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Regulation of CLOCK and MOP4 by Nuclear Hormone Receptors in the Vasculature: A Humoral Mechanism to Reset a Peripheral Clock

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Summary

Circadian clock genes are expressed in the suprachiasmatic nucleus and in peripheral tissues to regulate cyclically physiological processes. Synchronization of peripheral oscillators is thought to involve humoral signals, but the mechanisms by which these are mediated and integrated are poorly understood. We report a hormone-dependent interaction of the nuclear receptors, RARα and RXRα, with CLOCK and MOP4. These interactions negatively regulate CLOCK/MOP4:BMAL1-mediated transcriptional activation of clock gene expression in vascular cells. MOP4 exhibits a robust rhythm in the vasculature, and retinoic acid can phase shift *Per2* mRNA rhythmicity in vivo and in serum-induced smooth muscle cells in vitro, providing a molecular mechanism for hormonal control of clock gene expression. We propose that circadian or periodic availability of nuclear hormones may play a critical role in resetting a peripheral vascular clock.

Introduction

The internal circadian clock is a molecular time-keeping mechanism that generates a biological rhythm, regulating diverse physiological processes such as blood pressure, sleep-wake cycles, and body temperature in mammals. The master pacemaker resides in the suprachiasmatic nucleus (SCN) in mammals (Reppert and Weaver, 2001). The SCN consists of multiple, autonomous single cell circadian oscillators, that are synchronized to fire rhythmically, generating a coordinated, circadian, rhythmic output in intact animals (Welsh et al., 1995; Liu et al., 1997).

Interacting positive and negative transcriptional-translational feedback loops drive circadian oscillations in both *Drosophila* and mammals. The best-characterized feedback loop, in the mouse, involves the regulation of three *Period* genes (mPER1–3) and two *Cryptochrome* genes (mCRY1 and 2) (Reppert and Weaver, 2001). Currently, it is believed that transcription of *mPer* and *mCry* is driven by accumulating CLOCK:BMAL (MOP3) heterodimers, which, in turn bind to consensus E box elements (King et al., 1997; Darlington et al., 1998; Gekakis et al., 1998; Hogenesch et al., 1998; Jin et al., 1999). Subsequently, complexes of mPER and mCRY proteins enter the nucleus, where they shut off CLOCK:BMAL1-mediated transcription. At the same time, mPER2 upregulates levels of *Bmal1* RNA through an as yet uncharacterized mechanism leading to the formation of CLOCK:BMAL1 heterodimers which drive *mPer/mCry* transcription and restart the cycle (Kume et al., 1999; Shearman et al., 2000). MOP4 (also termed NPAS2) shares high homology at the amino acid level with CLOCK (Hogenesch et al., 1997; Zhou et al., 1997) and like CLOCK, forms heterodimers with BMAL1 (Hogenesch et al., 1998), promotes E box activation of genes such as *Per1* and *vasopressin*, and is negatively regulated by CRY1 and CRY2 (Kume et al., 1999). However, the low level of MOP4 expression (Hogenesch et al., 1998) and lack of expression in the SCN of neonatal and adult mice (Shearman et al., 1999) has limited consideration of its involvement in the core circadian feedback loop.

Recently, molecular clocks similar to those operating in SCN neurons have been uncovered in peripheral tissues (Zylka et al., 1998) and in immortalized rat-1 fibroblast cell lines (Balsalobre et al., 1998). In peripheral tissues, such as the liver, kidney, and heart, circadian rhythms in RNA abundance are apparent for each of the *mPer* genes, although the phase of oscillation is delayed 3–9 hr relative to the oscillation in the SCN (Zylka et al., 1998). Clock gene oscillations are lost in SCN-lesioned animals (Sakamoto et al., 1998). Furthermore, gene oscillations dampen more rapidly in cultures of peripheral tissues than SCN cells in vitro, where they are sustained for weeks (Yamazaki et al., 2000). This suggests that the peripheral oscillations may be driven or synchronized by the SCN. It has been suggested that the SCN clock may synchronize peripheral clocks via both neural and hormonal signals (Ishida et al., 1999; Akashi and Nishida, 2000).

Steroid hormones and vitamins regulate diverse developmental and physiological processes by activating intracellular members of the nuclear receptor superfamily, which function as ligand-dependent DNA binding transcription factors (Perlmann and Evans, 1997). Retinoids act through retinoic acid receptors (RAR) and retinoid X receptors (RXR). While RAR binds both all-*trans*-retinoic acid (tRA) and 9-*cis*-retinoic acid (9*cis*RA), RXR only binds 9*cis*RA (Heyman et al., 1992). In contrast to steroid receptors which typically function as homodimers, retinoid X receptors (RXRs) form heterodimers with numerous members of the family including retinoic acid receptors (RARs), thyroid hormone receptors (TRs), and peroxisome proliferator-activated receptor (PPARs). The DNA binding domain of these receptors provides gene specificity, and the ligand binding domain provides the transcriptional regulatory activity in addition to ligand binding specificity (Mangelsdorf and Evans, 1995; Perlmann and Evans, 1997).

Circulating concentrations of some steroids undergo circadian variability (Tronche et al., 1998; Czeisler and Klerman, 1999; Muller, 1999). In addition, recent observations suggest that feeding cycles can entrain periph-

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eral clocks independent of light entrainment (Damiola et al., 2000; Stokkan et al., 2001). While the mechanism remains unknown, examples of hormonal phase-shifting of circadian genes in peripheral organs have begun to emerge (Balsalobre et al., 2000). Together, these observations suggest that steroid hormones and vitamins may serve as candidate regulators of peripheral clocks.

We were interested in addressing the underlying mechanisms which regulate peripheral clocks in the vasculature. Although molecular evidence of a vascular clock was not extant, there is circumstantial evidence that it may exist. For example, blood pressure undergoes a marked circadian variability, which is increased in patients with hypertension (Millar-Craig et al., 1978; Panza et al., 1991) and coincides with a temporal variability in the incidence of acute vascular events, such as myocardial infarction, sudden cardiac death, and stroke (Marshall, 1977; Tsementzis et al., 1985; Ridker et al., 1990). Additional to the variability in blood pressure, we have previously described a circadian variability in the local vascular response to pressor hormones (Hossmann et al., 1980).

Here we demonstrate the presence of a molecular clock in the vasculature and identify a mechanism that would account for synchronization of this clock by circulating humoral signals. We report that the retinoid receptors, RXRα and RARα, can interact with MOP4 and CLOCK, in a hormone-dependent manner. Furthermore, these nuclear hormone receptors inhibit CLOCK:BMAL1 and MOP4:BMAL1 heterodimer-mediated expression of circadian responsive genes. Finally, we show that MOP4 displays a robust cyclical expression pattern in the vasculature and demonstrate that retinoic acid can phase-shift *Per2* oscillations, suggesting a role for nuclear receptor-mediated humoral signaling in the regulation of a peripheral clock in the vasculature.

Results

Identification of MOP4 and CLOCK as Retinoid Receptor Interacting Proteins

We identified the bHLH-PAS protein, MOP4, as an RXRα interacting clone using the ligand binding domain of hRXRα as a bait in a two-hybrid screen of a yeast Gal4 activation domain (AD) fusion cDNA library from human aortic vascular smooth muscle cells (Figure 1A). We also tested RXRα and RARα for possible interactions with full-length cDNAs for MOP4, CLOCK, and BMAL1 because of the high homology shared by MOP4 and CLOCK and the fact that both proteins can heterodimerize with BMAL1. Both RXRα and RARα interacted strongly with CLOCK and MOP4, but not their heterodimeric partner, BMAL1, indicating the specificity of the interaction. Similarly, no interaction was observed between MOP4, CLOCK, or BMAL1 and other nuclear receptor constructs which were analyzed (Figure 1A).

A liquid β-galactosidase activity assay was utilized to assess the effect of ligands on these novel interactions. While RXRα and RARα alone interacted with CLOCK and MOP4, the strength of these interactions was increased up to 15-fold in the presence of ligand (Figure 1B). Consistent with the yeast two-hybrid results, CLOCK and MOP4 interaction with VP16 fusions of RXRα and RARα was observed in mammalian cells (Figure 1C). No interactions with other nuclear receptors analyzed in the presence or absence of ligand was observed (Figures 1A and 1C), although they were functionally expressed (Figure 1D and data not shown). Together these results suggest that RXRα and RARα interact with MOP4 and CLOCK in intact yeast and mammalian cells and that these interactions are stimulated by the presence of cognate nuclear receptor ligands.

In agreement with cell-based assays (Figure 1), in vitro binding assays also showed nuclear receptor ligands stimulated the interaction of both MOP4 and CLOCK but not BMAL1, with GST-RXRα and GST-RARα-glutathione sepharose affinity matrices (Figure 2A). To further investigate the role of ligand in the interaction we utilized C-terminally truncated AF2 mutants RXRΔ443 and RARΔ404 (Schulman et al., 1996), which are defective in ligand-dependent function, in the interaction assays. Consistent with the ability of ligand to enhance complex formation, these mutant receptors were unable to form complexes with CLOCK and MOP4 (Figure 2B).

The ability of BMAL1 to heterodimerize with both MOP4 and CLOCK prompted us to address what effect the presence of BMAL1 would have on their interaction with nuclear receptors. While BMAL1 does not bind to RXRα, it did not significantly diminish the ability of MOP4 to interact with RXRα (Figure 2C, lanes 4 and 5). The addition of unlabeled MOP4 to the BMAL1-GSTRXRα binding reaction facilitated retention of BMAL1, suggesting that MOP4, BMAL1, and RXRα may exist as a trimeric complex in vitro (Figure 2C, lane 6). Even at limiting amounts of MOP4, the presence of BMAL1 did not prevent its association with GSTRXRα (Figure 2D). Thus, BMAL1 does not significantly alter the affinity of MOP4 for RXRα. Similar results were also obtained for CLOCK (data not shown). Additionally, incubation of anti-CLOCK or anti-RAR antibodies with in vitro translated CLOCK and RARα led to the coimmunoprecipiation of CLOCK and RARα in a ligand-enhanced manner (Figure 2E, panels I and II). Furthermore, in vivo ligand-dependent association between CLOCK and RARα was detected in cell lysates from NIH3T3 cells transiently cotransfected with expression plasmids encoding hCLOCK and hRARα (Figure 2E, panels III and IV). Together these results demonstrate that either CLOCK or MOP4, free or in its dimeric complex with BMAL1, can associate with the ligand binding domain (LBD) of nuclear receptors in vitro and in vivo in a ligand-enhanced manner.

Mapping of the Nuclear Receptor Interaction Domains of CLOCK and MOP4

We generated the N- and C-terminal truncation mutants of both CLOCK and MOP4 as Gal4BD fusions (Figure 3A) and used them in mammalian two-hybrid assays to map broadly the nuclear receptor interaction domain (NRID). While the N-terminal bHLH-PAS domain, which interacts with BMAL1 (3A, lane 2, panels I and II), showed no interaction, a very strong interaction between the C-terminal region (350–824) of MOP4 or CLOCK (370–846) with RXRα and RARα was observed (panels I and II, lanes 7 and 8). These results localize the NRIDs to the C termini of MOP4 and CLOCK. For finer mapping, serial

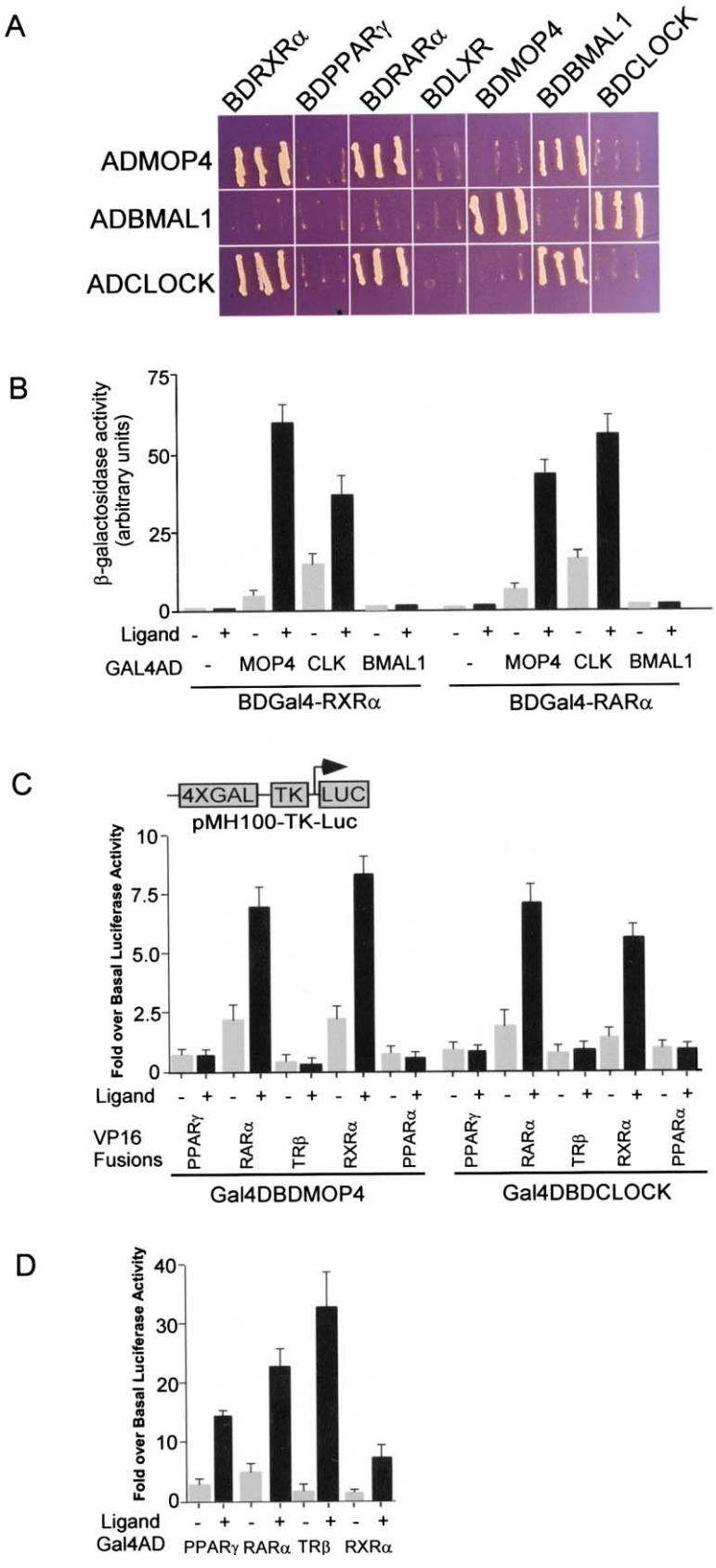

Figure 1. Identification of MOP4 and CLOCK as Retinoid Receptor Interacting Proteins

(A) MOP4 and CLOCK interact with RXRα and RARα in yeast. Three independent YRG2 clones containing Gal4AD fusions of MOP4, CLOCK, or BMAL1 transformed with Gal4BD fusions of RXRα, PPARγ, RARα, LXR, MOP4, CLOCK, or BMAL1 were patched onto minimal media lacking Trp, Leu, and His and assessed for their ability to grow.

(B) Retinoid receptor ligands increase the interaction of MOP4 and CLOCK with RXRα and RARα in YRG2 cells. Liquid β-galactosidase assays of YRG2 clones containing Gal4BD fusions of RXRα or RARα ligand binding domains and Gal4AD fusions of MOP4, CLK, or BMAL1 are shown. All-trans retinoic acid (tRA, 1 μM), in the case of RARα, and 9-*cis*-retinoic acid (9*cis*RA, 1 μM), in the case of RXRα, were added where indicated.

(C) CLOCK and MOP4 interact with RXRα and RARα in a ligand-dependent fashion in mammalian cells. CV-1 cells were transiently transfected with the reporter construct pMH100-TK-LUC, and various DNAs as shown. The ligands referred to in (C) are 15dPGJ$_2$, tRA, 9*cis*RA, T3 (1 μM), and ETYA (10 μM) for PPARγ, RARα, RXRα, TRβ, and PPARα, respectively.

(D) PPARγ, TRβ, RXRα, and RARα fused to the Gal4 DNA binding domain are all transcriptionally active. CV-1 cells were transiently transfected with the reporter construct pMH100-TK-LUC, and various DNAs and grown as indicated.

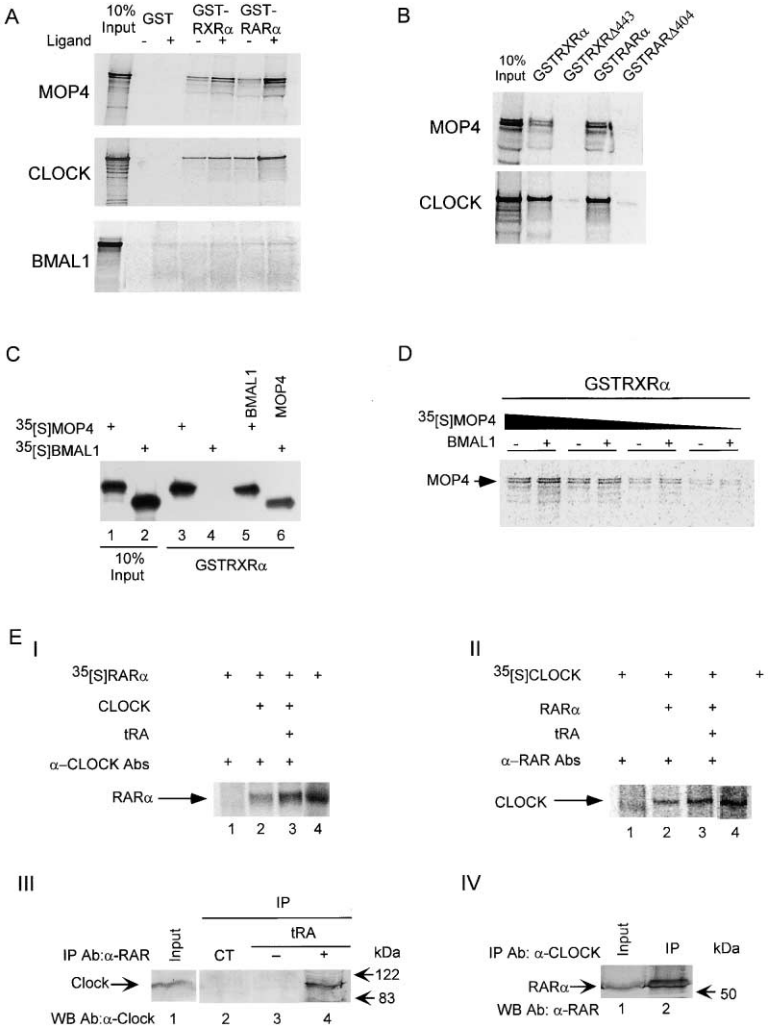

Figure 2. Characterization of MOP4/CLOCK: RXRα/RARα Interaction In Vitro

(A) MOP4 and CLOCK interact with RXRα and RARα in a ligand-enhanced fashion. In vitro translated, ^{35}[S]methionine-labeled MOP4, CLOCK, and BMAL1 were incubated with glutathione sepharose bead bound GST, GSTRXRα, or GSTRARα ± tRA or 9*cis*RA (1 μM). Reaction products were separated by SDS-PAGE and analyzed by phosphorimager. (B) AF2 mutants of RXRα and RARα are unable to bind MOP4 or CLOCK. ^{35}[S]MOP4 and ^{35}[S]CLOCK were incubated with the indicated GSTRXR and RAR constructs. (C) MOP4, BMAL1 and RXRα can exist in a trimeric complex. ^{35}[S]MOP4 or ^{35}[S]BMAL1 (lanes 3 and 4), ^{35}[S]MOP4 and unlabeled BMAL1 (lane 5) and ^{35}[S]BMAL1 and MOP4 (lane 6) were incubated with GSTRXRαLBD. (D) BMAL1 does not alter the affinity of MOP4 for RXRα. Decreasing concentrations of ^{35}[S]MOP4 ± BMAL1 were incubated with GSTRXRαLBD. All of the reactions in (B), (C), and (D) were carried out in the presence of 9*cis*RA (1 μM) in the case of RXRα or tRA (1 μM) in the case of RARα, and all reaction products were separated by SDS-PAGE and analyzed by phosphorimager. (E) CLOCK interacts with RARα in vitro and in vivo. ^{35}[S]RARα (panel I) or ^{35}[S]-CLOCK (panel II) were incubated without (lane 1) or with (lanes 2 and 3) CLOCK and RARα proteins, respectively, immunoprecipitated, and probed with anti-CLOCK or anti-RAR antibodies as indicated. Lane 3 in both panels I and II indicates ligand treatment. Lane 4 of each panel shows 10% input. In panels III and IV, cells were cotransfected with CMXRARα and CMXCLOCK and incubated for 48 hr. Cells were incubated with (panels III and IV) or without (panel III) tRA (1 μM). Cell lysates were DTSSP-cross-linked, immunoprecipitated, and Western blotted with anti-RAR, anti-CLOCK or control antibodies (CT) as indicated. Input refers to lysates from the transfected cells which were directly probed with anti-CLOCK antibodies (panel III, lane 1) or anti-RAR antibodies (panel IV, lane 1).

C-terminal deletion mutants of MOP4 were generated (Figure 3B, panel I), in vitro labeled, and analyzed for their ability to interact with nuclear receptors by GST pulldown assays. MOP4Δ727, but not MOP4Δ624 was retained on sepharose beads coupled to a GST fusion of the ligand binding domain of RXRα (Figure 3B, panel II). Deletion of aa 665–680 severely reduced the ability of MOP4 to interact with RXRα (Figure 3B, panel III, lane 3). Together, these assays localized the region of MOP4 necessary for interaction to aa 665–680. Similar analysis localized the region of CLOCK necessary for NR interaction to aa 370–509 (Figure 3C, panels I and II).

The region of MOP4 that bound to RXRα contained an LxxLL motif (aa 670–675). LxxLL motifs are present in nuclear receptor cofactors and in the LBDs of some nuclear receptors and are necessary for their interactions (Glass and Rosenfeld, 2000; Westin et al., 2000). We mutated the conserved leucines to alanines in the LxxLL motif of MOP4 to determine its role in nuclear receptor associations. Mutating these three leucines to alanines also prevented MOP4 from associating with GSTRXRα, suggesting a novel role for LxxLL motifs of MOP4 in modulating MOP4-RAR/RXR interactions (Figure 3B, panel III, lane 2).

Ligand-Bound Nuclear Receptors Block E Box Binding by CLOCK:BMAL1 and MOP4:BMAL1 Heterodimers

In *Drosophila* the interaction of CLOCK and CYCLE (*Drosopohila* homolog of BMAL1) with negative regulators PER and TIM does not disrupt the CLOCK/CYCLE heterodimer, but prevents it from binding to an E box (Lee et al., 1999). We performed EMSA analyses to determine how interaction with nuclear receptors might influence the ability of the heterodimer to bind to an E box element. The assembly of the bHLH-PAS heterodimer on the E box consensus sequence (Figures 4A and 4B, lanes 5 and 4 respectively) was severely inhibited in the presence of RXRα or RARα and their cognate ligands (Figures 4A, lanes 6–7, 4B, lanes 5–6, and 4C, compare lanes 2 and 3). These results indicate that interaction of liganded RXRα or RARα with the MOP4/BMAL1 or the CLOCK/BMAL1 heterodimer reduces their ability to bind DNA (Figure 4D).

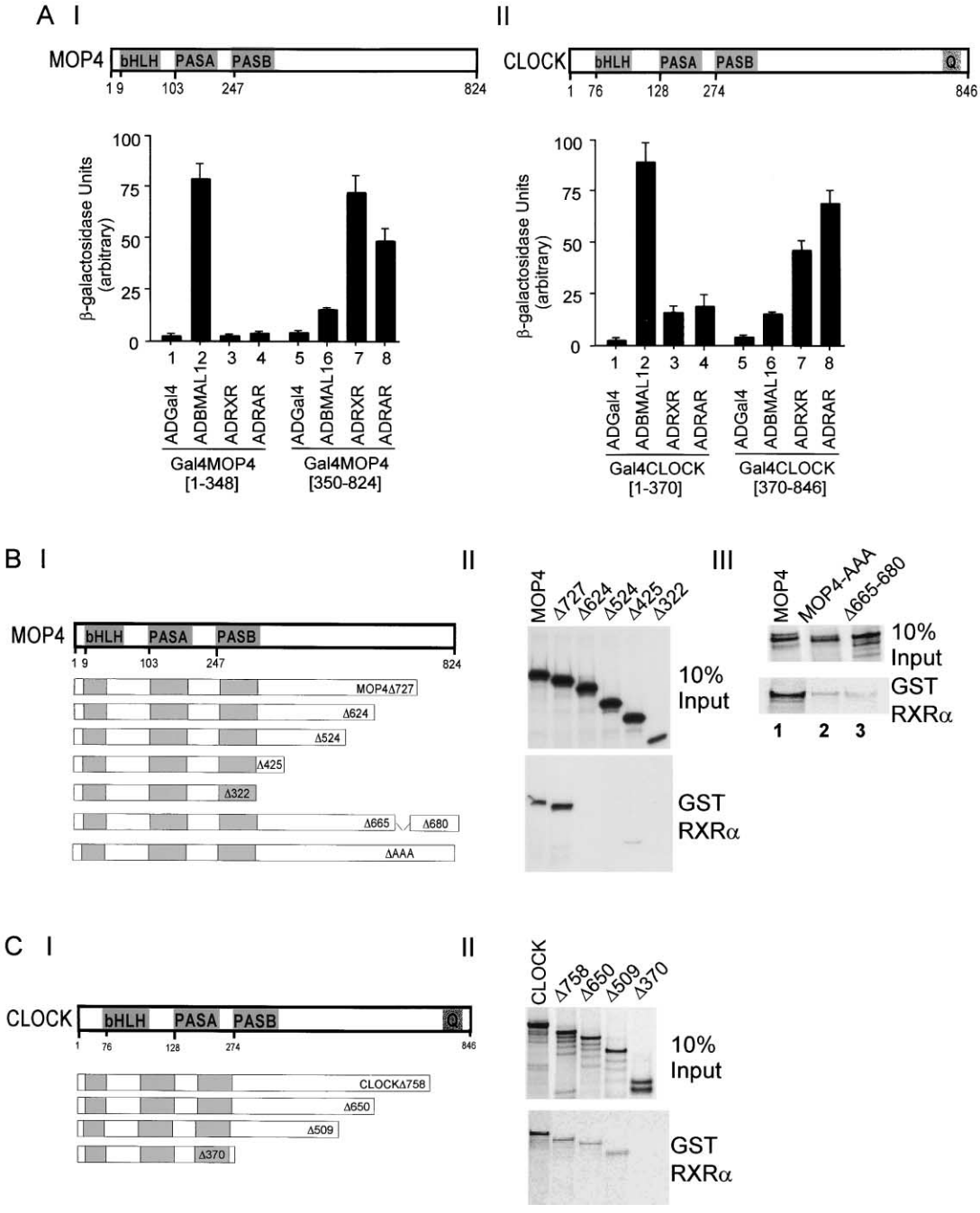

Figure 3. Mapping the Nuclear Receptor Interaction Domains of MOP4 and CLOCK

(A) C-terminal regions of MOP4 (panel I) and CLOCK (panel II) interact with RXRα and RARα. Liquid β-galactosidase assays were performed with YRG2 cells expressing MOP4, CLOCK, BMAL1, RXRα, or RARα as indicated. (B) NR interaction domain of MOP4 maps to aa 665–680. Panel I illustrates the regions of MOP4 which correspond to the deletion mutants. In vitro translated MOP4, full-length and deletion mutants (panel II and III) were incubated with GSTRXRα as indicated. (C) Panel I illustrates the regions of CLOCK which correspond to the C-terminal deletion mutants. In panel II, full-length CLOCK and CLOCK truncation mutants were incubated with GSTRXRαLBD as indicated. In (B) and (C), all reactions were carried out in the presence of 9*cis*RA (1μM), separated by SDS-PAGE, and analyzed by phosphorimager.

RXRα and RARα Block CLOCK/MOP4:BMAL1-Induced Transcription in NIH3T3 Cells

The effect of RXRα and RARα on E box binding of MOP4 and CLOCK is consistent with a role for nuclear receptors in the negative limb of the circadian feedback loop. We examined this hypothesis directly by analyzing the effect of overexpression of the retinoid receptors on E box-induced gene transcription in intact cells. Both MOP4:BMAL1 and CLOCK:BMAL1 activate transcription via an E box-dependent mechanism (Kume et al., 1999). Importantly, consistent with previous interaction specificity assays (Figure 1), overexpression of RXRα and RARα, but not PPARγ or TRβ, inhibited MOP4- and CLOCK-mediated luciferase reporter gene activation in

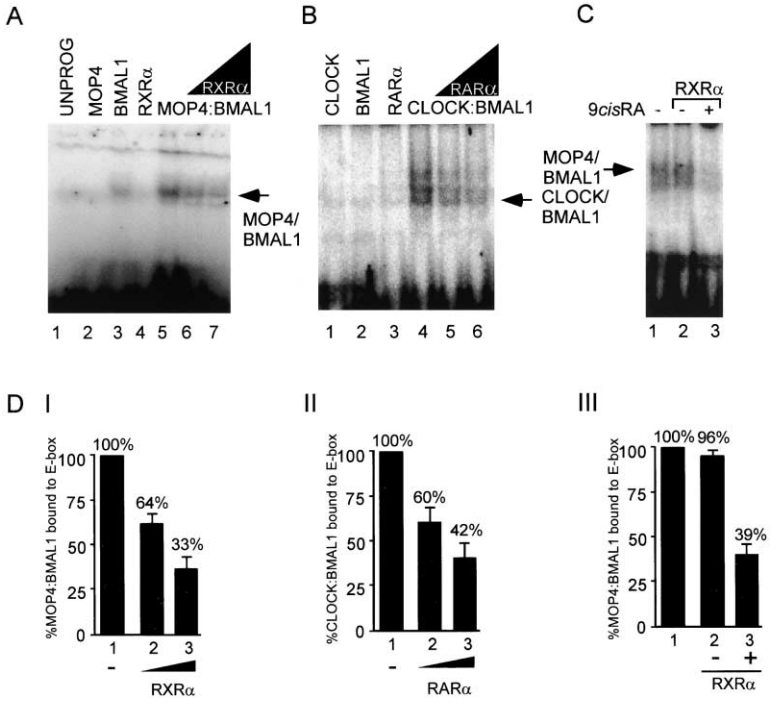

Figure 4. Ligand-Bound RXRα and RARα Prevent MOP4/CLOCK:BMAL1 Heterodimers from Binding to a Consensus E Box Element

(A and B) Interaction of MOP4:BMAL1 or CLOCK:BMAL1 with a radiolabeled E box element is inhibited in the presence of ligand-bound RXRα or RARα, respectively. EMSA analysis was performed using the radiolabeled consensus E box response element and unprogrammed lysate, or in vitro translated, unlabeled proteins as indicated. Binding of MOP4:BMAL1 or CLOCK:BMAL1 dimers to the E box was assayed in the presence of increasing concentrations of RXRα (A, lanes 6 and 7) or RARα (B, lanes 5 and 6) along with their respective ligands, 9*cis*RA, and tRA (both at 1 μM).

(C) Inhibition of the MOP4:BMAL1 heterodimer/E box interaction by RXRα is a ligand-dependent event. EMSA analysis was performed using the radiolabeled consensus E box response element and the MOP4:BMAL1 heterodimer. RXRα was included in the binding reaction − (lane 2) and + (lane 3) 9*cis*RA (1 μM).

(D) Results from each of three separate experiments from (A), (B), and (C) were quantitated by phosphorimager analysis and ImageQuant software. The inhibition of the MOP4/CLOCK:BMAL1 heterodimer binding to the radiolabeled E box by ligand-bound RXRα or RARα was calculated and is shown in panels I–III and expressed as % inhibition ± SEM.

a dose- and ligand-dependent fashion (Figures 5A, 5B, 5C, and 5D). Finally, we analyzed the effects of RXRα on the ability of mutant forms of MOP4 to activate reporter gene expression (Figure 5E). As expected, MOP4Δ727 and MOP4Δ665-680 retained the ability to activate fully the reporter in the presence of BMAL1 (Figure 5E, lanes 3 and 7), while MOP4Δ624 and MOPΔAAA were roughly 30% less effective (Figure 5E, lanes 5 and 9). MOP4Δ727 remained susceptible to inhibition by RXRα, which is consistent with this mutant having an intact NRID (Figure 5E, lane 4). In contrast, MOP4Δ624, MOP4Δ665-680 and MOP4ΔAAA, which showed significantly reduced interaction with RARα and RXRα, were significantly less sensitive to nuclear receptor-mediated inhibition (Figure 5E, lanes 6, 8, and 10 respectively). Such diminution of the inhibitory effects of RXRα, in the absence of the NR interaction domain in MOP4, are consistent with the GST-pulldown assay results and indicate that the carboxyl terminus of MOP4 is necessary for its interaction with nuclear receptors and for nuclear receptor-mediated inhibition (Figure 3B).

Clock Genes Are Expressed and Cycle in the Vasculature

Previous reports have demonstrated the expression of most of the subtypes of RXRα and RARα in vascular smooth muscle cells (Miano et al., 1996). Strong signals for *Clock*, *Mop4*, *Bmal1*, and *Cry2* were detected in vascular smooth muscle cells (Figure 6A). *Mop4* was present in brain tissue and in spinal chord as previously described (Zhou et al., 1997). *Bmal1* expression has been described to cycle in rat central and peripheral organs (Oishi et al., 1998), while *Clock* mRNA reportedly does not display a robust rhythm in the SCN of mice (Shearman et al., 1999). Ribonuclease protection analysis was performed after serum shock to determine whether *Mop4* expression fluctuates rhythmically in vascular smooth muscle cells (Balsalobre et al., 1998). *Mop4* mRNA expression cycled in a circadian pattern with peaks at 28 and 52 hr in VSMC, consistent with it having a putative role in a vascular clock (Figure 6B, panels I and II). However, in agreement with a previous report (Shearman et al., 1999), *Clock* did not display a rhythmic expression profile (Figure 6B, panel II).

After establishing that *Mop4* cycles in hVSMC in culture following serum shock, we next examined whether such an oscillation occurs in VSM in the whole animal. Aortae were harvested at 4 hr intervals over a 48 hr period from balb/c mice maintained either in 12 hr:12 hr light:dark (LD) cycles or in constant darkness (DD). RNA was extracted from these aortae and analyzed for the temporal expression profile of *Mop4*. *Mop4* mRNA was observed to exhibit a robust cyclical expression pattern under both LD and DD conditions (Figure 6C, panels I and III). *Per2* expression was also observed to cycle rhythmically under both conditions (Figure 6C, panels I and III) but in a pattern antiphase to that of *Mop4*. The temporal expression patterns of the clock genes *Bmal1*, *Clock, Cry1* were also analyzed under DD conditions and mRNA cycling was observed for *Bmal1* and *Cry1* but not *Clock* (Figure 6C, panels III and IV). *Mop4* and *Bmal1* mRNA cycled with a similar phase, both peaking 8 hr before *Per2*, which is consistent with the observation that MOP4:BMAL1 heterodimers drive expression of *Per2* mRNA (Figure 6C, panels II and IV). Together these results suggest the existence of a vascu-

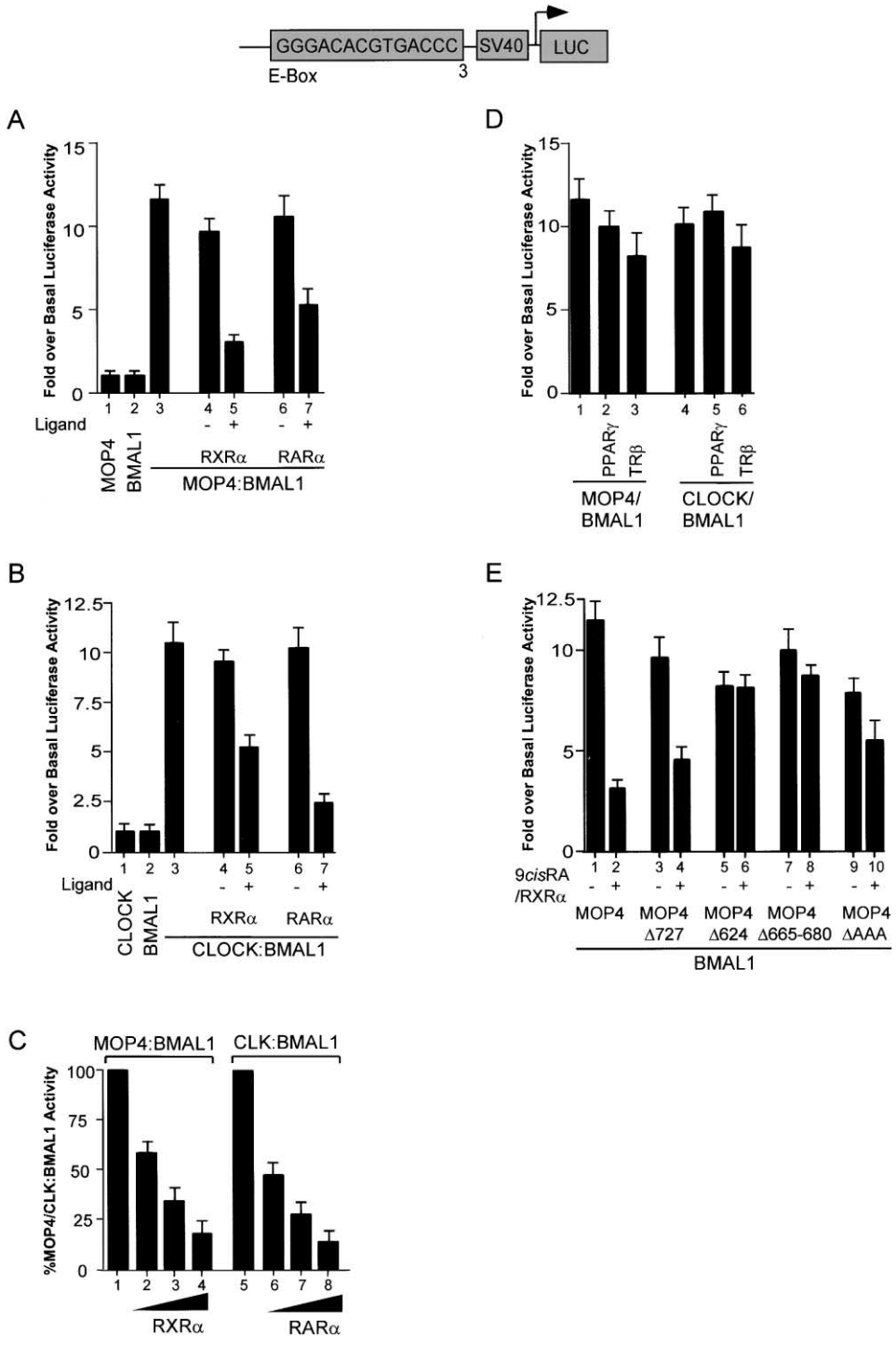

Figure 5. Overexpression of RXRα and RARα Inhibits CLOCK/MOP4:BMAL1-Dependent E Box-Mediated Transcription in NIH3T3 Cells

(A and B) MOP4:BMAL1 and CLOCK:BMAL1 mediated E box transactivation is inhibited by RXRα and RARα. NIH3T3 cells were transfected with pGL3M34-Luc, CMXBMAL1 and either CMXMOP4 or CMXCLOCK alone (A and B, lanes 1 and 2) or together (lane 3) and cotransfected with CMXRXRα (A and B, lanes 4 and 5) or CMXRARα (A and B, lanes 6 and 7).

(C) RXRα and RARα inhibition of CLOCK and MOP4 E box transactivation is dose dependent. NIH3T3 cells were transfected as described in (A) and (B) except that increasing concentrations of RXRα (lanes 2–4) or RARα (lanes 6–8) were introduced and incubated in the presence of ligand. MOP4/CLOCK:BMAL1 activity was normalized relative to the reporter activity (100%) of MOP4:BMAL1 and CLOCK:BMAL1 in the absence of cotransfected nuclear receptor (lanes 1 and 5).

(D) PPARγ and TRβ do not block MOP4/CLOCK:BMAL1-mediated E box transactivation. Cells were transfected with pGL3M34-Luc, CMXBMAL1, and either CMXMOP4 or CMXCLOCK (lanes 1 and 4) alone or cotransfected with CMXPPARγ (lanes 2 and 5) or CMXTRβ (lanes 3 and 6). Following transfection, cells were incubated in the presence of 15dPGJ$_2$ or T3 (1 μM).

(E) MOP4 NRID mutants are not sensitive to inhibition by ligand-bound RXRα. NIH3T3 cells were cotransfected with CMXBMAL1 and CMXMOP4 or various MOP4 deletion mutants in the presence or absence of CMXRXRα. Samples cotransfected with CMXRXRα were treated with 9cisRA (1 μM).

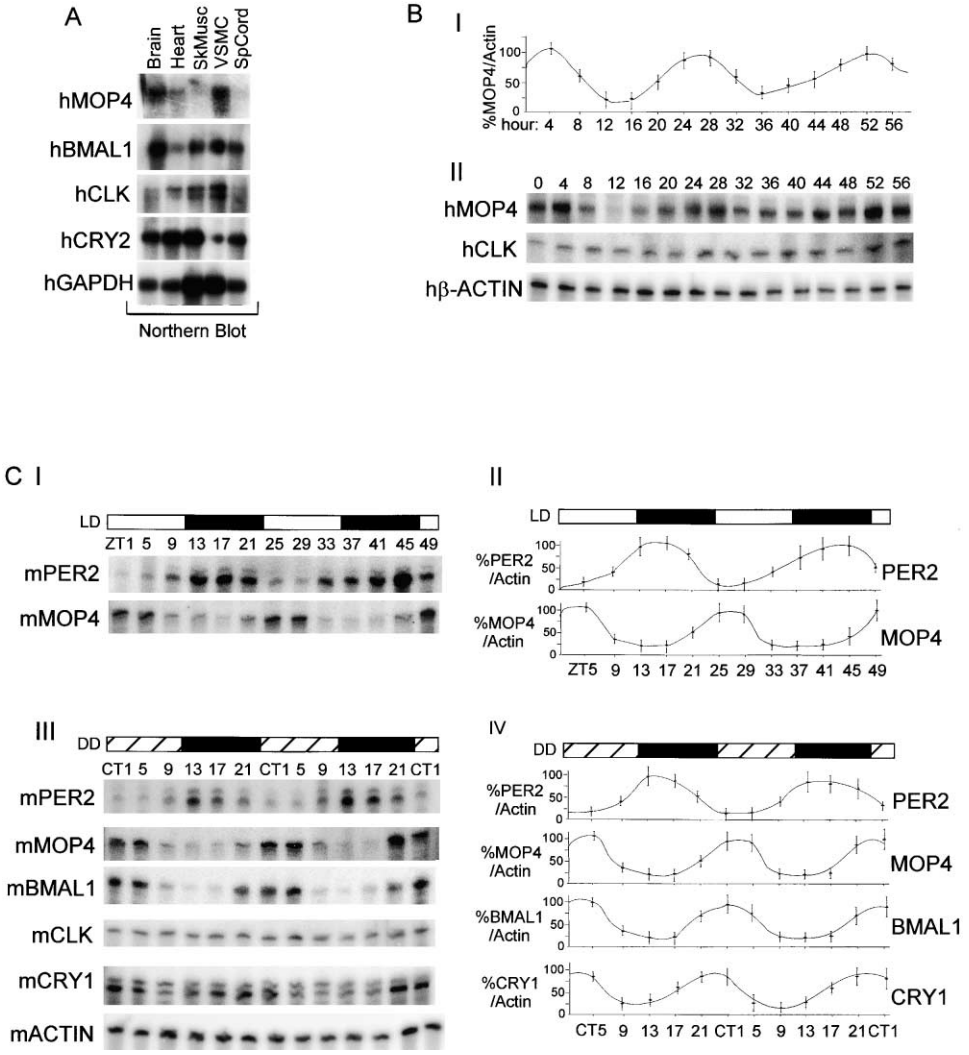

Figure 6. Analysis of Clock Gene Expression in the Vasculature

(A) Clock genes are expressed in VSMC. Northern blot of human brain, heart, skeletal muscle, vascular smooth muscle cell (VSMC), and spinal chord poly[A^+] RNA (3 μg) was probed with hMOP4, hBMAL1, hCRY2, hCLOCK, hGAPDH as indicated. Autoradiograph images were digitized using Adobe Photoshop® scanning software.

(B) RPA of hVSMC mRNA following serum shock. The temporal expression profile of *hMop4* mRNA after serum shock is represented in panel I and expressed as *Mop4/Actin* mRNA signal ratios on the *y* axis normalized by using the level of *hMop4* 4 hr after serum shock as 100%. Panel II shows hVSMC RPA gels analyzed for *hMop4*, *hClock*, and *hβ-Actin* expression.

(C) *mMop4* cycles in vivo in the vasculature. Aortae harvested over 48 hr from balb/c mice maintained in a 12 hr:12 hr LD cycle (panel I) and from mice in their 2nd and 3rd day of constant darkness (panel III) were analyzed for temporal expression of *mMop4, mPer2, mBmal1, mClock, mCry1, mβ-Actin*. Zeitgeber time (ZT) denotes the entrainment regime with ZT0 = "lights on" and ZT12 = "lights off." The corresponding time in constant darkness is denoted by circadian time (CT) in panel III. Results from RPA analysis of clock gene expression from three separate animals were quantitated using ImageQuant software and plotted as *target/Actin* mRNA signal ratios (panels II and IV) normalized using peak levels as 100%.

lar clock and provide in vivo evidence which place MOP4 in the core clock feedback loop in the vasculature.

Retinoic Acid Phase Shifts PER2 mRNA Rhythms

Liganded nuclear receptors interact with CLOCK and MOP4 to prevent their E box-mediated gene activation. This suggests that circadian variability or periodic availability in steroid hormones and vitamins, such as retinoids, might phase shift or reset a peripheral clock in the vasculature. We first determined whether oscillating clock gene expression could be induced by serum shock in hVSMC as has been reported in Rat1 and NIH3T3 fibroblasts (Balsalobre et al., 1998; Akashi and Nishida, 2000). Following addition of 50% serum for 2 hr, *hPer2* mRNA was induced very rapidly at T4 (4 hr after serum shock) and entered a cyclical expression pattern, peaking at T24 and T48 (Figure 7A, panels I and II). All-trans-retinoic acid (tRA) was added to the serum induced cells at four different time points, T6, T12, T18, and T24 for 2 hr intervals. Treatment at T12 delayed the *hPer2* mRNA from T24 to T28 (Figure 7A, panels I and IIc). tRA treatment at T6 and T24, however, had no effect on the *hPer2*

peak (Figure 7A, panels I, IIb, and IIe). The effect of tRA when administered at T18 was similar to treatment at T12; the *hPer2* peak was phase-shifted by 4 hr and peaked at T28. Consistent with a 4 hr shift, the second cycle peaked at T52 (Figure 7A, panels I and IId). Thus, tRA phase delayed the *hPer2* oscillation by 4 hr, which is consistent with the observation that ligand bound RXRα/RARα prevents the MOP4/CLK:BMAL1 heterodimer from activating transcription through its E box. Removal of the ligand restored the ability of the MOP4/CLK:BMAL1 heterodimer to induce *Per2* expression, but the phase of the cycle was delayed.

To address the specificity of this *Per2* phase delaying effect by retinoic acid, the effect of other nuclear hormone receptor ligands on *hPer2* oscillations was subsequently examined. The synthetic glucocorticoid, dexamethasone, which has been reported to phase shift *mPer2* rhythms in the liver (Balsalobre et al., 2000), was added at T18. Dexamethasone treatment phase advanced the *hPer2* peak to T20 resulting in the second peak the following day at T44 rather than T48 (Figure 7B, panels I and IIb), which is consistent with the response observed by Schibler and colleagues (Balsalobre et al., 2000), but suggests that the dexamethasone/GR effect on resetting the liver clock may occur by a different mechanism. T3 and $15dPGJ_2$, which are ligands for TRβ and PPARγ respectively, had no significant effect on the phase of the *hPer2* oscillation (Figure 7B, panels I, IIc, and IId), which is consistent with our in vitro interaction and transcriptional activation data (Figures 1 and 5).

We performed the tRA phase shift experiment in the presence of cyclohexamide (Figure 7B, panel I), to determine whether the effect of ligand-bound RXRα and RARα on MOP4/CLOCK function is direct and independent of protein synthesis. VSMC were pretreated with cyclohexamide at T17, 1 hr before treatment with tRA. Analysis of the *hPer2* oscillation demonstrated that cyclohexamide did not prevent tRA from phase delaying the *hPer2* oscillation to T28 (Figure 7B, panel I). Cyclohexamide treatment alone had a minimal effect on the vehicle-treated *hPer2* oscillation, slightly delaying the drop of the *hPer2* peak, which has been previously reported (Balsalobre et al., 1998).

Finally, mice maintained in constant darkness were injected intraperitoneally with tRA to address whether it could delay *mPer2* oscillations in vivo. tRA dissolved in DMSO or DMSO alone was injected at circadian time (CT) 9, and hearts and aortae were harvested at CT9, CT12, CT15, CT18, and CT21. RPA analysis of *mPer2* levels in retinoic acid-treated animals compared to vehicle- treated animals revealed that the *mPer2* peak was delayed in both the aorta and heart by 3 hr upon tRA treatment (Figure 7C).

Discussion

We report a mechanism whereby peripheral circadian oscillators may be regulated by humoral factors. This involves a novel, ligand-dependent interaction between the retinoid receptors, RXRα and RARα, and the bHLH-PAS circadian transcription factors, CLOCK and MOP4, which directly affects clock function. This mechanism pertains to a peripheral vascular clock, which is of potential importance in both vascular physiology and clinical vascular events.

A novel finding in this study is that MOP4 is an intrinsic component of the clock system, at least in the vasculature. The expression of MOP4 is reportedly absent in the SCN (Shearman et al., 1999), and there are no reports of its cyclical expression, making it difficult to place MOP4 in the core oscillatory loop. Deletion of the *MOP4* gene results in deficits in long-term memory of the cued and contextual fear task (Garcia et al., 2000); this is interesting, considering reports that conditioned fear may modulate circadian rhythms (Amir and Stewart, 1998). We now report that MOP4 is strongly expressed in human vascular smooth muscle cells and in the murine vasculature. MOP4 cycles in the vasculature in vivo under light/dark conditions and in constant darkness, and cycles in vascular smooth muscle cells in vitro after serum shock. We show that both MOP4 and CLOCK are sensitive to nuclear receptor-mediated inhibition. These observations place MOP4 in the peripheral circadian feedback loop.

The physiological relevance of nuclear receptor-mediated inhibition of CLOCK and MOP4 transcriptional gene activation comes from the demonstration that both serum-induced and in vivo cycling of *Per2* can be phase shifted by a nuclear receptor ligand, depending on the time of hormone treatment. Based on our results, we propose that RXRα and RARα play novel roles in the negative limb of the circadian feedback loop by binding to CLOCK and MOP4 and preventing their ability to drive E box-mediated *Per* transcription. It remains to be determined whether this effect is restricted to the periphery or is also operant in the brain. Involvement of MOP4 in this peripheral oscillator, but not in the SCN clock, illustrates that some components of the feedback loop exhibit tissue-specific expression, perhaps facilitating site-specific responses to humoral stimuli. Dexamethasone, which has been recently reported to phase shift the *mPer* rhythm in peripheral organs (Balsalobre et al., 2000), phase advances *hPer2* in our system, whereas tRA phase delays the *Per2* oscillation. These results suggest that glucocorticoids and retinoids may have opposing effects and therefore may utilize different mechanisms to reset peripheral clock oscillations.

Previous observations have hinted at a circadian role for retinoid receptors and vitamin A. For example, targeted disruption of the retinoid-related orphan receptor, RORβ, extends the period length (tau) of the free-running activity rhythm in mice and mildly affects circadian rhythmicity (Andre et al., 1998). Additionally the interphotoreceptor retinol binding protein (IRBP) has been shown to undergo circadian variation in zebrafish (Rajendran et al., 1996), and recently murine cellular retinol binding protein (mCRBP1), whose function is essential for vitamin A homeostasis, was observed to be a clock-controlled gene in the liver (Zheng et al., 2001). Interestingly, RBP, a retinoid binding protein is a member of the lipocalin protein family which resembles the novel *Drosophila takeout* (TO) gene superfamily, at least one member of which is controlled by the clock and affects feeding behavior (Sarov-Blat et al., 2000). Some of these observations indicate a role for retinoids, not only in peripheral circadian physiology, but also in functions that may be directly controlled by the brain. Our work

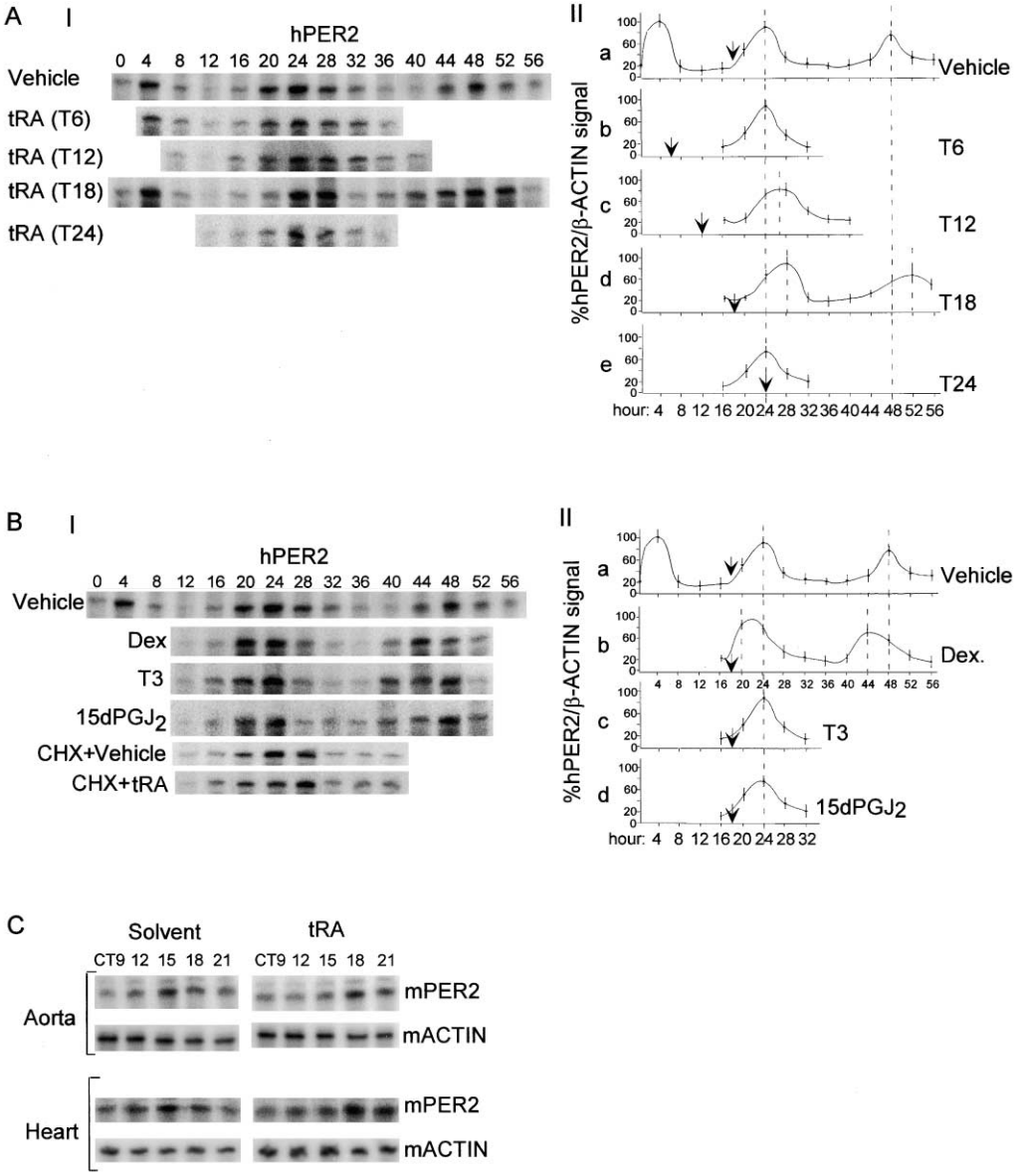

Figure 7. Hormonal Resetting of the Peripheral Clock

(A) Retinoic acid induces a phase delay in the *hPer2* rhythm. RPA of *hPer2* mRNA in serum-induced hVSMC treated with either vehicle at T18, or tRA (1 μM) at T6, T12, T18, and T24. Panel I shows representative scanned gels analyzed for *hPer2* expression after each treatment. Panels IIa, b, c, d, and e represent vehicle treatment at T18 and tRA treatment at T6, T12, T18, and T24, respectively.

(B) The effect of treatment with dex, $15dPGJ_2$, T3, and CHX/tRA on the *hPer2* rhythm. RPA analysis of *hPer2* mRNA in serum-induced hVSMC treated with either vehicle, dexamethasone (0.1 μM), T3 (1 μM), $15dPGJ_2$ (1 μM) at T18, or cyclohexamide (CHX) at T17 ± tRA (1 μM) treatment at T18. Panel I shows representative scanned gels analyzed for *hPer2* expression after each treatment. Panels IIa, b, c, and d represent vehicle, Dex, T3, and $15dPGJ_2$ treatment at T18, respectively. The relative levels of *hPer2* transcripts for treatments shown in (A) and (B), panel II are calculated as *hPer2/Actin* mRNA signal ratios on the *y* axis of each graph using level of *hPer2* 4 hr after serum shock as 100%.

(C) tRA phase delays the *hPer2* rhythm in vivo. Aortae and heart harvested over 12 hr from c57Bl/6 mice maintained in constant darkness which were intraperitoneally injected at CT9 with tRA (10 mg/kg) dissolved in DMSO or DMSO alone were analyzed for temporal expression of *mPer2* and *mβ-Actin.* Representative scanned gels analyzed for *mPer2 and mActin* expression in aorta and heart after each treatment are shown.

provides a molecular framework implicating a role for circulating steroid hormones and vitamins in vascular smooth muscle peripheral circadian physiology.

The mammalian circadian system is organized such that self-sustained oscillators in the SCN entrain peripheral oscillators by releasing a continuous stream of rhythmic signals (Yamazaki et al., 2000). The phase and amplitude of peripheral clocks vary between different tissues and organs, in addition to differing from the phase and amplitude in the SCN. One of the functions of a peripheral clock might be to regenerate a weak or dampened SCN signal, thus amplifying the oscillation

of the signal in that peripheral tissue. A vascular clock may generate an amplified and synchronized vascular rhythm in response to uncoordinated blood-borne signals from central and peripheral clocks. These signals may include hemodynamic stress, the diurnal release of catecholamines and steroids, or the periodic bioavailability of signals, such as vitamins, associated with feeding and metabolic cycling (Damiola et al., 2000; Stokkan et al., 2001).

The existence of multiple oscillators is a common characteristic of all circadian systems so far described in multicellular organisms. Peripheral oscillators permit tuning of biological rhythms without difficulty to small, gradual changes in the phase of the input signal. Distorted environmental cues such as transatlantic air travel, the changeover from a day to a night work schedule, or a large hormonal release such as sympathoadrenal activation under conditions of severe stress may abolish the phase relationships between SCN and peripheral clocks resulting temporarily in a severely disorganized circadian system.

In conclusion, we describe a novel negative regulatory mechanism of the human circadian feedback loop. We report that RXRα and RARα can inhibit CLOCK:BMAL1- and MOP4:BMAL1-dependent transcriptional activation of an E box-containing circadian enhancer element. This is evidence of a direct, receptor-mediated negative regulation of the core clock oscillator. These findings should establish a paradigm for identifying other receptor-mediated, clock regulatory events in peripheral organs and suggest that circulating steroids and vitamins may play a critical role in normal and abnormal vascular physiology.

Experimental Procedures

Plasmids and Proteins

For yeast, bacterial, and eukaryotic expression constructs of MOP4, CLOCK, BMAL1, PER2, CRY2, and all of the nuclear receptor constructs the appropriate PCR-amplified fragments were cloned into pBDGal4, pADGal4 (Stratagene), pGEX4T (Amersham Pharmacia Molecular Dynamics), CMX-PL1, CMXGal4, pCDNA3.1 and pCRII vectors (Invitrogen). Full-length cDNAs for MOP4, MOP3, CLOCK were kindly provided by Dr J. Hogenesch, hNPAS2 is a gift from Dr. S.L. McKnight and hCRY2 was generously provided by Dr. A. Cashmore. pGL3M34-LUC is a gift from Dr. C.A. Bradfield and pMH100-TK-Luc is a gift from Drs. R. Evans and B. Forman (Forman et al., 1995). Sequences of all constructs surrounding the cloning sites were verified by automated sequencing. Recombinant proteins were expressed in BL(21) (DE53) *E. coli* cells (Novagen), purified using glutathione beads (Amersham Pharmacia Molecular Dynamics). MOP4ΔAAA was constructed using the Quick Change Site-Directed Mutagenesis Kit (Stratagene).

Animal Handling and Tissue Harvesting

8- to 16-week-old balb/c and c57Bl/6 wild-type mice were kept in a 12 hr/12 hr light/dark regimen. At indicated time intervals, mice were lightly anesthetized and then exsanguinated. Tissues were harvested, immediately flash-frozen in liquid nitrogen, and stored at −70°C. Mice were intraperitoneally injected with 10 mg per kg of body weight (10 mg/kg) of all-trans retinoic acid (tRA) [500 μg/ml in DMSO] (Sigma) or DMSO alone. Animal care was in accordance with the Institutional Animal Care and Use Committee guidelines.

Yeast Two-Hybrid Analysis

The yeast strain YRG2 (Stratagene), transformed with the yeast expression plasmid pBDGal4cam (Stratagene) fused to hRXRα, was used to screen a yeast Gal4 activation domain (AD) fusion cDNA library from human aortic vascular smooth muscle cells (detailed procedure of the library construction is available upon request). RXRα interacting clones were selected on media lacking Trp, Leu, and His, which contained 10 mM 3-amino-triazol. Surviving colonies were assayed for β-galactosidase activity using a colony filter lift assay and incubation in the presence of 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-GAL) as described (Estojak et al., 1995). The cDNAs from LacZ positive clones were sequenced across the Gal4/library cDNA boundary and analyzed using the BLAST algorithm at the NCBI. Liquid β-galactosidase assays were carried out as described (Estojak et al., 1995).

In Vitro Immunoprecipitation and Interaction Assays

^{35}[S]methionine-labeled and unlabeled MOP4, CLOCK, BMAL1, and all of the MOP4 and CLOCK truncation and deletion mutants and GSTRXRα, GSTRARα, GSTRXRΔ443 or GSTRARΔ404 were synthesized and used in GST-pulldown assays as described (Seo et al., 2001) in the presence and absence of ligands. For IP, ^{35}[S]methionine-labeled RAR and CLOCK were incubated with purified CLOCK and RAR protein and immunoprecipitated using anti-CLOCK and anti-RAR antibodies (SantaCruz) as described (Seo et al., 2001).

Coimmunoprecipitations

NIH3T3 cells cotransfected with CMXCLOCK and CMXRAR, treated with tRA (1 μM) or vehicle, were lysed as described (Seo et al., 2001). After centrifugation the supernatant was incubated with cross-linker, DTSSP (final conc. 3 mM, Pierce) at room temperature for 30 min. Anti-Clock, Anti-RAR antibodies or goat-anti-rabbit control antibodies were added and coimmunoprecipitation was carried out as described (Seo et al., 2001) and probed with anti-RAR and anti-CLOCK antibodies, respectively.

EMSA Assay

A double-stranded ^{32}[P]-labeled consensus oligonucleotide, GGGA CACGTGACCC (Annovis Inc.) was incubated for 30 min at 4°C in a buffer containing 150 mM KCl, 20 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 200 ng poly(dI-dC) in the presence of in vitro translated MOP4, CLOCK, BMAL1 and RXRα or RARα in the presence of 1 μM tRA or 1μM 9*cis*RA (Sigma Chemicals) where indicated. The samples were loaded directly onto 4% polyacrylamide-TBE gels and complex formation analyzed by phosphorimager.

Transfection Studies and Mammalian Two-Hybrid Analysis

NIH3T3 cells were seeded at 20,000 cells/well of a 48-well dish as described (Seo et al., 2001) with internal control pRLSV40 (5 ng), pGL3M34 reporter, CMXMOP4 (100 ng) or CMXCLOCK (100 ng) and CMXBMAL1 (100 ng) in the presence or absence of CMXRXRα, CMXRARα (25 ng-100 ng), CMXPPARγ, or CMXTRβ. CMXMOP4-ΔAAA (100 ng), pCDNA3.1MOP4Δ727 (100 ng), pCDNA3.1-MOP4Δ624 (100 ng), and CMXMOP4Δ665-680(100 ng) were used instead of CMXMOP4 where indicated. tRA, 9*cis*RA, 3,3′,5-Triiodo-L-Tyronine (T3) and 15 deoxy Δ12- Prostaglandin J2 (15dPGJ$_2$) (all 1 μM; Sigma Chemicals) were added where indicated. Mammalian two-hybrid and modified one-hybrid analysis was identical as above except that CV-1 cells and the following DNA were used: MH100TK-LUC (100 ng) reporter, CMXGal4DBDMOP4 (20 ng), CMXGal4DBD-CLOCK (20 ng) in the presence of CMXVP16PPARγ (20 ng), CMXVP16RARα (20 ng), CMXVP16TRβ (20 ng), CMXRXRα (20 ng), or CMXVP16PPARα (20 ng). In addition, CMXGal4 expression constructs of RXRα, RARα, PPARγ, and TRβ were cotransfected with MH100TK-LUC (100 ng) with the same ligands as above plus 5,8,11,14-Eicosatetraynoic Acid (ETYA; 10 μM) (Cayman Chemicals) where indicated. For all the figures, each value is the mean of at least three independent experiments ± SEM. Each experiment included six replicates from a single assay.

Northern Analysis

Polyadenylated (poly[A$^+$]) RNA was extracted from hVSMC (AoSMC, Clonetics, BioWhittaker Inc.) using FastTrack 2.0 kit (Invitrogen). Brain, spinal chord, heart, and skeletal muscle poly[A$^+$] RNA was purchased from Clontech. Following electrophoretic transfer, the blots were hybridized with ULTRAhyb hybridization solution (Ambion) and washed. Probes (1 kb) used were hMOP4, hBMAL1,

hCLOCK, hCRY2, and hGAPDH (Clontech). Blots were exposed at −80°C to BioMax film with intensifying screens.

Serum Shock

Human VSMC were cultured following suppliers' instructions (Clonetics, Biowhittaker Inc.) and plated on 10 cm^2 plates (3500 cells/cm^2) 6 days before the experiment and were serum starved for 48 hr when they reached confluence. Serum shock was carried out as described (Balsalobre et al., 1998); cells were harvested at the indicated time points and stored at −80°C.

RPA Analysis

Total RNAs from cells and mouse tissues were extracted using RNAwiz (Ambion) and Trizol (Life Technologies). RT-PCR products were prepared for hMOP4, hACTIN, hPER2, hCLOCK, mMOP4, mPER2, mBMAL1, mCRY1, mCLOCK (Reverse Transcription System, Promega; Expand High Fidelity Taq DNA polymerase, Roche Molecular Biochemicals) using Placenta total RNA (Ambion). The RT-PCR products were cloned into pCRII (Dual Promoter TOPO TA cloning kit, Invitrogen). Antisense RNA probes were prepared by in vitro transcription of the linearized templates with T7 or SP6 RNA polymerase using ^{32}P-labeled UTP (MAXIscript, In vitro transcription kit, Ambion). RNA from each time point was hybridized with probe and digested with RNaseA/T1 (RPAIII, Ambion). The samples were separated on a 4% denaturing-polyacrylamide gel (Sequagel system, National Diagnostics), dried, and analyzed by phosphorimager. The data were quantitated using ImageQuant version 1.2 software (Amersham Pharmacia). All results represent a mean of at least three independent experiments and are expressed as the ratio of the target signal divided by the *Actin* signal at the same time point normalized to peak levels of target (100%).

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