, 24, 26, 33). Yagita and Okamura (24) reported that forskolin induced phosphorylation of CREB in rat-1 cells. Forskolin directly activates adenylate cyclase, which enhances the synthesis of native cAMP and the indirect activation of PKA. This pathway activates CREB by phosphorylation. Moreover, they revealed that forskolin induced *rPer1* but not *rPer2* mRNA in rat-1 cells.

The human β -adrenoceptor belongs to a family of receptors with seven-transmembrane domains and is classified into β_1 , β_2 , and β_3 subgroups. In particular, β_2 -adrenoceptors are widely distributed in the respiratory tract and airway smooth muscle (8). Intracellular signaling following β_2 -adrenoceptor activation is influenced by Gs protein coupled to adenylate cyclase. Airway relaxation is induced by cAMP through phosphorylation of muscle regulatory proteins and CREB via PKA and through reduction of cellular Ca^{2+} concentrations (8). Agonists of β_2 -adrenoceptors have been used to reduce airway obstruction in patients with bronchial asthma (7, 8). However, the influence of β_2 -adrenoceptor agonists on clock genes has been unclear.

We have demonstrated that β_2 -adrenoceptor agonists strongly induced *hPer1* mRNA expression in the human bronchial epithelial cell line BEAS-2B, using real-time PCR analysis and detected phosphorylated CREB by immunoblotting (13). In brief, to detect phosphorylation of CREB in BEAS-2B cells by fenoterol or procaterol, immunoblotting was performed using an anti-phospho-CREB-specific antibody after exposure of the cells to β_2 -adrenoceptor agonists. Fenoterol or procaterol induced phosphorylation of CREB, although the total amount of CREB was unchanged (13). Figure 5 demonstrates induction of *hPer1* mRNA by fenoterol or procaterol (10^{-7} M) in BEAS-2B cells according to real-time PCR

Fig. 5. Effect of fenoterol or procaterol on the *hPer1* mRNA induction in BEAS-2B cells according to real-time PCR. Cells that had been pre-incubated with serum-free DMEM for 24 h were stimulated with fenoterol or procaterol for 1 h. Expression of *hPer1* mRNA was normalized to expression of β -actin mRNA in real-time PCR analysis. White and black bars indicate the results of fenoterol and procaterol treatment, respectively. Data are shown as means $\pm$ S.D. (n = 4 for fenoterol, n = 3 for procaterol).

analysis, with *hPer1* expression normalized to expression of β -actin. This demonstrated that β_2 -adrenoceptor agonists strongly induced *hPer1* mRNA expression in BEAS-2B cells via the cAMP-CREB signaling pathway (13). A previous report suggested that the changes in noradrenaline, an α - and β -adrenoceptor agonist, maintained *mPer1* and *mPer2* oscillation in mouse liver over the course of a day (26). In humans, the influence of β_2 -adrenoceptor agonists on clock genes in lungs is still unclear, requiring further study. Figure 6 shows the signaling pathway leading to expression of *Per1* via its CRE.

We also examined induction of human clock genes *in vivo* by exogenous β_2 -adrenoceptor agonists in healthy subjects. When a β_2 -adrenoceptor agonist is administered at night, *Per1* mRNA expression could be greatly influenced (data not shown). Recently, a transdermal system for once-daily evening dosing of tulobuterol was developed in Japan (Hokunarin tape[®]; Abbott Japan Pharmaceutical, Tokyo). The tulobuterol patch attached to the skin in early evening then achieves a peak blood concentration after 9 to 12 h (34). This slow-release drug

Fig. 6. Signaling pathway leading to *Per1* mRNA expression via the cAMP response element (CRE).

delivery system is designed for use as chronotherapy for nocturnal asthma (7). We found that the influence of tulobuterol transdermal patch on *hPer1* mRNA expression in whole peripheral blood cells sampled at night was less than that of oral tulobuterol tablet (data not shown).

Conclusions

In summary, human peripheral blood cells may be used as a surrogate for clock gene mRNA expression in other peripheral tissues. Moreover, monitoring of clock genes rhythmicity in peripheral blood cells may be useful for choosing the most appropriate time of day for giving certain medications to patients with bronchial asthma, aiming to increase therapeutic effects and/or reduce adverse effects. Specialized drug delivery systems may attenuate excessive influence of some drugs on clock genes. However, the actual physiologic impacts on clock genes by medications given to patients with asthma remain a matter for future study.

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