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HEME REVERSIBLY DAMPS PERIOD2 RHYTHMS IN MOUSE SUPRACHIASMATIC NUCLEUS EXPLANTS

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Abstract—The hypothalamic suprachiasmatic nucleus (SCN), which in mammals serves as the master circadian pacemaker by synchronizing autonomous clocks in peripheral tissues, is composed of coupled single-cell oscillators that are driven by interlocking positive/negative transcriptional/translational feedback loops. Several studies have suggested that heme, a common prosthetic group that is synthesized and degraded in a circadian manner in the SCN, may modulate the function of several feedback loop components, including the REV-ERB nuclear receptors and PERIOD2 (PER2). We found that ferric heme (hemin, 3–100 μM) dose-dependently and reversibly damped luminescence rhythms in SCN explants from mice expressing a PER2::LUCIFERASE (PER2::LUC) fusion protein. Inhibitors of heme oxygenases (HOs, which degrade heme to biliverdin, carbon monoxide, and iron) mimicked heme's effects on PER2 rhythms. In contrast, heme and HO inhibition did not damp luminescence rhythms in thymus and esophagus explants and had only a small effect on PER2::LUC damping in spleen explants, suggesting that heme's effects are tissue-specific. Analysis of the effects of heme's degradation products on SCN PER2::LUC rhythms indicated that they probably were not responsible for heme's effects on rhythms. The heme synthesis inhibitor N-methylprotoporphyrinIX (NMP) lengthened the circadian period of SCN PER2::LUC rhythms by about an hour. These data are consistent with an important role for heme in the circadian system.

Key words: circadian, tissue culture, desynchronization, luciferase, NR1D1, clock genes.

In mammals, circadian rhythms in physiology and behavior are generated by a master pacemaker in the hypothalamic suprachiasmatic nucleus (SCN), which is composed of thousands of coupled single-cell oscillators (Welsh et al., 1995; Yamaguchi et al., 2003). Rhythms in individual cells are sustained by a series of interacting positive and negative transcriptional/translational feedback loops (Ko and Takahashi, 2006). Among these, CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers drive transcription of the *Period* (*mPer1-3* in mice) and *cryptochrome* (*mCry1-2* in mice) genes and of the genes for the nuclear receptors REV-ERBα (NR1D1) and REV-ERBβ (NR2D2); the mCRYs and mPERs then feed back to inhibit CLOCK:BMAL1- and NPAS2:BMAL1-mediated transcription while the REV-ERBs repress *Bmal1* expression.

While models of the molecular basis of circadian rhythms have focused primarily on these transcriptional/translational feedback loops, small cytosolic signaling molecules, such as Ca^{2+} and cAMP, have recently emerged as fundamental components of the circadian clock mechanism (Ikeda et al., 2003; Lundkvist et al., 2005; Hastings et al., 2008; O'Neill et al., 2008). Heme, an ubiquitous molecule that is synthesized in virtually every cell in the body, has long been considered essential for normal cellular function, since it acts as a prosthetic group for several proteins involved in a broad range of physiological processes (Wagener et al., 2003). The recent finding that heme functions as a reversible REV-ERB ligand to repress transcription of REV-ERB target genes (Raghuram et al., 2007; Yin et al., 2007) suggests that heme may also act as a signaling molecule, and, more specifically, as a cytosolic regulator of the circadian clock. Consistent with this idea, heme application phase-shifted the electrical activity rhythm in acute SCN slices (Artinian et al., 2001) and peripheral heme administration altered clock gene expression in mouse liver (Kaasik and Lee, 2004). Additionally, hemin treatment has been shown to synchronize clock gene expression in mouse fibroblasts (Kaasik and Lee, 2004; Rogers et al., 2008).

We investigated if heme homeostasis is necessary for normal circadian clock function in mouse tissue explants and found that pharmacologically elevating intracellular heme levels reversibly and dose-dependently damps PER2::LUCIFERASE rhythms in the SCN. These data are consistent with an important role for heme in the mammalian circadian clock.

EXPERIMENTAL PROCEDURES

Animals

Male and female heterozygous ($mPer2^{Luc/+}$) and homozygous ($mPer2^{Luc/Luc}$) mPER2::LUCIFERASE knockin mice (Yoo et al., 2004) were drawn from an in-house colony (derived from founders generously provided by Joseph Takahashi, Northwestern University) and were housed in a 12 h:12 h light:dark cycle prior to dissection. All animal protocols were approved by the Smith College Institutional Animal Care and Use Committee.

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Abbreviations: CO, carbon monoxide; HO, heme oxygenase; LPS, lipopolysaccharide; NMP, N-methylprotoporphyrin IX; PPIX, protoporphyrin IX; RI, rhythmicity index; ROSs, reactive oxygen species; SCN, suprachiasmatic nucleus; SnPP, tin protoporphyrin IX; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α.

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Tissue preparation

SCN and peripheral tissue explants were prepared according to standard methods (Yoo et al., 2004). Subjects were overdosed with halothane or isoflurane anesthesia, and 300 μm coronal sections through the SCN were cut on a vibratome (Campden Istruments, Lafayette, IN, USA) in chilled Hank's balanced salt solution (Invitrogen, Carlsbad, CA, USA). Sections were trimmed by hand to the SCN and immediately surrounding tissue and were transferred to culture inserts (Millipore, Billerica, MA, USA) in 35 mm dishes [BD Falcon (Franklin Lakes, NJ, USA) or Electron Microscopy Sciences (Hatfield, PA, USA)]. Peripheral tissues were trimmed by hand to approximately 1–8 mm^3 and were cultured on nylon mesh (Small Parts, Miami Lake, FL, USA). Dishes contained 1.0–1.2 mL Dulbecco's modified Eagle's medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 1×B27 (Invitrogen), 4 mM L-glutamine (Invitrogen), 25 mM glucose (Sigma), 4.2 mM $NaHCO_3$ (Sigma), 10 mM HEPES (Sigma), 25 Units/mL penicillin-G sodium (Invitrogen), 34 μM streptomycin sulfate (Invitrogen), and 100 μM beetle luciferin (Promega, Madison, WI, USA). Dishes were sealed with hot glue (for CO experiments) or with vacuum grease (Dow, Midland, MI, USA; for all other experiments). As many as two SCN sections and four peripheral tissue sections (per tissue type) were collected from a single animal; multiple samples of the same tissue from a single animal were placed in different treatment groups.

Drug treatments

Biliverdin hydrochloride, hemin chloride, tin protoporphyrin IX, protoporphyrin IX, and N-methylprotoporphyrin IX [all from Sigma or Frontier (Logan, UT, USA)] were dissolved in sterile 0.085 M or 0.17 M Na_3PO_4 (Sigma). Ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2$] was dissolved in sterile water. Ketoconazole (Sigma) was dissolved in DMSO. Lipopolysaccharide (Sigma) was dissolved in Hank's balanced salt solution. Tumor necrosis factor-α (TNF-α; Peprotech, Rocky Hill, NJ, USA) was dissolved in water. Drugs were diluted 500-fold (ketoconazole), 10,000-fold (lipopolysaccharide), 2000-fold (TNF-α), or 1000-fold (all other drugs) into culture media at the time of dissection. For all porphyrin treatments, the final Na_3PO_4 concentration was 170 μM, which did not change the initial pH of the media or the media's pH after a week in culture (data not shown). Carbon monoxide (CO) was measured, mixed with air, and delivered using gas-tight syringes (Hamilton, Reno, NV, USA). To produce equilibrium aqueous CO concentrations of 30 μM and 100 μM, 50 mL of 4%:96% and 13%:87% v/v CO:Air mixtures (see R. Sander, http://www.henrys-law.org), respectively, were flushed through a hole in the top of each pre-sealed culture dish before rapidly sealing the hole with hot glue; in pre-

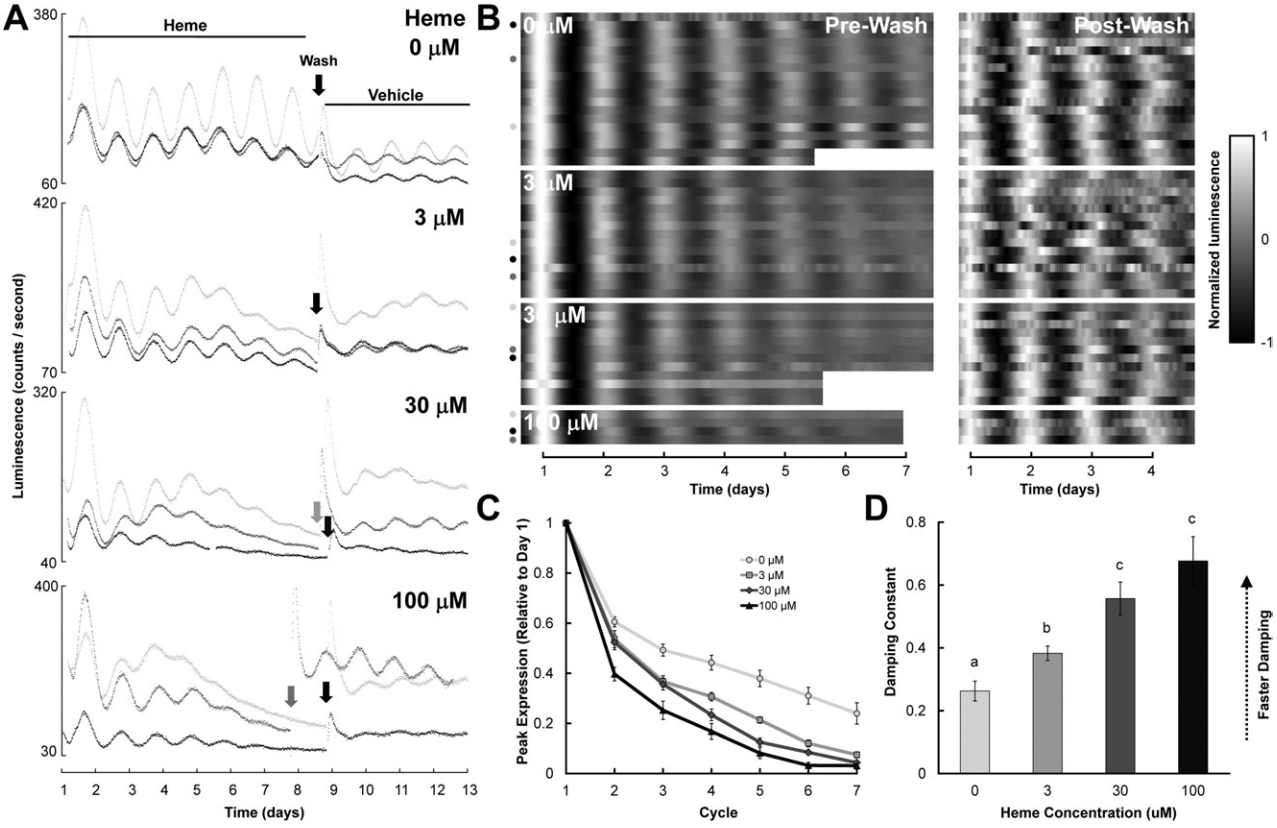

Fig. 1. Effects of heme on PER2::LUC rhythms in mouse SCN explants. (A), (B) Three representative raw bioluminescence traces (A) and all baseline-subtracted, smoothed data (B) for SCNs treated with 0–100 μM heme (left panels in B); cultures were subsequently washed and transferred to control media at the time indicated by the downward arrows in (A), and bioluminescence monitoring was continued (right panels in B). Tissue was dissected on day 0 (not shown). In B, each heatmap row represents a different culture, the x-axis indicates time (with t=1 corresponding to the time of peak luminescence on day 1 in the left panels and to the time of peak luminescence on the day following the media change in the right panels), colors represent luminescence level as indicated in the key to the right, pre- and post-media change data were normalized independently, and colored dots indicate the cultures illustrated by traces in part (A). (C) Mean peak PER2::LUC expression during each circadian cycle prior to the media change, normalized to peak expression on the day after dissection (Cycle 1). (D) Mean damping constants for recordings prior to the media change. In this and subsequent figures: groups with the same letters above error bars are statistically similar while groups with different letters are statistically different (Tukey's HSD, $P's<0.05$); all error bars indicate ±SEM.