Published in final edited form as:

Biochem Biophys Res Commun. 2008 April 18; 368(4): 955–958. doi:10.1016/j.bbrc.2008.02.031.

Relationship between circadian oscillations of *Rev-erba* expression and intracellular levels of its ligand, heme

Pamela M. Rogers, Ling Ying, and Thomas P. Burris*

Nuclear Receptor Biology Laboratory, Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Road, LA 70808, USA

Abstract

The nuclear hormone receptors, REV-ERB α [NR1D1] and REV-ERB β [NR1D2], were recently demonstrated to be receptors for the porphyrin, heme. Heme regulates the ability of these receptors to repress transcription of their target genes via modulation of the affinity of the receptor's ligand binding domain for the corepressor, NCoR. The REV-ERBs function as critical components of the mammalian clock and their expression oscillates in a circadian manner. Here, we show that in NIH3T3 cells intracellular heme levels also oscillate in a circadian fashion. These data are the first to show the temporal relationship of intracellular heme levels to the expression of its receptor, *Rev-erba*, and suggest that the rapid oscillations in heme levels may be an important component regulating REV-ERB transcriptional activity.

Keywords

Circadian; Steroid receptor; Nuclear receptor; Metabolism

Nuclear hormone receptors (NHRs) regulate many physiological processes ranging from growth and differentiation to metabolism and reproduction [1]. NHRs function as ligand regulated transcription factors and function as receptors for a variety of hydrophobic ligands including steroid hormones, thyroid hormones, and dietary lipids. Many of the 48 human NHRs are still characterized as orphan receptors due to the lack of an identified ligand [2]. Two of these receptors, which until recently were characterized as orphan receptors (REV-ERB α [NR1D1] and REV-ERB β [NR1D2]), are particularly interesting NHRs due to their role in regulation of both metabolism and the circadian rhythm [3,4].

Circadian rhythms are common in physiology and are essential for normal regulation of an array of process including metabolism, thermoregulation, blood pressure, renal function and the sleep-wake cycle. At the cellular level, the circadian rhythms are generated by feedback loops in the expression of clock genes where heterodimers of BMAL1 and CLOCK induce the expression of *Cryptochrome* (*Cry*) and *Period* (*Per*) genes [5]. When CRY and PER reach a crucial threshold they repress the stimulatory effects of the BMAL1/CLOCK heterodimers on expression of their own genes. REV-ERB α (a transcriptional repressor) and another NHR, ROR α [NR1F1] (a transcriptional activator) play critical roles in regulating the cyclic expression of *Bmal1* and are thus important components of the mammalian clock [6-8]. Additional feedback is contributed by the BMAL1/CLOCK heterodimer directly regulating *Rev-erba* expression via an E-box within its promoter [9,10]. *Rev-erba*^{-/-} mice express

Bmal1 in an abnormal fashion and the mice also exhibit alterations in the phase and period of their circadian behavior patterns [7].

Recently, the porphyrin heme was identified as a ligand for REV-ERBs [11,12]. Heme binds directly to the ligand binding domain of either REV-ERB and regulates the ability of the receptors to repress its target genes including *Bmal1* [11,12]. Interestingly, the level of expression of the rate limiting enzyme in heme production, δ -aminolevulinic acid synthase 1 (*Alas1*), is also regulated in a circadian fashion [13,14]. Components of the mammalian clock including NPAS2/BMAL1 and PER have been shown to be essential regulators of the circadian expression pattern of *Alas1* [13,14]. Although the circadian expression pattern of *Alas1* has been characterized, there have been no studies examining intracellular heme levels in circadian synchronized cells.

Materials and Methods

Cell culture

NIH3T3 cells were maintained in DMEM + 10% FBS as per ATCC guidelines. Twenty-four hours prior to synchronization, cells were plated in 6-well plates at a density of 10^6 cells/well. Cells were synchronized by a 50% horse serum shock [15-17] or 30 μ M hemin shock [14] for 2 h. Following the 2 h shock, cells were harvested for RNA ($n = 4$) every 4 h for 52 h. At the timepoints described for RNA isolation, cells were harvested for heme measurements ($n = 4$) as described below.

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described [18,19]. Primers have been previously described [20].

Heme measurements

Intracellular heme was measured as previously described [11].

Statistics

One-way ANOVA was utilized to analyze the alterations in gene expression and heme concentrations as a function of time. The Bonferroni test was utilized to assess significant ($P < 0.05$) differences between various times. A point was deemed significant if it was significantly different than the nadir point of the trough either just before or after the peak.

Results

Serum shock has been demonstrated synchronize the expression of mammalian clock genes in cultured cells revealing the diurnal pattern of expression of *Per2*, *Bmal1*, and *Rev-erba* among other genes [15-17]. We shocked NIH3T3 cells with 50% horse serum for 1 h and then examined the expression of *Per2*, *Bmal1*, and *Rev-erba* by qPCR every 4 h for 2 days post serum shock. As illustrated in Fig. 1 *Per2* and *Bmal1* expression displays a circadian pattern with opposing phases as expected indicating that the serum shock successfully synchronized the cells. *Per2* expression varied as much as 40-fold (Fig. 1A) while *Bmal1* expression varied approximately threefold (Fig. 1B). Both displayed the expected period of approximately 24 h. *Rev-erba* expression also displayed the expected circadian pattern of expression with an approximate 22-fold variation in expression (Fig. 2A). Interestingly, when we monitored the intracellular levels of heme in NIH3T3 cells we observed rapid oscillations in heme concentrations with a frequency much greater (period~8 h) than the 24 h period normally observed for clock genes (Fig. 2B). Heme concentrations varied within the range of 0.8 to 2.3 μ M/mg protein, which is well within the range found to modulate REV-ERB target genes such

as *Bmal1* as much as threefold in HepG2 cells (0.7 to 1.5 $\mu\text{M}/\text{mg}$ protein) [11]. This pattern was maintained for ~ 32 h before the oscillations appeared to dampen, possibly due to desynchronization within the cultured cells. No oscillations in heme levels were observed in cells that had not been synchronized with the 50% horse serum shock (data not shown).

A heme shock (30 μM hemin) has also been shown to have the ability to synchronize the circadian rhythm in cultured cells [14]. We also performed a heme shock and measured *Per2* expression to monitor synchronization of the cells as had been performed previously [14]. We noted circadian oscillations in *Per2* expression that were very similar to those noted by Kassik and Lee [14] (Fig. 3A). We also monitored intracellular heme levels in NIH3T3 cells that had been synchronized with the 30 μM heme shock. As illustrated in Fig. 3B, intracellular heme levels were initially quite elevated compared to the concentration range noted in the serum shocked cells (~ 5 -fold greater). This is consistent with the elevated levels of *heme oxygenase-1* expression that we noted in these cells (data not shown). As the intracellular heme levels decreased to levels similar to non-heme shocked cells we noted what appeared to be oscillations similar to those observed in the serum shock; however, multiple significant oscillations were not achieved. (Fig. 2B).

Discussion

Although circadian oscillations in the expression of the rate limiting enzyme for heme production, *Alas1*, have been previously noted [13,14] no direct observation of oscillations of intracellular heme levels have been observed until now. The fact that *Alas1* oscillations have been observed in multiple models along with our observation of intracellular heme oscillations suggests that this may be a common physiological phenomenon. The role heme plays in the circadian rhythm is unclear, but heme coordinating protein components of the circadian oscillator such as NPAS2 have been described [21,22]. After we and others found that REV-ERB α and REV-ERB β act as heme receptors [11,12], we sought to determine the relative levels of intracellular heme during the cellular circadian rhythm following synchronization with a serum shock. Both *ROR α* and *Rev-erba* exhibit circadian patterns of expression with a period of approximately 24 h; however, they are 180° out of phase with one another. They often compete for the identical DNA response element as exemplified by the elements within the *Bmal1* promoter and since ROR α is a constitutive activator of transcription and REV-ERB α is a constitutive repressor of transcription the oscillations in expression of these two transcription factors may underlie the oscillations in observed *Bmal1* expression [3,23]. However, we now know that the repressor activity of REV-ERB α requires heme binding and based as demonstrated in this study, intracellular heme levels also oscillate. Since intracellular heme levels oscillate at a much higher frequency than that of REV-ERB α , this indicates that it is likely that the transcriptional repressor activity of REV-ERB α varies even during the time when it is the dominant factor occupying the REV-ERB α /ROR α response elements such as those found in the *Bmal1* promoter (Fig. 4). The frequency of heme oscillations may vary, and the high frequency oscillations that we observed may be specific to the cell-type and entraining signal we utilized. In fact, when we entrained with a heme shock we observed a very different response in terms of intracellular heme concentrations. The significance of the heme oscillations is unclear, but there are several possibilities that can be suggested including that the heme oscillations are required for mammalian clock function. However, this appears unlikely since we observed *Per2* oscillations prior to initiation of intracellular heme oscillations (Fig. 3) suggesting that the oscillations in intracellular heme are not required for oscillations of at least some components of the mammalian clock. More likely is the possibility that intracellular heme levels offer a point of access for modification of the characteristics of the circadian rhythm. There are a number of examples of regulatory inputs that modify *Alas1* expression including bile acids [24], nutritional status via PGC-1 α [25], and xenobiotics [26, 27]. Additionally, regulation of heme oxygenase, which controls heme degradation, is another

mechanism that may be used to modify the mammalian clock via modulation of intracellular heme levels.

References

1. Savkur RS, Bramlett KS, Clawson D, Burris TP. Pharmacology of nuclear receptor-coregulator recognition. *Nuclear Receptor Coregulators* 2004;145–183.
2. Kliewer SA, Lehmann JM, Willson TM. Orphan nuclear receptors: shifting endocrinology into reverse. *Science* 1999;284:757–760. [PubMed: 10221899]
3. Fontaine C, Staels B. The orphan nuclear receptor Rev-erb alpha: a transcriptional link between circadian rhythmicity and cardiometabolic disease. *Current Opinion in Lipidology* 2007;18:141–146. [PubMed: 17353661]
4. Burris TP. Nuclear hormone receptors for heme: REV-ERB{alpha} and REV-ERB{beta} are ligand-regulated components of the mammalian clock. *Molecular Endocrinology*. 2008
5. Grimaldi B, Sassone-Corsi P. Circadian rhythms: metabolic clockwork. *Nature* 2007;447:386–387. [PubMed: 17522662]
6. Guillaumond F, Dardente H, Giguere V, Cermakian N. Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *Journal of Biological Rhythms* 2005;20:391–403. [PubMed: 16267379]
7. Preitner N, Damiola F, Molina LL, Zakany J, Duboule D, Albrecht U, Schibler U. The orphan nuclear receptor REV-ERB alpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 2002;110:251–260. [PubMed: 12150932]
8. Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, Fitzgerald GA, Kay SA, Hogenesch JB. A functional genomics strategy reveals rora as a component of the mammalian circadian clock. *Neuron* 2004;43:527–537. [PubMed: 15312651]
9. Triqueneaux G, Thenot S, Kakizawa T, Antoch MP, Safi R, Takahashi JS, Delaunay F, Laudet V. The orphan receptor Rev-erb alpha gene is a target of the circadian clock pacemaker. *Journal of Molecular Endocrinology* 2004;33:585–608. [PubMed: 15591021]
10. Ripperger JA. Mapping of binding regions for the circadian regulators BMAL1 and CLOCK within the mouse Rev-erbalpha gene. *Chronobiology International* 2006;23:135–142. [PubMed: 16687287]
11. Raghuram S, Stayrook KR, Huang P, Rogers PM, Nosie AK, McClure DB, Burris LL, Khorasanizadeh S, Burris TP, Rastinejad F. Identification of heme as the ligand for the orphan nuclear receptors REV-ERB[alpha] and REV-ERB[beta]. *Nature Structural Molecular Biology* 2007;14:1207–1213.
12. Yin L, Wu N, Curtin JC, Qatanani M, Szwergold NR, Reid RA, Waitt GM, Parks DJ, Pearce KH, Wisely GB, Lazar MA. Rev-erb{alpha}, a heme sensor that coordinates metabolic and circadian pathways. *Science* 2007;318:1786–1789. [PubMed: 18006707]
13. Zheng BH, Albrecht U, Kaasik K, Sage M, Lu WQ, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, Lee CC. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell* 2001;105:683–694. [PubMed: 11389837]
14. Kaasik K, Lee CC. Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. *Nature* 2004;430:467–471. [PubMed: 15269772]
15. Nagoshi E, Saini C, Bauer C, Laroche T, Naef F, Schibler U. Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* 2004;119:693–705. [PubMed: 15550250]
16. Balsalobre A, Marcacci L, Schibler U. Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Current Biology* 2000;10:1291–1294. [PubMed: 11069111]
17. Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* 1998;93:929–937. [PubMed: 9635423]
18. Stayrook KR, Bramlett KS, Savkur RS, Ficorilli J, Cook T, Christe ME, Michael LF, Burris TP. Regulation of carbohydrate metabolism by the farnesoid X receptor. *Endocrinology* 2005;146:984–991. [PubMed: 15564327]

19. Savkur RS, Bramlett KS, Michael LF, Burris TP. Regulation of pyruvate dehydrogenase kinase expression by the farnesoid X receptor. *Biochemical and Biophysical Research Communications* 2005;329:391–396. [PubMed: 15721319]
20. Zvonic S, Ptitsyn AA, Conrad SA, Scott LK, Floyd ZE, Kilroy G, Wu XY, Goh BC, Mynatt RL, Gimble JM. Characterization of peripheral circadian clocks in adipose tissues. *Diabetes* 2006;55:962–970. [PubMed: 16567517]
21. Dioum EM, Rutter J, Tuckerman JR, Gonzalez G, Gilles-Gonzalez MA, McKnight SL. NPAS2: a gas-responsive transcription factor. *Science* 2002;298:2385–2387. [PubMed: 12446832]
22. Rutter J, Reick M, Wu LC, McKnight SL. Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science* 2001;293:510–514. [PubMed: 11441146]
23. Staels B. When the clock stops, ticking metabolic syndrome explodes. *Nature Medicine* 2006;12:54–55.
24. Peyer AK, Jung D, Beer M, Gnerre C, Keogh A, Stroka D, Zavolan M, Meyer UA. Regulation of human liver delta-aminolevulinic acid synthase by bile acids. *Hepatology* 2007;46:1960–1970. [PubMed: 17975826]
25. Handschin C, Lin JD, Rhee J, Peyer AK, Chin S, Wu PH, Meyer UA, Spiegelman BM. Nutritional regulation of hepatic heme biosynthesis and porphyria through PGC-1 alpha. *Cell* 2005;122:505–515. [PubMed: 16122419]
26. Kakizaki S, Yamamoto Y, Ueda A, Moore R, Sueyoshi T, Negishi M. Phenobarbital induction of drug/steroid-metabolizing enzymes and nuclear receptor CAR. *Biochimica et Biophysica Acta* 2003;1619:239–242. [PubMed: 12573483]
27. Fraser DJ, Zumsteg A, Meyer UA. Nuclear receptors constitutive androstane receptor and pregnane X receptor activate a drug-responsive enhancer of the murine 5-aminolevulinic acid synthase gene. *Journal of Biological Chemistry* 2003;278:39392–39401. [PubMed: 12881517]

Fig. 1.

Circadian oscillations of *Per2* and *Bmal1* expression in NIH3T3 cells following serum shock. Cells were synchronized with a 50% horse serum shock followed by analysis of *Per2* and *Bmal1* mRNA by qPCR at the times indicated. * $P < 0.05$.

Fig. 2. Circadian oscillations of *Rev-erba* expression and intracellular [Heme] in NIH3T3 cells following serum shock. Cells were synchronized with a 50% horse serum shock followed by analysis of *Rev-erba* mRNA by qPCR and intracellular [Heme] at the times indicated. * $P < 0.05$.

Fig. 3.

Circadian oscillations of *Per2* expression and intracellular [Heme] in NIH3T3 cells following heme shock. Cells were synchronized with a 30 mM hemin shock followed by analysis of *Per2* mRNA by qPCR and intracellular [Heme] at the times indicated. * $P < 0.05$.

Variable Repression Due To Ligand Availability?

Fig. 4.

Model comparing the oscillations in *Rev-erba* and intracellular [Heme] suggesting the possibility that there may be variability in the level of receptor occupancy and thus repressor activity while REV-ERB α is elevated during the circadian rhythm.