MAP kinase-dependent induction of clock gene expression by α_1-adrenergic receptor activation

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Abstract While peripheral oscillators can be reset by humoral factors such as glucocorticoid hormones, indirect neural communications involving sympathetic and parasympathetic neurons from the suprachiasmatic nucleus to various peripheral tissues suggest that autonomic nerve innervations also function in the resetting and synchronization of peripheral tissues. To study the role of sympathetic adrenergic signaling on clock gene expression, we constructed NIH3T3 cells that stably expressed each of three α_1-adrenergic receptor subtypes (α_{1A}, α_{1B} and α_{1D}). We found that noradrenaline transiently induced the expression of *mPer1*, *mPer2*, and *mE4bp4* 1–2 h after α_1-receptor activation. The extent and time course of clock gene mRNA induction by noradrenaline or the α_1-receptor agonist phenylephrine (PE) was similar to that seen by 50% horse serum shock. Clock gene mRNA induction by PE was inhibited by U0126, a MEK inhibitor, suggesting involvement of the mitogen-activated protein kinase signaling pathway. We also found that both *mPer1* and *mPer2* mRNAs were induced in the mouse liver 60 min after PE injection. These results suggest that although humoral factors are important for entrainment of the peripheral clock, the autonomic nervous system may also be involved in the process.

Key words: α_1-Adrenergic receptor; Mitogen-activated protein kinase; *mPer1*; *mPer2*; NIH3T3; Phenylephrine

1. Introduction

A main oscillator located in the suprachiasmatic nucleus (SCN) regulates a variety of behavioral and physiological phenomena by modulating the function of other brain regions and various peripheral organs [1]. In peripheral tissues, several humoral entrainment signals from retinoic acid [2] and glucocorticoid hormones [3] can reset clock gene expression. Furthermore, several chemical cues from phorbol myristate acetate, forskolin [4], or 12-*O*-tetradecanoylphorbol 13-acetate [5] can elicit rhythmic gene expression in cultured cells. Thus, the SCN pacemaker may use multiple chemical cues for synchronization of the peripheral oscillators in vivo. On the other hand, indirect neural communications of sympathetic and parasympathetic neurons from the SCN to various peripheral organs such as the liver and pancreas were shown through use of a viral transneuronal tract tracer [6]. These findings indicate the possible involvement of autonomic nerve innervations from the SCN to peripheral tissues in the entrainment of peripheral tissues.

Several functions of the sympathetic nervous system are mediated by a catecholamine-adrenergic receptor signaling mechanism. Adrenergic receptors belong to the G protein-coupled receptor family and are activated by catecholamines, namely, adrenaline or noradrenaline. Our focus was on the α_1-adrenergic receptors since they mediate a variety of important physiological functions such as vascular smooth muscle contraction, glyconeogenesis, and myocardial ionotropic response [7]. In mammals, there are at least three subtypes of α_1-receptors, α_{1A}-, α_{1B}-, and α_{1D}-receptors. Activation of all receptor subtypes induces rapid inositol triphosphate formation and subsequent release of Ca^{2+} from intracellular stores [7]. To study the role of α_1-receptor activation on clock gene expression, we constructed mouse α_1-adrenergic receptor-expressing NIH3T3 cells and compared the results with those of found in vivo.

2. Materials and methods

2.1. Cell culture

NIH3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and 100 U/ml streptomycin at 37°C with 5% CO_2. The medium was replaced every 2 days. For various assays, quiescent cells were made by treatment in serum-free DMEM for 24 h.

2.2. Stable transfection of NIH3T3 cells with an α_1-receptor-expressing vector

Full-length open reading frames of the mouse α_{1A}-, α_{1B}-, and α_{1D}-adrenergic receptor genes were amplified from mouse liver cDNA and inserted into the pcDNA3.1/V5-His-TOPO vector by using the TOPO TA Expression Kit (Invitrogen, San Diego, CA, USA). Sequences of the expression vectors were verified by the ABI310 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

For stable transfection, NIH3T3 cells (1.6×10^6) were seeded in 100-mm^2 culture dishes containing DMEM and 10% FBS. The following day, each α_1-receptor subtype expression vector (4 μg) was transfected according to the manufacturer's instructions using Poly-Fect Transfection Reagent (Qiagen, Chatsworth, CA, USA). Forty-eight hours later, the transfected cells were reseeded into ten 10-mm^2 dishes and the culture medium was changed to a medium containing

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NA, noradrenaline; PE, phenylephrine; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time

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500 μg/ml Geneticin (Sigma, St. Louis, MO, USA). After 10 days, the surviving colonies were isolated and grown in medium containing Geneticin. Cells that expressed similar mRNA levels of each α_1-adrenergic receptor subtype were selected and used for further experiments.

2.3. Drug treatment and serum shock

Noradrenaline (L-noradrenaline bitartrate; NA) and phenylephrine ((*R*)-(−)-phenylephrine hydrochloride; PE) were purchased from Wako Chemicals (Osaka, Japan). Before each experiment, cells were grown to confluence and incubated with serum-free DMEM for 24 h. For cell culture analysis, all drugs were dissolved in dimethylsulfoxide (DMSO) and directly added to the medium. In all experiments, the same DMSO concentrations were used for control groups. To block β-adrenergic receptor activity in the cells, 1 μM timolol was added 30 min before NA or PE treatment for all culture experiments. For serum shock, the medium was changed to DMEM containing 50% horse serum.

2.4. RNA isolation and semiquantitative RT-PCR

The total RNA of liver or cultured cells ($n = 3$–4 per time point) was extracted using Isogen (Wako Chemicals). Remaining DNA was completely removed by RNase-free DNase treatment. 100 ng of total RNA was reverse-transcribed and amplified using the Superscript One-Step RT-PCR System (Invitrogen). Specific primer pairs for each mouse gene were designed based on published data in GenBank as follows: *actin* (452 bp): GAGGGAAATCGTGCGTGACAT and ACATCTGCTGGAAGGTGGACA; c-*fos* (181 bp): GCGTCAATGTTCATTGTCATG and CCACATGTCGAAAGACCTCA; *mPer1* (289 bp): CAAGTGGCAATGAGTCCAACG and CGAAGTTTGAGCTCCCGAAGT; *mPer2* (381 bp): GACTCATGATGACAGAGG and GATGTACAGGATCTTCCC; *mE4bp4* (267 bp): TTTGTGGACGAGCATGAGCCTG and GTGGGAGTAAGTGGAGAAAGAGC; *Adra1a* (α_{1A}) (269 bp): GCTGCCGTTCTTCCTCGTGA and CCATGCCTCTGTGCTGTTCCTCTA; *Adra1b* (α_{1B}) (257 bp): ACCTGCGCCCAATGACGACA and CCTTGGCCTTGGTACTGCTG; and *Adra1d* (α_{1D}) (270 bp): GCAGCCACTAGCGCCAAAGGAA and GGTTCACACAGCTATTG.

Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed under the conditions described previously [8]. In brief, cDNA synthesis at 50°C for 30 min and then 94°C for 2 min was followed by PCR amplification for 24 cycles and denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min. The PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide, and analyzed by an EDAS-290 system (Kodak). Target clock gene cDNA was always co-amplified with *β-actin* cDNA in a single PCR tube. The intensity of the PCR product of the target gene was normalized to the intensity of *β-actin*.

2.5. Western blotting

Cells (35-mm culture dish) were washed twice with ice-cold PBS and dissolved directly in 100 μl of ice-cold 2× sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris–HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol, 0.004% bromophenol blue, and 4% SDS). After brief sonication, samples were boiled for 5 min and stored at −80°C. Equal amounts of each sample (15 μl) were electrophoresed and immunoblotted with anti-rabbit p44/42 mitogen-activated protein (MAP) kinase or anti-rabbit phospho-p44/42 (Thr202/Tyr204) MAP kinase antibody (Cell Signalling Technology, Beverly, MA, USA) as previously described [9].

2.6. Animals

We used 6-week-old male *ddY* mice (Takasugi Experimental Animals, Saitama, Japan). Animals were maintained on a light–dark cycle (12 h light, 12 h dark with lights on at 8.30 h, room temperature 23°C) and given food and water ad libitum. For injection, PE was dissolved in saline. All animals were treated in accordance with the Law (No. 105) and Notification (No. 6) of the Japanese Government.

2.7. Statistical analysis

The values are expressed as means ± S.E.M. The significance of the differences between groups at each point was determined by Student's *t*-test. The rhythmicity of each group was tested first by one-way analysis of variance (ANOVA) and then by Dunnett's two-tailed test.

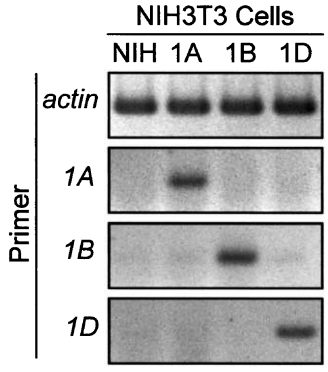

Fig. 1. α_1-Adrenergic receptor mRNA expression in stably transfected NIH3T3 cells. Total RNA was extracted from wild-type NIH3T3 (NIH) cells and cells stably expressing each of the α_1-receptor subtypes α_{1A} (1A), α_{1B} (1B), and α_{1D} (1D). RT-PCR using subtype-specific primer pairs was performed as described in Section 2 to confirm the expression of each α_1-receptor mRNA.

3. Results and discussion

3.1. Transient induction of c-fos and clock gene mRNA by α_1-adrenergic receptor subtypes in NIH3T3 cells

We transfected the expression vector of each mouse α_1-receptor (α_{1A}, α_{1B}, and α_{1D}) into NIH3T3 cells. After G418 selection, surviving cell colonies were isolated and tested for receptor expression by RT-PCR. Stably transfected NIH3T3 cells that expressed the receptor mRNAs at similar levels were used in this study (Fig. 1). The mRNA of c-*fos*, a proto-oncogene, is markedly induced by receptor activation in the rat aorta [10] as well as human α_1-receptor-transfected NIH3T3 [11] and Rat-1 [12] cells. Thus, c-*fos* expression was used in this experiment as a functional index of the biologically significant expression of α_1-receptors. When wild-type and stably α_1-receptor-transfected NIH3T3 cells were treated with NA (10 μM), c-*fos* mRNA was transiently induced only in α_1-receptor-transfected cells (Fig. 2).

Next, we tested whether clock genes (*mPer1*, *mPer2*, and

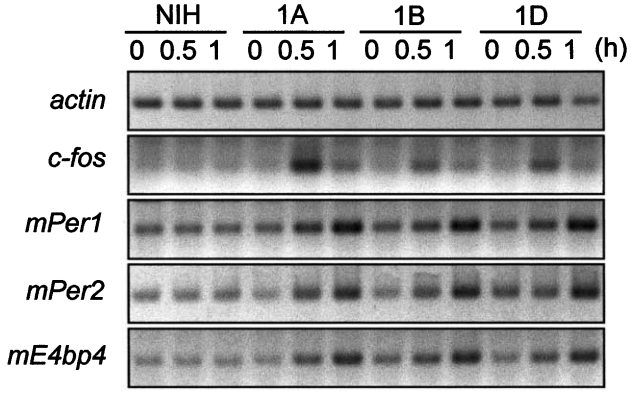

Fig. 2. NA-induced mRNA induction of c-*fos* and clock genes in α_1-receptor-expressing cells. Confluent wild-type NIH3T3 (NIH) cells and cells that stably expressed α_1-receptor subtypes α_{1A} (1A), α_{1B} (1B), and α_{1D} (1D) were incubated with serum-free DMEM for 24 h before the experiment. After NA (10 μM) treatment for 0, 0.5, and 1 h, cells were harvested for the isolation of total RNA, and RT-PCR was performed as described in Section 2 to examine the expression of c-*fos*, *mPer1*, *mPer2*, and *mE4bp4* mRNA. To block possible β-adrenergic receptor activity in the cells, 1 μM timolol was added 30 min before NA treatment.

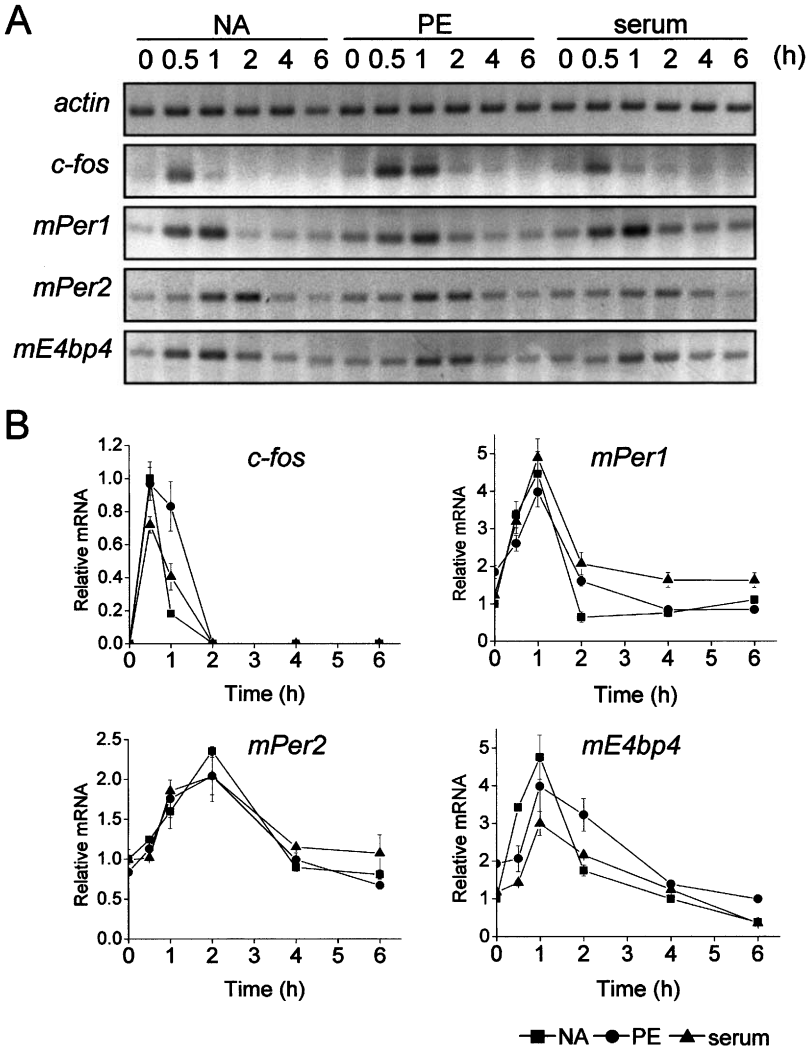

Fig. 3. Similar time-dependent induction of c-*fos* and clock gene mRNA by NA, PE and serum shock. A: Confluent NIH3T3 cells expressing the α_{1A}-receptor were incubated with serum-free DMEM for 24 h before the experiment and treated with NA (10 μM), PE (10 μM), and 50% horse serum. To block possible β-adrenergic receptor activity in the cells, 1 μM timolol was added 30 min before NA and PE treatment. After treatment for 0, 0.5, 1, 2, 4, and 6 h, cells were harvested for the isolation of total RNA. Expression of c-*fos* and clock gene (*mPer1*, *mPer2*, and *mE4bp4*) mRNA was examined by RT-PCR as described in Section 2. B: Relative mRNA abundance of c-*fos* and each clock gene was plotted. Relative mRNA level was normalized to *β-actin* expression ($n = 3$–4 at each time point). Basal expression (time = 0) of the serum-treated group was set at 1.0 for *mPer1*, *mPer2*, and *mE4bp4*. Since there was no c-*fos* mRNA expression at time = 0, the peak value (time = 0.5 h) of the serum-treated group was set at 1.0.

mE4bp4) were induced by α_1-receptor activation. All clock genes were transiently induced 1 h after receptor activation by NA (Fig. 2). Wild-type NIH3T3 cells, which barely express endogenous α_1-receptors (Fig. 1), did not induce c-*fos* or clock genes with NA treatment (Fig. 2). These results showed that activation of all three α_1-receptor subtypes induced clock gene mRNA in NIH3T3 cells.

3.2. *Time course of* c-fos *and clock gene mRNA induction by PE and serum shock*

For further analysis, we compared the time course of c-*fos* and clock gene mRNA induction after NA and PE (a specific α_1-receptor agonist) injection to that after serum shock, which is also known to induce c-*fos* and clock gene mRNA [13]. As shown in Fig. 3, all stimuli transiently induced c-*fos* mRNA in a similar time-dependent manner (peak time = 0.5 h). Clock genes (*mPer1*, *mPer2*, and *mE4bp4*) were also induced within a similar time course by these stimuli (peak time = 1 h for *mPer1* and *mE4bp4*, and 2 h for *mPer2*), but their return to normal level was slower than for c-*fos* (Fig. 3). The level of c-*fos* and clock gene mRNA induction by NA and PE was similar to that by 50% serum treatment (Fig. 3). c-*fos* and clock gene induction was also observed when Rat-1 cells were stimulated by multiple chemical cues coming from glucocorticoid hormones [3] or dibutyryl cAMP [4]. Interestingly, a calcium ionophore, calcymicin [4], and an endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin [14], both of which elevate cytoplasmic Ca^{2+} concentration, were shown to induce *mPer1* and *mPer2* expression in cultured cells. *mPer* gene expression in cerebellar granule cell culture was also induced by Ca^{2+} stimulus [9]. Since activation of α_1-receptors induces intracellular Ca^{2+} mobilization in many cells [15], the present results conform to those from previous experiments.

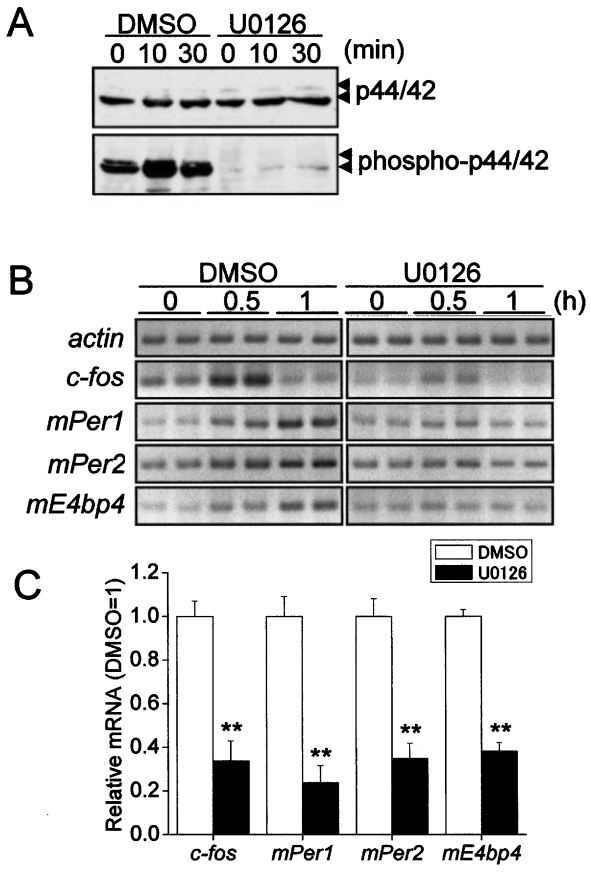

Fig. 4. Effect of the MEK inhibitor U0126 on the mRNA induction of c-*fos* and clock genes when α_{1A}-receptors are activated by PE. NIH3T3 cells expressing the α_{1A}-adrenergic receptor were incubated with serum-free DMEM for 24 h before experimentation and treated with DMSO or the MEK inhibitor U0126 (10 μM) for 15 min before PE (10 μM) treatment. To block possible β-adrenergic receptor activity in the cells, 1 μM timolol was added 30 min before PE treatment. A: 0, 10, and 30 min after treatment, cells were washed twice with ice-cold PBS and lysed directly in 2× sample buffer. The amount and phosphorylation of p44/p42 MAP kinase was examined by Western blotting. B: 0, 0.5, and 1 h after PE treatment, cells were harvested for isolation of total RNA, and RT-PCR was performed to examine the expression of c-*fos*, *mPer1*, *mPer2*, and *mE4bp4* genes. Representative electrophoresis photographs of each PCR products are shown. C: Expression of c-*fos*, *mPer1*, *mPer2*, and *mE4bp4* mRNA was plotted as relative mRNA level that was normalized to *β-actin* expression. Maximal expression of the vehicle-treated group (time = 0.5 h (c-*fos*) or 1.0 h (*mPer1*, *mPer2*, and *mE4bp4*)) was set at 1.0 ($n = 3$–4 at each time point) $^{**}P < 0.01$ vs. vehicle.

3.3. *Involvement of the MAP kinase pathway in transient* c-fos *and clock gene mRNA induction by* α_{1A}*-receptor activation in NIH3T3 cells*

While the precise signaling mechanisms underlying α_1-receptor-stimulated c-*fos* induction are still unclear, several signal transduction pathways including protein kinase A, protein kinase C, MAP kinase, and Ca^{2+}/calmodulin-dependent kinase are believed to be involved in c-*fos* induction by α_1-receptor activation [7]. Different multiple signaling and subtype-specific pathways may be implicated in each tissue or cell. For example, Chen et al. [12] reported that intracellular Ca^{2+} rather than MAP kinase and cAMP signaling pathways is important for c-*fos* induction by α_1-receptor activation in Rat-1 cells. On the other hand, Hu et al. [11] reported that MAP kinase and phosphoinositide 3′-kinase are important for c-*fos* induction by α_{1A}- and α_{1B}-receptor activation in NIH3T3 cells.

The MAP kinase signaling pathway is known to be involved in many downstream events related to intracellular Ca^{2+} release. In fact, PE treatment results in activation of MAP kinase in rat cardiomyocytes [16], α_{1A}- or α_{1B}-receptor-transfected Rat-1 cells [12], and α_{1B}-receptor-transfected COS-7 cells [17]. We tested whether c-*fos* and clock gene induction by α_{1A}-receptor activation in NIH3T3 cells was p44/42 MAP kinase-dependent. As shown in Fig. 4A, 10 min after α_{1A}-receptor activation by PE, phosphorylation of MAP kinase increased. Phosphorylation of MAP kinase then decreased but still remained comparatively high 30 min after treatment. The total amount of MAP kinase did not change with PE treatment. When cells were treated with the MEK inhibitor U0126 (10 μM) 10 min before PE treatment, both basal and PE-stimulated MAP kinase phosphorylation were completely inhibited (Fig. 4A).

Under our experimental conditions, c-*fos* and clock gene mRNA induction by PE were severely suppressed in α_{1A}-receptor-expressing cells (Fig. 4B,C) as well as α_{1B}- and α_{1D}-receptor-expressing cells. These results lead us to believe that the MAP kinase signaling pathway is involved in inducing c-*fos* and clock gene expression by α_{1A}-receptor activation in NIH3T3 cells. Oh-hashi et al. [14] revealed that NIH3T3 cells treated with thapsigargin, which also elevates intracellular Ca^{2+} levels by inhibiting endoplasmic reticulum Ca^{2+}-ATPase, induces *mPer1* and *mPer2* mRNA. In the SCN, light is conveyed to the SCN through the retinohypothalamic tract (RHT), which leads to *mPer1* and *mPer2* mRNA induction. Glutamate, a neurotransmitter of the RHT, and the Ca^{2+} influx through glutamate receptors are required for clock resetting [18]. Our paper and previous papers show that intracellular Ca^{2+} levels are also important for clock gene induction in cultured cells.

3.4. *Time-dependent induction of* mPer1 *and* mPer2 *mRNA in the mouse liver after PE injection*

We examined the effect of PE injection on clock gene expression in the mouse liver in which both α_{1A}- and α_{1B}-receptors were abundantly expressed [19]. Vehicle (saline) treatment did not induce *mPer1* and *mPer2* mRNA expression (data not shown) and instead showed a normal daily fluctuation during which *mPer1* expression was high at ZT12 (Zeitgeber time: ZT0 is defined as the lights-on time and ZT12 as the lights-off time) and *mPer2* was high at ZT12 and ZT18 (one-way ANOVA $P < 0.001$) (Fig. 5). If PE (5 mg/kg) was intraperitoneally injected when the basal *mPer1* and *mPer2* expression levels were low or moderate (ZT0, 6, and 18 for *mPer1*; ZT0 and 6 for *mPer2*), *mPer1* and *mPer2* were induced in the liver 1 h after injection (Fig. 5). However, if PE was injected when basal *mPer1* and *mPer2* mRNA levels were high, the *mPer1* and *mPer2* induction was not significant. These results show that PE-induced *mPer* in the liver was dependent on the time of day at which PE was injected.

It has been proposed that molecule diffusion from the transplanted SCN alone can sufficiently transfer circadian messages, without the need for synaptic contacts [20]. However, although a transplanted SCN is capable of restoring behavioral rhythms, it fails to restore endocrine rhythms such as cortisol and melatonin release [21,22]. Thus, the for-

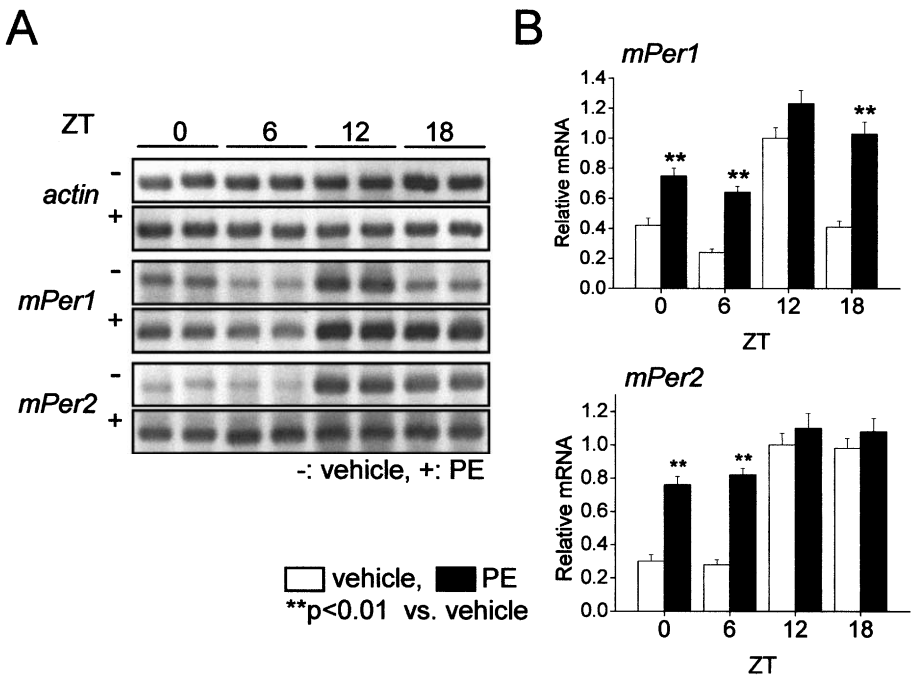

Fig. 5. Effect of PE injection on clock gene expression in the mouse liver. A: Vehicle (saline) or PE (5 mg/kg) was intraperitoneally injected. One hour after injection, mice were killed at ZT0, 6, 12, or 18. Total liver RNA was extracted and RT-PCR was performed to examine the *mPer1* and *mPer2* mRNA expression. Representative electrophoresis photographs of each PCR product are shown. B: Relative expression level of *mPer1* and *mPer2* was normalized to *β-actin* expression in the vehicle- or PE-injected mouse liver. *mPer1* and *mPer2* mRNA expression of the vehicle-treated group at ZT12 was set at 1.0. $^{**}P < 0.01$ vs. vehicle.

mation of precise connections between SCN neurons and SCN target areas is essential for the complete transmission of circadian messages from the biological clock. A multisynaptic autonomic connection from SCN neurons to various peripheral organs such as the liver, heart, pancreas, and adrenal cortex was shown through use of the viral tracing techniques [6]. Based on these findings, peripheral tissues seem to be indirectly regulated by the SCN through the autonomic nervous system.

The circadian rhythm of melatonin synthesis in the mammalian pineal gland is regulated by a β-adrenergic receptor and under the control of the SCN through a multisynaptic pathway including, successively, pre-autonomic neurons of the paraventricular nucleus, sympathetic preganglionic neurons in the spinal cord, and noradrenergic neurons of the superior cervical ganglion [23]. It is very plausible that the β-receptor is also involved in peripheral circadian regulation. Further study can help to determine the involvement of the autonomic nervous system on peripheral oscillator entrainment.

In conclusion, we demonstrated through the present results that several clock genes are induced by α_1-receptor activation via the MAP kinase-dependent pathway in NIH3T3 cells. *mPer1* and *mPer2* mRNA induction in the liver after PE injection led us to believe that not only humoral factors but also the adrenergic signaling pathway may be involved in peripheral oscillator entrainment in vivo.

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