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 β2-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 β2-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: β2-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.
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 $\beta_2$-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, CKIε, 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 increase 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).](image-url)
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.

**β₂-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 β₁, β₂, and β₃ subgroups. In particular, β₂-adrenoceptors are widely distributed in the respiratory tract and airway smooth muscle (8). Intracellular signaling following β₂-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 Ca²⁺ concentrations (8). Agonists of β₂-adrenoceptors have been used to reduce airway obstruction in patients with bronchial asthma (7, 8). However, the influence of β₂-adrenoceptor agonists on clock genes has been unclear.

We have demonstrated that β₂-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 β₂-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 analysis, with hPer1 expression normalized to expression of β-actin. This demonstrated that β₂-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 β₂-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 β₂-adrenoceptor agonists in healthy subjects. When a β₂-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®, Abott 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
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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