Reduced Nitric Oxide Causes Age-Associated Impairment of Circadian Rhythmicity

Takeshige Kunieda,* Tohru Minamino,* Kentaro Miura, Taro Katsuno, Kaoru Tateno, Hideyuki Miyauuchi, Shuichi Kaneko, Christopher A. Bradfield, Garret A. FitzGerald, Issei Komuro

Abstract—Impairment of circadian rhythmicity in the elderly has been suggested to cause age-associated diseases such as atherosclerosis and hypertension. Endothelium-derived nitric oxide (NO) is a critical regulator of cardiovascular homeostasis, but its production declines with aging, thereby inducing vascular dysfunction. We show here that impaired circadian rhythmicity is related to a decrease of NO production with aging. Treatment with an NO donor significantly upregulated the promoter activity of the clock gene Period via the cAMP response element–dependent and the E-box enhancer element–dependent pathways. Both phosphorylation and S-nitrosylation by NO are involved in this upregulation. In aged animals, endothelial NO synthase activity was markedly decreased during the daytime, along with impairment of clock gene expression and the circadian variation in blood pressure. Treatment of aged animals with an NO donor significantly improved the impairments. Inhibition of NO synthase activity also led to impairment of clock gene expression and blood pressure rhythm. These results suggest that NO is a key regulator of the circadian clock in the cardiovascular system and may be a novel target for the treatment of age-associated alteration of circadian rhythms. (Circ Res. 2008;102:607-614.)

Key Words: aging ● clock gene ● endothelial nitric oxide synthase

Aging is associated with various alterations of the circadian rhythms of the body,1,2 including changes of the rhythms for blood pressure, locomotor activity, core body temperature, and the sleep–waking cycle. However, the molecular mechanisms underlying these age-associated changes of circadian rhythmicity are not fully understood. In mammals, circadian rhythmicity is under the control of a molecular pacemaker that is composed of the products of clock genes.3-8 These gene products form an oscillatory mechanism that is based on self-sustained transcriptional–translational feedback loops, and the regulatory feedback loops can be divided into positive and negative limbs. The positive limb consists of 2 PAS helix–loop–helix transcription factors, BMAL1 and CLOCK, which form heterodimers and bind to the E-box enhancer element to regulate transcription of the period genes and cryptochrome genes. The PER and CRY proteins are components of the negative limb, which attenuates their own activation by BMAL1/CLOCK, thereby generating negative feedback. Exposure to light is a prominent stimulant that synchronizes endogenous circadian rhythmicity to the environmental light–dark cycle. It has been reported that cAMP response element (CRE)-binding protein (CREB)-dependent activation of clock genes is crucial for light-induced resetting of the pacemaker.9-13 In mammals, the master pacemaker controlling the circadian rhythm is located in the suprachiasmatic nuclei (SCN). Several lines of evidence indicate that various peripheral tissues such as the heart and blood vessels as well as various cells, including cardiovascular cells,14 also possess circadian oscillators and that impairment of the function of such peripheral clocks may contribute to the development of age-associated cardiovascular diseases.15-22

In the present study, we demonstrated a protective role of nitric oxide (NO) against the occurrence of age-associated alterations of the circadian rhythm. We found that NO regulates circadian signals through both the CRE-mediated and E-box enhancer element–mediated pathways. We also found that aged mice exhibited a decrease of NO production and had impaired clock gene rhythmicity, which was improved by treatment with an NO donor. These results provide some new insights into the treatment of age-associated changes of circadian rhythms.

Materials and Methods

Cell Culture

Human aortic vascular smooth muscle cells were purchased from Cambrex (Walkersville, Md) and were grown according to the instructions of the manufacturer. Cells from passages 5 to 7 were...
used for the experiments. NIH3T3 cells and HEK293 cells were cultured in DMEM supplemented with 10% FCS at 37°C in a mixture of 95% air and 5% CO2. Transfection was performed using fuge 6 (Roche, Indianapolis, Ind) according to the instructions of the manufacturer.

Animal Models
All animal experiments were approved by our institutional review board. Wild-type C57BL/6J mice were purchased from SLC and endothelial NO synthase (eNOS)-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, Me). The mice were maintained on a 12:12-hour light:dark (LD) cycle for at least 14 days before the experiments. To inhibit NO synthase, mice were treated with L-nitro-L-arginine methyl ester (L-NAME) (1 mg/kg per day). Continuous blood pressure was monitored in conscious, unrestrained mice using the telemetry system (Data Science, St Paul, Minn). Implantation of a telemeter (Data Science, TA11PA-C20) was performed as described, and telemetry recordings began on the morning of the 10th day.

Luciferase Assay
The reporter gene plasmid (1 μg) was transfected into NIH3T3 cells (2.5×104 cells per 12-well plate) at 24 hours before the luciferase assay. The control vector encoding Renilla luciferase (0.1 μg) was cotransfected as an internal control. The luciferase assay was carried out using a dual luciferase reporter assay system (Promega, Madison, Wis) according to the instructions of the manufacturer. The reporter gene constructs were kindly provided by Dr M. Izumo (University of Virginia, Charlottesville, Va) and Dr H. Shime no (Fukuoka University, Japan).

Preparation of Nuclear Extracts
Samples were homogenized in 5 volumes of buffer A (0.2% Nonidet P-40, 10 mMol/L Hepes [pH 7.9], 10 mMol/L KCl, 0.1 mMol/L EDTA, 0.1 mMol/L EGTA, 1 mMol/L dithiothreitol, and 0.1 mMol/L phenylmethylsulfonyl fluoride), and the crude nuclear pellet was isolated by centrifugation at 3000 g for 10 minutes. The pellet was resuspended in buffer C (20 mMol/L Hepes [pH 7.9], 0.4 mMol/L NaCl, 1 mMol/L EDTA, 1 mMol/L EGTA, 1 mMol/L dithiothreitol, 0.1 mMol/L phenylmethylsulfonyl fluoride, and 10% glycerol) and placed on ice for 1 hour. The mixture was centrifuged at 20 000 g for 10 minutes, and the supernatant was used as the nuclear extract.

Electrophoretic Mobility-Shift Assay
The double-stranded oligonucleotides used for the electrophoretic mobility-shift assay (EMSA) consisted of the stem/Per1 E-box sequence (5’-cgcgCAAGTCCACGTGCAGGGAtcga-3’). Nuclear extracts were incubated with 32P-labeled oligonucleotides in binding buffer containing 10 mMol/L Tris–HCl (pH 8.0), 0.5 mMol/L dithiothreitol, 10% glycerol, 50 μg/mL poly(dI-dC), 50 mMol/L NaCl, and 5 mMol/L MgCl2. In competition experiments, a 200-fold molar excess of unlabeled E-box or mutant E-box oligonucleotides (5’-cgcgCAAGTCtcgctcCAGGGAtcga-3’) was added to the reaction mixture. The mixtures were subjected to electrophoresis on 5% non-denaturing polyacrylamide gels.

Northern Blot Analysis
Total RNA (20 to 30 μg) was isolated by using RNAZol-B (Molecular Research Center, Cincinnati, Ohio), separated on a formaldehyde denaturing gel, and transferred to a nylon membrane (Amersham, Buckinghamshire, UK). The blot was then hybridized with a radiolabeled cDNA probe using Quickhyb hybridization solution (Stratagene, Tokyo, Japan) according to the instructions of the manufacturer.

Quantitative Real-Time RT-PCR
Primers were designed by PRIMER EXPRESS 1.0 (PE Applied Biosystems), and the DNA-intercalating SYBR green reagent (Applied Biosystems) was used for detection. Primer sequences were as follows: Per1, 5’-TCGAAACAGGACACCTTTCTCT-3’ and 5’-ACACAAAGCCCCACGGG-3’; Per2, 5’-GCTGCCCATCCACAGGAAG-3’ and 5’-ATAAGGGTAACTGAAGGCG-3’; GAPDH, 5’-CGATTTGGCGTATGG-3’ and 5’-CTTCCACC-TCTAACACGGTG-3’.

Western Blot Analysis
Whole-cell lysates (30 to 50 μg) or nuclear extracts (10 to 20 μg) were resolved by SDS-PAGE. Proteins were transferred onto a poly(vinylidene difluoride) membrane (Millipore, Bedford, Mass) and incubated with the primary antibody, followed by horseradish peroxidase–conjugated anti-rabbit IgG or anti-mouse IgG antibodies (Jackson, West Grove, Pa). Specific proteins were detected by using enhanced chemiluminescence (Amersham). The primary antibodies used for Western blotting were as follows: anti–phospho-eNOS, phospho-CREB antibody (Upstate Cell Signaling, Charlottesville, Va); anti-FLAG, β-actin antibody (Sigma); and anti-eNOS antibody (Santa Cruz Biotechnology, Santa Cruz, Calif); anti-Per1 antibody (Abcam, Cambridge, Mass). The biotin switch assay was performed to detect S-nitrosylated Bmal-1 as described previously.

Statistical Analysis
Data are shown as means±SEM. Differences between groups were examined by Student’s t test or ANOVA, followed by Bonferroni’s correction for comparison of means. In all analyses, P<0.05 was considered statistically significant.

Results
NO Increases Per1 Promoter Activity via Both CRE-Mediated and E-Box Element–Mediated Pathways
NO plays a crucial role in cardiovascular physiology, and its downregulation has been thought to underlie age-related development of cardiovascular disease. To investigate the relationship between NO and age-associated changes of circadian rhythmicity, we first examined the effect of NO on Per1 promoter activity. The Per1 promoter contains CRE and E-box enhancer elements, both of which have been reported to be involved in the regulation of Per1 expression. We transfected a construct of the Per1 promoter region linked to the luciferase reporter gene (Per1-WT, Figure 1A), which contained a single CRE and three E-box enhancer elements. Treatment with an NO donor, S-nitrosothioglutathione (GSNO), significantly increased the promoter activity compared with the control (Figure 1B). To determine which element was involved in mediating NO-induced activation of the Per1 promoter, we generated Per1 reporter constructs that lacked either the E-box element (Per1-CRE) or CRE (Per1–E-box; Figure 1A). Treatment with GSNO significantly increased reporter activity driven by the E-box element–deficient or CRE-deficient promoters, but these effects were significantly smaller than the increase of Per1-WT activity (Figure 1B). These results suggest that NO causes an increase of Per1 promoter activity via both CRE- and E-box–mediated pathways.

Mechanisms Underlying Upregulation of Clock Genes by NO
The CRE-mediated transcriptional pathways in the central clock have been shown to be activated by multiple kinases,
including protein kinase (PK)G, and NO is known to upregulate PKG activity.27 GSNO treatment markedly increased the level of phospho-CREB, thereby increasing Per1 promoter activity, and this effect was attenuated by a specific PKG inhibitor (KT5823) (Figure 2A). Introduction of a dominant-negative form of CREB significantly suppressed activation of the Per1 promoter by NO, indicating that NO partly upregulates clock gene expression via PKG/CREB-mediated pathways (Figure 2B). In contrast, a PKA inhibitor (H89) did not suppress NO-induced upregulation of Per1 promoter activity (Figure 2A in the online data supplement, available http://circres.ahajournals.org). Treatment with GSNO also upregulated Bmal1 expression by increasing its stability (Figure 2C and 2D) and thereby enhanced binding activity for the E-box element (Figure 2E), whereas GSNO did not affect clock expression (data not shown). Disruption of Bmal1 expression completely inhibited the increase of Per1–E-box activity induced by NO (Figure 2F), suggesting an essential role of Bmal1 in NO-induced upregulation of Per1. In contrast to the effect of NO on CREB, this upregulation was not inhibited by treatment with KT5823 (Figure 2G and 2H), indicating that PKG-independent mechanisms were involved. S-nitrosylation involves the covalent binding of NO moieties to the sulphhydryl residues of proteins, resulting in the formation of S-nitrosothiols. Accumulating evidence has suggested that NO exerts some of its effects on the cardiovascular system as S-nitrosylation biotin switch assay.29,30 Treatment with NEM (Figure 2I) markedly inhibited NO-induced upregulation of Bmal1 (Figure 2J), suggesting that S-nitrosylated Bmal1 was involved in NO-induced upregulation of clock gene expression.

**NO Regulates the Circadian Rhythm of PER2 Expression**

We next examined the effect of NO on the circadian rhythm of clock genes by using serum-shocked cells, a well-known in vitro model for circadian expression of clock genes.31 Human aortic smooth muscle cells cultured without serum for 48 hours were transferred to serum-rich medium for 2 hours and again returned to serum-free medium. Subsequently, we harvested the cells at several time points and examined the expression of PER2 by Northern blot analysis. Serum stimulation induced a transient increase of PER2 expression, which was followed by downregulation and sequential upregulation (Figure 3A and data not shown). Treatment with GSNO significantly enhanced PER2 expression, resulting in a phase advance (Figure 3A). Conversely, inhibition of NO production by L-NAME reduced PER2 expression and thereby delayed the phase of its rhythmic expression (Figure 3B). Likewise, expression of PER1 was significantly increased by treatment with GSNO (supplemental Figure IB). This upregulation appeared to be essential for the effect of NO on PER2 expression because GSNO treatment failed to induce the expression in Per1-deficient vascular cells (supplementary Figure IC). These results suggest that NO can regulate the circadian rhythm of clock genes in cultured cells.

**NO Production and Circadian Rhythmicity in Aged Animals**

Basal production of NO by the cardiovascular system decreases with aging and this contributes to age-related cardiovascular dysfunction.32 To examine the relationship between NO and age-related changes of circadian rhythms, we first measured NO production in aged mice. Consistent with previous reports, the levels of urinary NO metabolites, ie, nitrite/nitrate (NOx), were significantly lower in aged mice (8 weeks of age) than in young animals (8 weeks of age; Figure 4B). Western blot analysis showed that the extent of phosphorylation of eNOS displayed robust cyclic changes with a peak at ZT12 in the young mice (Figure 4B). In contrast, daytime phospho-eNOS levels were significantly lower and circadian rhythmicity was impaired in the aged animals (Figure 4B). To investigate whether reduced production of NO affects the circadian clock in aged animals, we examined the daily changes of phospho-CREB levels and binding activity for the E-box element. Western blot analysis demonstrated rhythmic changes of phospho-CREB in young mice, but this circadian rhythm was markedly impaired in aged mice (Figure 4C). Similar to the changes of phospho-eNOS, the phase of the phospho-CREB circadian rhythm was delayed in aged mice.
EMSAs were performed to detect E-box binding activity. GSH (GSNO) or GSNO (1 mmol/L), after which Western blot analysis. I, HEK293 cells were transfected with the expression vector encoding Flag-tagged Bmal1 and were incubated with GSH (GSNO) or GSNO (1 mmol/L). Luciferase activity was measured at 24 hours after transfection. DNCREB indicates dominant-negative form of CREB. Data are means±SEM (n=5). *P<0.05 vs GSNO or DNCREB. #P<0.05 vs Flag-Bmal1 and treated with GSNO (1 mmol/L) or GSH (1 mmol/L). At 24 hours after transfection, the cells were treated with cycloheximide (CHX) (50 µmol/L). Cells were harvested at the indicated time points after cycloheximide treatment, and the level of Flag-tagged Bmal1 was examined by Western blot analysis. Data are means±SEM (n=5). *P<0.05 vs control (GSH) treatment. E, HEK293 cells were transfected with Flag-Bmal1 and treated with GSNO (1 mmol/L) or GSH (1 mmol/L). Expression of Flag-tagged Bmal1 was examined by Western blot analysis. Data are means±SEM (n=5). *P<0.05 vs control (GSH) treatment. D, HEK293 cells were transfected with Flag-Bmal1 and treated with GSNO (1 mmol/L) or GSH (1 mmol/L). Nuclear extracts were prepared, and the EMSA for E-box–binding activity was performed as described in Materials and Methods. Competition experiments showed the specificity of this assay. F, Embryonic fibroblasts prepared from wild-type (WT) or Bmal1–deficient mice (KO) were transfected with Per1–E-box and were incubated with GSH (GSNO) or GSNO (1 mmol/L). Luciferase activity was measured at 24 hours after transfection. Data are means±SEM (n=6). *P<0.05 vs WT/GSNO, #P<0.05 vs WT/GSNO. G, NIH3T3 cells were transfected with Per1–E-box and were incubated with GSH (GSNO) or GSNO (1 mmol/L) in the presence or absence of KT5823 (10 µmol/L). Luciferase activity was measured at 24 hours after transfection. Data are means±SEM (n=5). *P<0.05 vs GSNO. H, HEK293 cells were transfected with the expression vector encoding Flag-tagged Bmal1 and were incubated with GSH (GSNO) or GSNO (1 mmol/L) in the presence or absence of KT5823 (10 µmol/L). Luciferase activity was measured at 24 hours after transfection. Data are means±SEM (n=5). *P<0.05 vs GSNO. I, HEK293 cells were transfected with the expression vector encoding Flag-tagged Bmal1 and were incubated with GSH (GSNO) or GSNO (1 mmol/L), after which S-nitrosylated Bmal1 levels were examined by the biotin switch assay. J, NIH3T3 cells were transfected with Per1–E-box and incubated with GSH (GSNO) or GSNO (1 mmol/L) in the presence or absence of NEM (40 µmol/L). Luciferase activity was measured at 24 hours after transfection. Data are means±SEM (n=3). *P<0.05 vs GSNO. K, HEK293 cells were transfected with the expression vector encoding Flag-tagged Bmal1 and were incubated with GSH (GSNO) or GSNO (1 mmol/L) in the presence or absence of NEM (40 µmol/L). Flag-tagged Bmal1 levels were examined by Western blot analysis. L, HEK293 cells were transfected with Flag-tagged Bmal1 and HA-tagged Clock and were incubated with GSH (GSNO) or GSNO (1 mmol/L) in the presence of NEM (0–100 µmol/L). Nuclear extracts were prepared, and EMSA was performed to detect E-box binding activity.

compared with young mice (Figure 4C). In the young animals, binding activity for the E-box element increased to reach a peak at ZT6 and decreased thereafter, whereas it peaked at ZT12 in aged mice (Figure 4D). These results suggest that NO activates both CRE-mediated and E-box element–mediated pathways during the day and that impairment of NO production results in a phase shift of the circadian clock.

NO Induces Phase Shift of Clock Gene Expression
To further investigate the relationship between NO production and regulation of the circadian clock, we examined clock gene expression in the aortas of mice housed under the above LD cycle. As reported previously,18 there was marked cyclic expression of Per2 in young mice with a peak at ZT12 (Figure 5A). In aged mice, the circadian phase of Per2 expression was significantly delayed (Figure 5A). Treatment of young
animals with L-NAME reduced Per2 expression during the daytime, resulting in phase delay (Figure 5B). Disruption of eNOS also impaired the daytime expression of clock genes such as Per1, Per2, and Bmal1 and thereby induced a phase shift of expression of these genes, and this impairment was improved by treatment with GSNO (Figure 5C and supplemental Figure IIA and IIB). These results suggest that reduced production of NO contributes to age-associated impairment of clock gene expression. Finally, we examined whether treatment with an NO donor could restore clock gene expression in aged mice. Administration of GSNO (1 mg/kg) during the daytime significantly enhanced Per2 expression and eliminated the phase delay (Figure 5D). Similarly, Per1 expression in aged mice was significantly increased by treatment with GSNO (supplemental Figure IIC and IID), indicating that supply of exogenous NO may be a useful treatment for age-associated impairment of circadian rhythmicity.

**NO and Circadian Variation of Blood Pressure**

One aspect of the age-related impairments of circadian rhythmicity in the cardiovascular system is nondipper hypertension, in which the normal nocturnal decrease of blood pressure is diminished. Nondippers show impaired endothelial-dependent vasodilation attributable to a decrease of NO production and are known to have a higher risk of cardiovascular diseases.33–35 To further investigate a relationship between NO and age-related impairment of circadian rhythmicity in the cardiovascular system, we monitored blood pressure in unrestrained mice using the telemetry system. A robust diurnal variation in blood pressure was apparent in young mice (Figure 6A and 6D). Blood pressure was decreased during the daytime (corresponding to the nighttime for humans) relative to the nighttime (Figure 6A and 6D). The decline of blood pressure during the daytime was less prominent in aged mice, and this change was ameliorated by treatment with an NO donor (Figure 6B and 6D). Treatment of young animals with L-NAME conversely reduced the daytime fall of blood pressure (Figure 6A and 6D). Similar to aged mice, the circadian variation in blood pressure was markedly decreased in Bmal1-deficient mice,36 but treatment with an NO donor could not improve this change (Figure 6C and 6D).

**Figure 3.** Effect of NO on circadian expression of PER2 in vitro. A, Human aortic smooth muscle cells were deprived of serum for 48 hours, transferred to serum-rich medium (time = 0), and incubated for another 2 hours. Then the medium was replaced with serum-free medium. The cells were treated with GSNO (1 mmol/L) or GSH (1 mmol/L) after 15 hours (arrow) and were harvested at the indicated time points. Total RNA (30 μg) was prepared, and PER2 expression was examined by Northern blot analysis. Data are means±SEM (n = 3). *P<0.05 vs GSH treatment. B, Serum shock treatment of human aortic smooth muscle cells was performed as described in A, and the cells were treated with L-NAME (1 mmol/L) after 15 hours (arrow). Total RNA (30 μg) was prepared, and PER2 expression was examined by Northern blot analysis. Data are means±SEM (n = 3). *P<0.05 vs saline treatment.

**Figure 4.** Age-associated impairment of clock gene regulation. A, Total urinary NOx was evaluated by ELISA. Data are means±SEM (n=4). *P<0.05 vs young mice. B and C, Young (8-week-old) or aged (90-week-old) mice were maintained on the LD cycle described in Materials and Methods for at least 2 weeks, and their aortas were harvested at the indicated time points. Protein extracts (100 μg) were prepared from the aortas, and daily changes of phospho-eNOS (B) and phospho-CREB (C) were examined by Western blot analysis. The nonphosphorylated forms were used as a control, and relative values are plotted in the graphs. Data are means±SEM (n=3 to 4). *P<0.05 vs young mice. D, Hearts were harvested from young or aged mice at the indicated time points, and nuclear proteins (10 μg) were extracted. Then binding activity for E-box elements was examined by EMSA.
Discussion

Our present results suggest that the reduced production of NO is responsible for impairment of various circadian rhythms in aged animals for the following reasons. First, administration of an NO donor enhanced clock gene expression, whereas inhibition of NO production reduced such gene expression in vitro. Second, daytime NOS activity was reduced in aged animals and clock gene expression was downregulated. Third, genetic or pharmacological disruption of NOS activity also downregulated daytime clock gene expression in young mice. Finally, administration of an NO donor reversed the changes of clock gene expression in aged mice.

We propose the following mechanism of clock gene regulation by NO. S-Nitrosylation has recently been recognized as an important mechanism for posttranslational regulation of various proteins, including enzymes, signaling molecules, and membrane receptors. Recent studies have demonstrated that NO also modulates transcription factors and their regulatory partners by S-nitrosylation. This regulation by NO is often exerted at the level of ubiquitinylation-dependent proteosomal targeting. Consistent with these reports, we found that an NO donor caused the S-nitrosylation of BMAL1 protein and thus increased its stability, with these changes being inhibited by an S-nitrosylation inhibitor. Bmal1 protein has 14 cysteine residues, and electrospray ionization mass spectrometry identified 6 putative cysteine residues involved in S-nitrosylation (supplemental Table I), but no single mutation of these residues alters the effect of NO on its transcriptional activity (unpublished data, 2007), suggesting that at least 2 cysteine residues may be involved in this regulation. Although there is evidence indicating that NO regulates gene expression via DNA methylation, we did not find any effects of NO on CpG methylation in the promoter region of the Per1 gene (unpublished data, 2007).

The NO/PKG pathways in the SCN are postulated to mediate the phase-advancing effects of exposure to light on the circadian clock because pharmacological inhibition of these signaling molecules blocks light-induced phase shifts. Although the mechanisms acting downstream of the NO/PKG pathways in the SCN have not been completely elucidated, it has been reported that CRE-mediated transcription plays a crucial role in phase resetting of the clock by NO. A recent study showed that sildenafil, an inhibitor of cGMP-specific phosphodiesterase 5, enhances light-induced phase advances in the SCN. We also found that NO induced the phosphorylation of CREB, thereby upregulating the transcription of Per1 via a PKG-dependent mechanism. Reduced NO production was associated with a delay in the phase of cyclic alterations of phospho-CREB levels and clock gene expres-
sion, whereas treatment with an NO donor advanced the phase of the circadian rhythm in the cardiovascular system, suggesting that NO may be able to improve impaired rhythmicity associated with cardiovascular disease in the elderly.

A physiological decrease of blood pressure during the daytime was attenuated in Bmal1-deficient mice as well as aged wild-type mice. An NO donor ameliorated this impairment in aged mice but not in Bmal1-deficient mice, suggesting that the NO-Bmal1 axis plays a crucial role in regulating circadian variation of blood pressure. Interestingly, single injection of an NO donor improved the circadian rhythm of blood pressure over 3 days after treatment. Because we have previously reported that sympathoadrenal function is disrupted in Bmal1-deficient mice, the NO-Bmal1 axis may modulate expression of genes relevant to catecholamine biosynthesis. We also demonstrated that eNOS showed rhythmic changes of phosphorylation in the aorta, but the underlying mechanisms are unclear. eNOS activity is tightly controlled by co- and posttranslational lipid modifications, phosphorylation of multiple residues, and regulatory protein–protein interactions. Thus, both systemic signals (hormones and growth factors) and local signals (metabolism and shear stress) may influence the circadian rhythm of the peripheral clock in the cardiovascular system by regulating eNOS activity. Further studies on regulation of the cardiovascular clock should help to provide novel insights into the treatment of impaired rhythmicity associated with cardiovascular disease.

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Disclosures
None.

References


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Supplementary Methods

Nano electrospray ionization mass spectrometry (ESI-MS)
HEK293 cells were transfected with the expression vector encoding Flag-tagged Bmal1 and were incubated with GSH (GSNO –) or GSNO (1 mM). Nuclear extracts were immunoprecipitated with anti-Flag (Sigma) antibody and separated by electrophoresis. Gel was fixed and stained with Coomassie Brilliant Blue G-250. Selected gel bands were excised manually and placed into eppendorf tubes. The reduced and alkylated samples were digested by trypsin overnight at 35 °C, after which the peptide mixture was analyzed by nano ESI-MS analysis (Q-Tof2, Waters Micromass, UK).

Analysis of DNA methylation
We analysed DNA methylation using the EZ DNA Methylation-Gold kit (Zymo Research Corp., Orange, CA) according to the manufacturer’s instructions. The following primers were used for PCR; (-1721 to -1444), 5'-AGAAGGTAAATGAGGGGTAGG-3' and 5'-AATTAACACCTAAAACCCA-3'; (-732 to -444), 5'-TGGTTAGTATAGGATTGGTTT-3' and 5'-CCCACCTAATCTAAAACCTCTA-3'. The PCR products were cloned into the pCR4-TOPO vector (Invitrogen), and two independent clones from each sample were sequenced.
Supplementary Figure legends

Supplementary Figure 1 Effects of NO in vitro.

(A) NIH3T3 cells were transfected with Per1-CRE and were incubated with GSH (GSNO –) or GSNO (1 mM) in the presence or absence of H89 (10 µM). Luciferase activity was measured at 24 hours after transfection. Data are the mean ± SEM (n=5). *p<0.05 vs. GSNO (–) H89 (–); #p<0.05 vs. GSNO (-) H89 (+). (B) Human aortic smooth muscle cells were deprived of serum for 48 hours, transferred to serum rich-medium (time=0), and incubated for another 2 hours. Then the medium was replaced with serum-free medium. The cells were treated with GSNO (1 mM) or GSH (1 mM) after 15 hours and were harvested at the indicated time points. Total RNA was prepared and PER1 expression was examined by real time RT-PCR. Data are the mean ± SEM (n=3). *p<0.05 vs. GSH treatment. Relative PER1 mRNA levels corrected by GAPDH expression were plotted in the graph. (C) Vascular smooth muscle cells prepared from wild-type (WT) or Per1-deficient mice (KO) were deprived of serum for 48 hours, transferred to serum rich-medium (time=0), and incubated for another 2 hours. Then the medium was replaced with serum-free medium. The cells were treated with GSNO (1 mM) or GSH (1 mM) after 15 hours and were harvested at 21 hours. Total RNA was prepared and Per2 expression was examined by real time RT-PCR. Data are the mean ± SEM (n=6). *p<0.05 vs. WT/GSNO (-); #p<0.05 vs. WT/GSNO (+). Relative Per2 mRNA levels corrected by Gapdh expression were plotted in the graph.

Supplementary Figure 2 Effects of NO in vivo.

(A) Wild-type (Wild) or eNOS-deficient mice (eNOS KO, 8 weeks old) were maintained on the same LD cycle for at least 2 weeks. Their aortas were harvested at the indicated time points and Per1 and Bmal1 expression was examined by Northern
blot analysis. (B) eNOS-deficient mice (eNOS KO, 8 weeks old) were treated with GSNO (1 mg/kg) at 6 hours. Their aortas were harvested at 8 hours and Per1 expression was examined by real time RT-PCR. Data are the mean ± SEM (n=4). *p<0.05 vs. vehicle treatment. (C), (D) Aged mice (90 weeks old) were treated with GSNO (1 mg/kg) at 6 hours. Their aortas were harvested at 8 hours and Per1 expression was examined by real time RT-PCR (C) and Western blot analysis (D). Data are the mean ± SEM (n=5). *p<0.05 vs. vehicle treatment.
**Supplementary Table. List of putative S-nitrosylation sites identified by nano ESI-MS analysis.**

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<td>Cys-249</td>
</tr>
<tr>
<td>277-289</td>
<td>R.KSFCTIHSTGYLK.S</td>
<td>757.3776</td>
<td>757.3897</td>
<td>Cys-280</td>
</tr>
<tr>
<td>278-289</td>
<td>K.SFCTIHSTGYLK.S</td>
<td>693.3123</td>
<td>699.3301</td>
<td>Cys-280</td>
</tr>
</tbody>
</table>
| 290-319         | K.SWPPTKMGLDEDNEPDNEGCNLSCLVAIGR.L | 1659.7272     | 1659.8141  | Cys-309,
|                 |                                    |               |            | Cys-313 |
| 290-319         | K.SWPPTKMGLDEDNEPDNEGCNLSCLVAIGR.L | 1667.7246     | 1667.7675  | Cys-309,
|                 |                                    |               |            | Cys-313 |
Supplementary Figure 1

A

B

C

Relative luciferase levels

Relative PER1 levels

Relative Per2 levels

GSNO - + - + +
H89 - + - + +

GSNO

WT

KO

**gs**

Supplementary Figure 1

Relative luciferase levels

Relative PER1 levels

Relative Per2 levels

GSNO - + - + +
H89 - + - + +

GSNO

WT

KO

**gs**
Supplementary Figure 2

A

Bmal1

WT eNOS KO

Per1

WT eNOS KO

ZT (h) 0 6 12 18 24 30

B

Relative Per1 levels

GSNO - +

ZT (h) 0 6 12 18 24 30

C

Relative Per1 levels

GSNO - +

D

GSNO - +

Per1 Actin