# Restricted feeding induces daily expression of clock genes and *Pai-1* mRNA in the heart of *Clock* mutant mice

Yoichi Minami, Kazumasa Horikawa, Masashi Akiyama, Shigenobu Shibata*

*Department of Pharmacology and Brain Science, School of Human Sciences, Waseda University, Tokorozawa, Saitama 359-1192, Japan*

Received 27 June 2002; revised 29 July 2002; accepted 30 July 2002

First published online 7 August 2002

Edited by Felix Wieland

**Abstract Plasminogen activator inhibitor-1 (PAI-1) is a key factor of fibrinolytic activity. The activity and mRNA abundance show a daily rhythm. To elucidate the mechanism of daily *Pai-1* gene expression, the expression of *Pai-1* and several clock genes was examined in the heart of homozygous *Clock* mutant (*Clock*/*Clock*) mice. Damping of the daily oscillation of *Pai-1* gene expression in *Clock*/*Clock* mice was accompanied with damped or attenuated oscillations of *mPer1*, *mPer2*, *mBmal1*, and *mNpas2* mRNA. Daily restricted feeding induced a daily mRNA rhythm of all clock genes and *Pai-1* mRNA in *Clock*/*Clock* mice as well as wild-type mice. The peaks of clock genes and *Pai-1* mRNA were phase-advanced in the heart of both genotypes after 6 days of restricted feeding. The present results demonstrate that daily *Pai-1* gene expression depends on clock gene expression in the heart and that a functional *Clock* gene is not required for restricted feeding-induced resetting of the peripheral clock.** **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

*Key words:* Circadian; *Clock* mutation; Heart; Plasminogen activator inhibitor-1; Restricted feeding

## 1. Introduction

An endogenous circadian rhythm controls various physiological and behavioral phenomena. The molecular circadian clock system is thought to be based on transcriptional/translational feedback loops consisting of 'clock' genes and their products [1]. In mammals, CLOCK, a member of the bHLH-PAS transcription factors, heterodimerizes with BMAL1 and binds to E-box DNA motifs in the promoter of *Per1/2* and *Cry1/2*, thereby activating their transcription. On the other hand, CRY1/2 proteins negatively regulate the E-box binding ability of CLOCK:BMAL1. Concurrently, PER2 enhances *Bmal1* transcription [1]. Moreover, when BMAL2 (also called CLIF), which is highly homologous to BMAL1 [2], is expressed with CLOCK, E-box mediated transcription is accelerated [3]. NPAS2, a transcription factor that is highly homologous to CLOCK, heterodimerizes with BMAL1 and binds to the E-box motif, thereby activating *Per1/2* transcription [4]. Both NPAS2:BMAL1 and CLOCK:BMAL2 might also function as positive regulators in place of CLOCK:BMAL1. These clock genes are expressed not only in the suprachiasmatic nucleus (SCN) of the hypothalamus where the master clock exists, but also in other brain regions and various peripheral tissues. For example, in the rat heart, these clock genes are abundantly expressed and show clear circadian rhythms [5,6].

In previous studies, an abnormal circadian rhythmicity was demonstrated in *Clock* mutant mice. Heterozygous *Clock* mutant mice (*Clock*/+) demonstrated a lengthened and less stable circadian period, whereas homozygous mutant mice (*Clock*/*Clock*) showed a gradual loss of circadian rhythmicity in constant darkness (DD) [7]. Positional cloning and subsequent analysis showed that mutant CLOCK lacks 51 amino acids [8]. Furthermore, mutant CLOCK failed to activate E-box mediated transcription [9].

The onset of myocardial infarction frequently occurs from 6 a.m. to 12 p.m. in humans [10]. This phenomenon results in part because of a down-regulation of fibrinolytic activity. Plasminogen activator inhibitor type 1 (PAI-1) is the primary regulator of the fibrinolytic cascade [11,12], and its activity changes in a circadian fashion such that it is high in the morning and low in the evening [13]. *Pai-1* mRNA expression appears in the adiposities, the liver, the kidneys, and abundantly in the heart [14]. The *Pai-1* gene has two functional E-box motifs in the promoter [15]. Maemura et al. [3] demonstrated that *Pai-1* mRNA shows a daily rhythm in vivo, and *Pai-1* transcription is up-regulated by CLOCK:BMAL2 through E-box sites and down-regulated by mPER2 and mCRY1 in cultured cells. To confirm the importance of the *Clock* gene in circadian expression of the *Pai-1* gene, we investigated *Pai-1* gene expression in the heart of *Clock*/*Clock* mice.

Under restricted feeding (RF) conditions, animals are allowed daily access to food for only a limited time. RF-induced anticipatory locomotor activity rhythm is known to be SCN-independent because SCN-lesioned animals still maintain this activity rhythm [16]. Recent reports demonstrate that circadian clocks expressed in peripheral tissues such as the liver are reset by daily RF during the day in both normal and SCN-lesioned animals [17–19]. We examined whether an RF-induced phase advance in the expression of clock genes can

*Corresponding author. Fax: (81)-42-947 6806.
*E-mail address:* shibata@human.waseda.ac.jp (S. Shibata).

*Abbreviations:* DD, constant dark; LD, light–dark; PAI-1, plasminogen activator inhibitor-1; RF, restricted feeding; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time

0014-5793/02/$22.00 © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0014-5793(02)03159-9

also induce a phase advance of *Pai-1* mRNA expression. We further investigated whether normal *Clock* gene function is required for RF-induced resetting of the peripheral clock by using *Clock*/*Clock* mice.

## 2. Materials and methods

### 2.1. Animals and housing

*Clock* mutant mice were purchased from Jackson Laboratory (Stock No. 002923) (Bar Harbor, ME, USA) and interbred in our laboratory. Genotypes were determined by PCR [20]. Animals were maintained on a light–dark (LD) cycle (12 h light, 12 h dark, with lights on at 8:30 a.m.) at a room temperature of 23°C and given food and water *ad libitum* except for RF experiments. All animals were treated in accordance with the Law (No. 105) and Notification (No. 6) of the Japanese Government. Some mice were transferred to DD conditions at ZT12 (Zeitgeber time; ZT0 is defined as lights-on time and ZT12 as lights-off time), and sampling was initiated 24 h after transfer.

### 2.2. RNA Isolation and RT-PCR

Mice ($n = 3$–6 for each time point) were deeply anesthetized with ether and intracardially perfused with ice-cold saline. After perfusion, the heart was rapidly isolated, frozen in liquid nitrogen, and stored at −80°C until RNA isolation. Total RNA was extracted using ISOGEN Reagent (Nippon Gene, Tokyo, Japan). 100 ng of total RNA was reverse transcribed and amplified using the Superscript One-Step RT-PCR System (Invitrogen, CA, USA) in a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). Specific primer pairs were designed from published data of the *mPer1*, *mPer2*, *mBmal1*, *mNpas2*, *Pai-1*, and *β-actin* genes in GenBank as follows: *mPer1* [289 bp]: 5′-CAAGTGGCAATGAGTCCAACG-3′ (forward) and 5′-CGAAGTTTGAGCTCCCGAAGT-3′ (reverse); *mPer2* [381 bp]: 5′-CAGACTCATGATGACAGAGG-3′ (forward) and 5′-GAGATGTACAGGATCTTCCC-3′ (reverse); *mBmal1* [344 bp]: 5′-CACTGACTACCAAGAAAGTATG-3′ (forward) and 5′-ATCCATCTGCTGCCCTGAGA-3′ (reverse); *mNpas2* [243 bp]: 5′-CTCAGTGGTCAGTTACGCAG-3′ (forward) and 5′-TGGAGGTGGGTTCTGACATG-3′ (reverse); *Pai-1* [539 bp]: 5′-TCAGAGCAACAAGTTCAACTACACTGAG-3′ (forward) and 5′-CCCACTGTCAAGGCTCCATCACTTGCCCCA-3′ (reverse); and *β-actin* [452 bp]: 5′-GAGGGAAATCGTGCGTGACAT-3′ (forward) and 5′-ACATCTGCTGGAAGGTGGACA-3′ (reverse). We used a semi-quantitative RT-PCR method for measuring the expression level of mRNA. All PCR products were placed under linear amplification from cycles 26 through 30; however, from the 32nd cycle products levels plateaued (data not shown). Therefore, we obtained PCR products in the 28th cycle for quantification. PCR was performed under the following conditions: cDNA synthesis at 50°C for 30 min followed by 94°C for 2 min, PCR amplification for 28 cycles with denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min. The target clock gene cDNA was co-amplified with *β-actin* cDNA in a single PCR tube. The PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide, and analyzed by an EDAS-290 system (Kodak, NY, USA). The intensity of PCR product of the target gene was normalized to the intensity of *β-actin*. The amplitude (ratio of peak and trough) and phase determined by this method were reproduced in a different experiment and found comparable as determined by Northern blot [5], which suggests that the present experimental conditions can detect a circadian change of *mPer1* and *mPer2* gene expression in the mouse liver.

### 2.3. RF experiment

The RF experiment was performed as previously described [22]. In brief, after 1 day of fasting (termed as day 0), mice were allowed access to food for 4 h from ZT5 to ZT9 for 6 consecutive days (day 1 to day 6). On day 7, food was again withdrawn for the entire day. Animals were sacrificed at ZT5, 11, 17, and 23 on day 6, and ZT5 on day 7.

### 2.4. Statistical analysis

The values are expressed as means ± S.E.M. For statistical analysis, one-way ANOVA was applied followed by Dunnett's two-tailed test or the Student's *t*-test.

## 3. Results

### 3.1. Daily expression of mPer1, mPer2, mBmal1, mNpas2, and Pai-1 genes in the heart

Using the semi-quantitative RT-PCR method, all clock genes examined in the mouse heart under LD conditions showed a clear daily mRNA rhythm (Fig. 1 and Table 1). *mBmal1* (Fig. 1B) and *mNpas2* (Fig. 1C) mRNA levels peaked at night, while *mPer1* (Fig. 1D) and *mPer2* (Fig. 1E) mRNA levels peaked during the day. *Pai-1* mRNA, the basal expression of which is thought to be under the control of a circadian clock, also showed a clear daily change in the heart, with a peak at late evening (Fig. 1F). On the other hand, *mBmal2* (*Clif*) expression did not show a daily oscillation as previously reported (data not shown) [3].

The daily rhythm of clock genes under LD conditions was attenuated or even absent in the heart of *Clock*/*Clock* mice (Fig. 1 and Table 1). *mBmal1* mRNA did not show significant daily variation in *Clock*/*Clock* mice; as previously shown, there was a constant high expression [21]. The *mPer1* mRNA rhythm was also dampened. *mPer2* mRNA was still expressed in a circadian fashion under our experimental conditions, but there was a severely dampened amplitude and delayed peak compared to wild-type mice (Fig. 1E) *mNpas2* mRNA also showed a weak but significant daily rhythm (Fig. 1C), whereas *Pai-1* mRNA expression lacked significant daily variation in *Clock*/*Clock* mice (Fig. 1F).

To rule out the effect of light, the circadian patterns of clock genes and *Pai-1* gene expression were also studied under DD conditions. Similar to LD conditions, circadian expression of these genes was observed under DD conditions in wild-type mice (Table 1). In *Clock*/*Clock* mice, *mPer1, mPer2,* and *Pai-1* gene expression exhibited no significant rhythmicity when assessed by one-way ANOVA (Table 1).

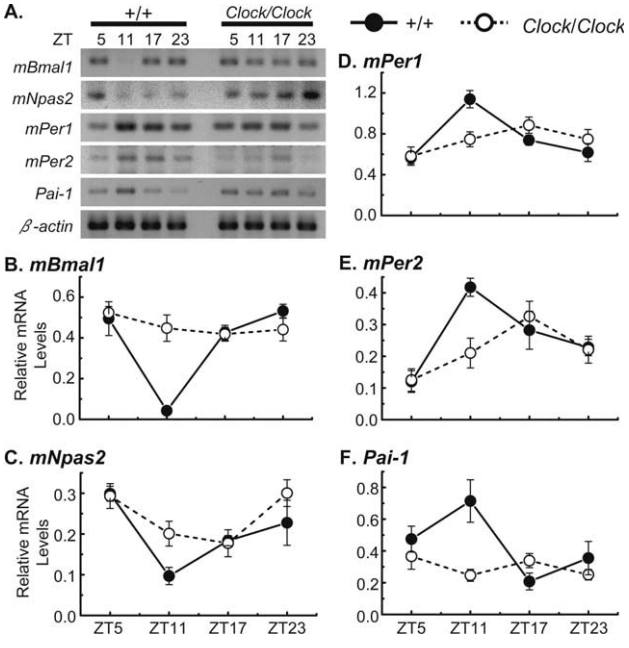


Fig. 1. Daily fluctuation of *mPer1*, *mPer2*, *mBmal1*, *mNpas2*, and *Pai-1* mRNA in the heart of wild-type and *Clock*/*Clock* mice. A: Representative electrophoresis photographs of PCR products from each genotype (wild-type and *Clock*/*Clock*) at ZT 5, 11, 17, and 23. B: Daily mRNA abundance of each clock gene was plotted as a relative mRNA level that was normalized to *β-actin* mRNA ($n = 4$–6).

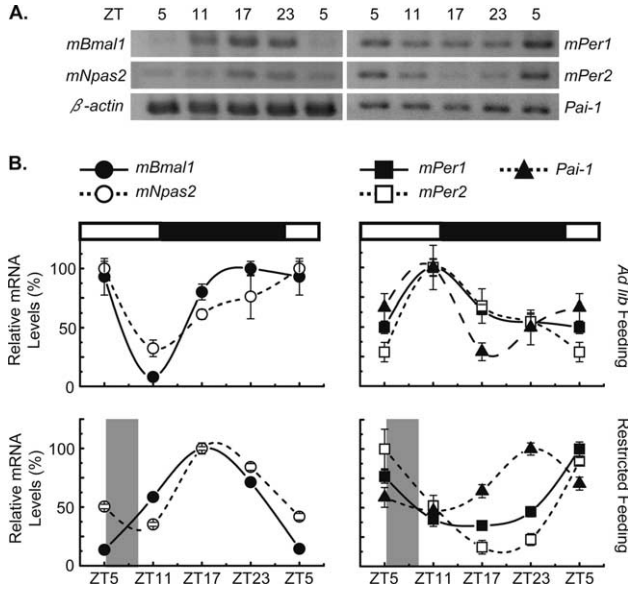


Fig. 2. Effect of RF on the daily clock gene expression in the heart of wild-type mice. A: Representative electrophoresis photographs of PCR products from wild-type mice at ZT5, 11, 17, and 23 on day 6 (final day of RF) and at ZT 5 on day 7. B: Expression of clock genes of wild-type was plotted as a relative mRNA level that was normalized to *β-actin* expression. The peak value was set to 100% ($n = 3$–6). Points from the *ad libitum* feeding schedule at ZT5 on day 7 were re-plotted as data at ZT5 on day 6.

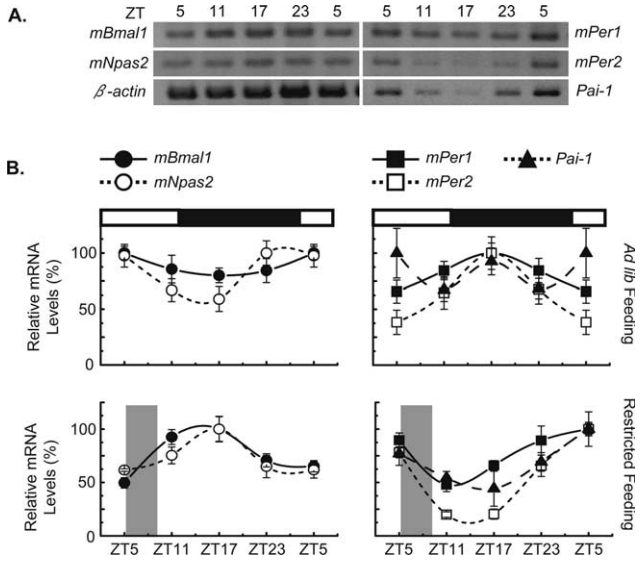


Fig. 3. Effect of RF on daily clock gene expression in the heart of *Clock*/*Clock* mice. A: Representative electrophoresis photographs of PCR products from *Clock*/*Clock* at ZT5, 11, 17, and 23 on day 6 (final day of RF) and at ZT5 on day 7. B: Expression of clock genes of *Clock*/*Clock* was plotted as a relative mRNA level that was normalized to *β-actin* expression, and the peak value was set to 100% ($n = 3$–6). Points from the *ad libitum* feeding schedule at ZT5 on day 7 were re-plotted as data at ZT5 on day 6.

### 3.2. RF-induced entrainment of clock genes and Pai-1 gene expression in Clock/Clock mice

Wild-type and *Clock*/*Clock* mice were placed on a RF schedule under LD conditions. As shown in Fig. 2, wild-type mice placed under RF for 6 days showed a daily variation in all clock genes as well as *Pai-1* gene expression in the heart (lower panels). Compared to mice with free access to food (upper panels), the phase of the peaks and troughs of clock genes was advanced by 6–12 h with RF. The *Pai-1* mRNA expression pattern also showed a 12-h change under an RF schedule.

When *Clock*/*Clock* mice followed a RF schedule, both *mPer2* and *mNpas2*, that showed a weak daily rhythmicity under *ad libitum* conditions, exhibited a robust phase-advanced rhythmicity in the heart. Interestingly, *mPer1*, *mBmal1*, and *Pai-1* mRNAs exhibiting no significant rhythmicity under *ad libitum* conditions, showed a clear daily rhythmicity under RF conditions in *Clock*/*Clock* mice. The peak expression of these mRNAs also showed a phase-advanced rhythmicity that was similar to wild-type mice under RF conditions (Fig. 3 and Table 1).

## 4. Discussion

In the present experiment, we found that the amplitude of daily oscillations of *mBmal1* and *mPer1* gene expression was blunted in the heart of *Clock*/*Clock* mice. In addition, *Pai-1* mRNA oscillation completely disappeared in the heart of mutant mice, suggesting that expression of the *Pai-1* gene is closely clock-controlled in the heart. The present in vivo results support those of a recent paper in which CLOCK: BMAL2 up-regulated the *Pai-1* gene and PER2 and CRY1 down-regulated *Pai-1* in cultured cells [3]. As PAI-1 activity regulates the fibrinolytic cascade [11], possibly a circadian change of *Pai-1* expression underlies the circadian rhythmicity of myocardial infarction occurrence.

In the present experiment, we demonstrated a daily expression of *mNpas2* in the wild-type mouse heart. However, the oscillation of *mNpas2* expression in *Clock*/*Clock* mice is not as severely dampened as that of *mBmal1* expression, which is lost under LD conditions. NPAS2 possesses high homology to CLOCK, and co-expression of NPAS2 and BMAL1 activates transcription of *Per1*, *Per2*, and *Cry1* genes, suggesting that

Table 1
Daily variation and peak time of clock genes and *Pai-1* mRNA expression

| | Feeding conditions | Lighting conditions | Positive regulator | | Negative regulator | | Output |
|---|---|---|---|---|---|---|---|
| | | | *mBmal1* | *mNpas2* | *mPer1* | *mPer2* | *Pai-1* |
| +/+ | *Ad lib* | DD | pZT23*** | pZT23*** | pZT11*** | pZT11*** | pZT17** |
| | *Ad lib* | LD | ZT23*** | ZT5*** | ZT11*** | ZT11*** | ZT11* |
| | RF | LD | ZT17*** | ZT17*** | ZT 5*** | ZT 5*** | ZT23* |
| *Clock*/*Clock* | *Ad lib* | DD | pZT 5*** | pZT 5* | – | – | – |
| | *Ad lib* | LD | – | ZT23* | – | ZT17* | – |
| | RF | LD | ZT11-17*** | ZT17* | ZT5*** | ZT5*** | ZT23* |

*Ad lib*: free feeding schedule (four time points); pZT: projected ZT; RF: RF schedule (five time points). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. –: not significant.

NPAS2 is capable of replacing CLOCK as a BMAL1 partner in areas other than the SCN [4]. Therefore, *mPer1* and other clock genes in which the expression is regulated through the E-box are not only controlled by CLOCK:BMAL1 but also by NPAS2:BMAL1 (or CLOCK:BMAL2) in the heart.

Since RF does not affect the daily or circadian expression of clock genes in the SCN [17,22], RF-induced oscillation of the peripheral clock does not require oscillation of the master SCN clock. Furthermore, lesion of the SCN does not affect the RF-induced oscillation of *mPer* gene expression in the liver [19]; however, the necessity of *Clock* gene function in the RF-induced circadian rhythm had yet to be determined. We demonstrated that the rhythmicity of *mPer1*, *mBmal1*, and *Pai-1* mRNA, of which the daily oscillation dampened under *ad libitum* conditions, reappeared under RF conditions in *Clock*/*Clock* mice. Recently, it was shown that methamphetamine (MAP) restores locomotor activity rhythm in behaviorally arrhythmic *Clock*/*Clock* mice [23]. These results suggest that intact CLOCK is not necessary for inducing either wheel-running activity rhythm with chronic MAP treatment or food entrainment of the peripheral oscillator through RF. Interestingly, the MAP-induced locomotor activity rhythm is also independent of the circadian oscillator in the SCN [24,25]. Functional CLOCK might not be necessary for these SCN-independent rhythms. Perhaps, NPAS2 compensates for the functional deficit observed in *Clock*/*Clock* mice.

*Pai-1* mRNA expression showed a RF-induced phase advance in the mouse heart. This result indicates the possibility that the timing of fibrinolytic activity may be regulated through *Pai-1* gene expression by changing the feeding time schedule. Some $Ca^{2+}$ channel blockers [26] and β-adrenergic receptor antagonists [27] have reportedly caused a time of day-dependent effect on myocardial disease. Therefore, a controlled feeding time may help with the success of this type of chronopharmacological treatment. Further studies are needed to confirm the interaction of peripheral clock gene function and fibrinolytic activity through *Pai-1* gene expression.

In summary, we demonstrated that *Pai-1* mRNA expression had a daily rhythmicity in the mouse heart, the expression of which is under the control of clock genes. Furthermore, feeding schedule can affect the rhythmic phase of not only clock gene expression but also *Pai-1* gene expression in both wild-type and *Clock*/*Clock* mice. Thus, molecular clock resetting in the mouse heart under RF conditions may not require the functional *Clock* gene.

*Acknowledgements:* This work was partially supported by grants awarded to S.S. from the Japanese Ministry of Education, Sports, and Culture (11170248, 11233207, 12877385, 13470016), The Special Coordination Funds of the Japanese Science and Technology Agency, Waseda University, and Kyowa Hakko, Inc.

## References

[1] Reppert, S.M. and Weaver, D.R. (2001) Annu. Rev. Physiol. 63, 647–676.
[2] Ikeda, M., Yu, W., Hirai, M., Ebisawa, T., Honma, S., Yoshimura, K., Honma, K.I. and Nomura, M. (2000) Biochem. Biophys. Res. Commun. 275, 493–502.
[3] Maemura, K., de la Monte, S.M., Chin, M.T., Layne, M.D., Hsieh, C.M., Yet, S.F., Perrella, M.A. and Lee, M.E. (2000) J. Biol. Chem. 275, 36847–36851.
[4] Reick, M., Garcia, J.A., Dudley, C. and McKnight, S.L. (2001) Science 293, 506–509.
[5] Sakamoto, K. and Ishida, N. (2000) Eur. J. Neurosci. 12, 4003–4006.
[6] Young, E.M., Razeghi, P. and Taegtmeyer, H. (2001) Circ. Res. 88, 1142–1150.
[7] Vitaterna, M.H., King, D.P., Chang, A.M., Kornhauser, J.M., Lowrey, P.L., McDonald, J.D., Dove, W.F., Pinto, L.H., Turek, F.W. and Takahashi, J.S. (1994) Science 264, 719–725.
[8] King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D., Vitaterna, M.H., Kornhauser, J.M., Lowrey, P.L., Turek, F.W. and Takahashi, J.S. (1997) Cell 89, 641–653.
[9] Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S. and Weitz, C.J. (1998) Science 280, 1564–1569.
[10] Muller, J.E., Ludmer, P.L., Willich, S.N., Tofler, G.H., Aylmer, G., Klangos, I. and Stone, P.H. (1987) Circulation 75, 131–138.
[11] Schleef, R.R., Higgins, D.L., Pillemer, E. and Levitt, J.J. (1989) J. Clin. Invest. 83, 1747–1752.
[12] Fay, W.P., Shapiro, A.D., Shih, J.L., Schleef, R.R. and Ginsburg, D. (1992) N. Engl. J. Med. 327, 1729–1733.
[13] Andreotti, F., Davies, G.J., Hackett, D.R., Khan, M.I., DeBart, A.C., Aber, V.R., Maseri, A. and Kluft, C. (1988) Am. J. Cardiol. 62, 635–637.
[14] Sawdey, M.S. and Loskutoff, D.J. (1991) J. Clin. Invest. 88, 1346–1353.
[15] Hua, X., Miller, Z.A., Wu, G., Shi, Y. and Lodish, H.F. (1999) Proc. Natl. Acad. Sci. USA 96, 13130–13135.
[16] Mistlberger, R.E. (1994) Neurosci. Biobehav. Rev. 18, 171–195.
[17] Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F. and Schibler, U. (2000) Genes Dev. 14, 2950–2961.
[18] Stokkan, K.A., Yamazaki, S., Tei, H., Sakaki, Y. and Menaker, M. (2001) Science 291, 490–493.
[19] Hara, R., Wan, K., Wakamatsu, H., Aida, R., Moriya, T., Akiyama, M. and Shibata, S. (2001) Genes Cells 6, 269–278.
[20] Jin, X., Shearman, L.P., Weaver, D.R., Zylka, M.J., deVries, G.J. and Reppert, S.M. (1999) Cell 96, 57–68.
[21] Oishi, K., Fukui, H. and Ishida, N. (2000) Biochem. Biophys. Res. Commun. 268, 164–171.
[22] Wakamatsu, H., Yoshinobu, Y., Aida, R., Moriya, T., Akiyama, M. and Shibata, S. (2001) Eur. J. Neurosci. 13, 1190–1196.
[23] Masubuchi, S., Honma, S., Abe, H., Nakamura, W. and Honma, K. (2001) Eur. J. Neurosci. 14, 1177–1180.
[24] Honma, K., Honma, S. and Hiroshige, T. (1986) Physiol. Behav. 38, 687–695.
[25] Honma, K., Honma, S. and Hiroshige, T. (1987) Physiol. Behav. 40, 767–774.
[26] Fujimura, A. and Ebihara, A. (1988) Life Sci. 42, 1431–1437.
[27] Mulcahy, D., Keegan, J., Cunningham, D., Quyyumi, A., Crean, P., Park, A., Wright, C. and Fox, K. (1988) Lancet 2, 755–775.