

The Orphan Nuclear Receptor Rev-erb α Regulates Circadian Expression of Plasminogen Activator Inhibitor Type 1*

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Jing Wang¹, Lei Yin, and Mitchell A. Lazar²

From the Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, and the Institute for Diabetes, Obesity, and Metabolism, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Plasminogen activator inhibitor type 1 (PAI-1) is a major physiologic regulator of the fibrinolytic system and has recently gained recognition as a modulator of inflammation and atherosclerosis. PAI-1 exhibits circadian rhythmicity in its expression, peaking in the early morning, which is associated with increased risk for cardiovascular events. However, the mechanisms that determine PAI-1 circadian rhythmicity remain poorly understood. We discovered that the orphan nuclear receptor Rev-erb α , a core component of the circadian loop, represses human *PAI-1* gene expression through two Rev-erb α binding sites in the *PAI-1* promoter. Mutations of these sites, as well as RNA interference targeting endogenous Rev-erb α and its corepressors, led to increased expression of the *PAI-1* gene. Furthermore, glycogen synthase kinase 3 β (GSK3 β) contributes to *pai-1* repression by phosphorylating and stabilizing Rev-erb α protein, which can be blocked by lithium. Interestingly, serum shock generated circadian oscillations in *PAI-1* mRNA in NIH3T3 cells, suggesting that *PAI-1* is a direct output gene of the circadian loop. Ectopic expression of a stabilized form of Rev-erb α that mimics GSK3 β phosphorylation dramatically dampened *PAI-1* circadian oscillations. Thus, our results suggest that Rev-erb α is a major determinant of the circadian *PAI-1* expression and a potential modulator of the morning susceptibility to myocardial infarction.

Circadian clocks are present in cells throughout the body and drive many physiologic and disease processes. The cardiovascular system displays circadian rhythms in many of its normal functions, including platelet activation, fibrinolytic activity, and blood pressure (1), as well as in the timing of acute cardiac events such as myocardial infarction and stroke, both of which peak in the early morning (2–4). The morning excess of cardiac events may partly result from a natural circadian variation in fibrinolytic activity. Plasminogen activator inhibitor type 1 (PAI-1)³ is a major inhibitor of fibrinolysis and exhibits a diurnal

pattern in its expression (5). The morning peak of plasma PAI-1 corresponds to a nadir in net fibrinolysis, suggesting a role in the onset of acute thrombotic events (6, 7).

Several lines of evidence suggest that elevated PAI-1 levels may indeed promote the development of atherothrombosis (8). Reduced fibrinolysis as result of increased plasma PAI-1 may lead directly to thrombosis and ischemia, as transgenic mice expressing a stable form of human PAI-1 develop spontaneous coronary thrombosis and myocardial infarction (9–12). PAI-1 may also play a role in vascular remodeling and the propagation of inflammatory signals. PAI-1 interacts with the extracellular matrix protein vitronectin to inhibit endothelial cell migration, potentially compromising wound healing and neointima formation after vascular injury (13–15). Excess PAI-1 has been found in atherosclerotic plaques in humans and is further elevated in subjects with diabetes (16). PAI-1 is induced in both acute and chronic inflammatory states such as sepsis (17) and obesity (18) and may contribute to thrombotic tendencies in these diseases. PAI-1 deficiency, on the other hand, appears to be cardioprotective, as the lack of the *Pai-1* gene in ApoE^{-/-} mice delays thrombus formation following atherosclerotic plaque rupture (19). Disruption of the *Pai-1* gene also reduces adiposity and improves metabolic profile in diabetic or high fat diet-fed mice (20, 21), which may provide secondary protection against cardiovascular disease.

Basal expression of the *PAI-1* gene is known to be regulated by an array of factors, but mechanisms determining *PAI-1* circadian rhythm are less understood. Endogenous sources of PAI-1, including the liver, adipose tissue, and the vascular endothelium, all contain robust circadian clocks that govern gene expression. Therefore it is possible that *PAI-1* is a direct output gene of the circadian clock. Indeed, the *PAI-1* promoter contains E-box enhancers that mediate transcription activation by CLOCK·BMAL heterodimers, which are the positive limb of the circadian feedback loop (12, 22). However, little is known about negative regulation of the *PAI-1* promoter by core circadian clock proteins.

The orphan nuclear receptor Rev-erb α is a key negative feedback regulator of the circadian clock (23). Rev-erb α is expressed in liver and adipose tissues (24–26), which also express PAI-1 (27, 28), and is itself expressed in a circadian manner that is finely controlled both transcriptionally and

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² To whom correspondence should be addressed: University of Pennsylvania School of Medicine, 611 Clinical Research Bldg., 415 Curie Blvd., Philadelphia, PA 19104-6149. Tel.: 215-898-0198; Fax: 215-898-5408; E-mail: lazar@mail.med.upenn.edu.

³ The abbreviations used are: PAI-1, plasminogen activator inhibitor type 1;

N-CoR, nuclear receptor corepressor; ChIP, chromatin immunoprecipitation; ROR, retinoic acid receptor-related orphan receptor; RORE, ROR response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; GSK3 β , glycogen synthase kinase 3 β ; HDAC3, histone deacetylase 3; WT, wild type; SMRT, silencing mediator of retinoid and thyroid hormone receptors; GFP, green fluorescent protein.

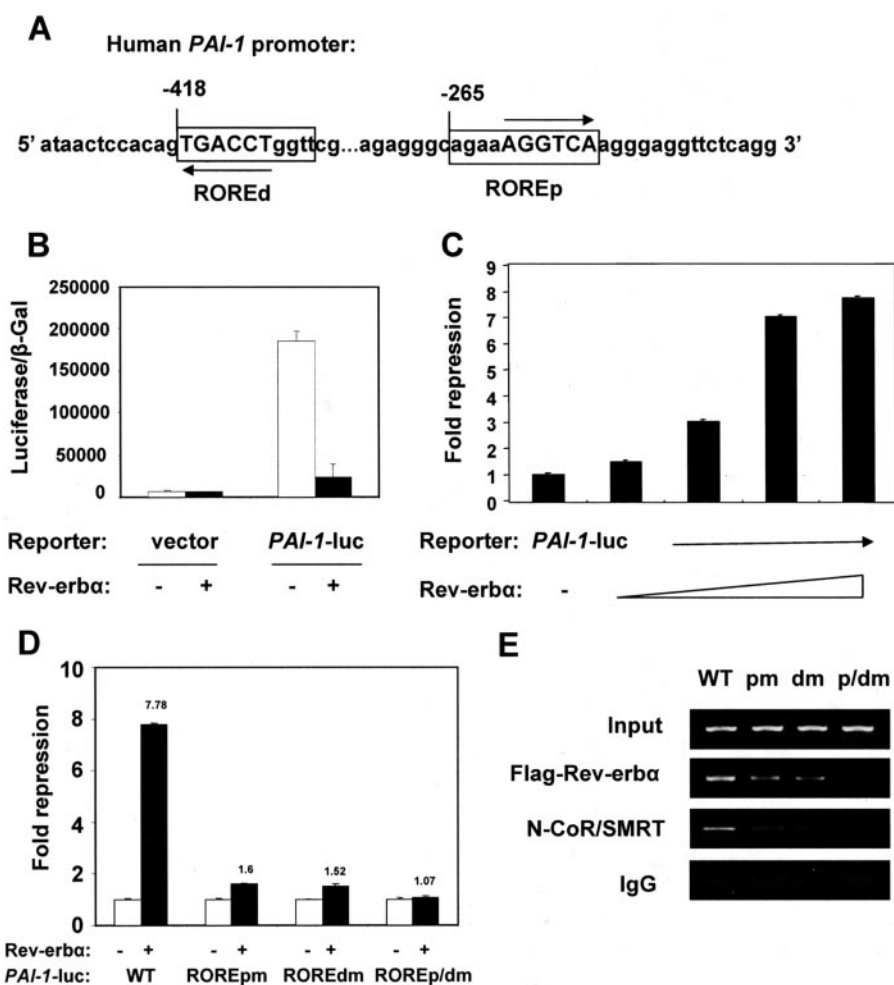


FIGURE 1. Rev-erba represses the human PAI-1 promoter. *A*, schematic representation of the human PAI-1 promoter sequence including two Rev-erba monomeric binding sites separated by 153 bases. ROREd, distal RORE; ROREp, proximal RORE. *B*, Rev-erba suppresses pai-1-luciferase reporter activity in 293T cells. Control was the pGL4 backbone vector. *C*, dose-dependent repression by Rev-erba. 200 ng of PAI-1-luciferase reporter along with 0.1, 0.5, 1, or 2 μ g of Rev-erba expression vector were transfected into 293T cells. Plasmid dosage was kept constant by the addition of empty pcDNA expression vector. *D*, mutation of either Rev-erba binding site diminishes repression. The AGGTCA half-site was changed to ACATCA by site-directed mutagenesis of nucleotides -259(G to A)/-260(G to C) and -414(G to C)/-415(G to A). *E*, mutations of PAI-1 ROREs reduce Rev-erba binding and abolish N-CoR/SMRT recruitment. ChIP was performed using antibodies that detect exogenous Rev-erba (FLAG epitope) and endogenous N-CoR/SMRT. pm, proximal mutant; dm, distal mutant; p/dm, proximal/distal double mutant.

post-transcriptionally (23, 29). Rev-erba constitutively represses transcription of its target genes, which include Rev-erba itself (30), by binding to target promoters and recruiting repression complexes containing the nuclear receptor corepressor (N-CoR) (31–33) and histone deacetylase 3 (HDAC3) (34). In addition, Rev-erba competitively inhibits gene activation by ROR α , a constitutively active orphan nuclear receptor that recognizes the same DNA response element (35, 36), and has been shown to regulate cardiovascular risk factors and atherosclerosis (37–40).

Here we demonstrate that Rev-erba is a direct repressor of the PAI-1 gene. We show that Rev-erba potently represses the PAI-1 promoter both by recruiting corepressors and by blocking ROR α -mediated activation. Furthermore, repression by Rev-erba is an important determinant of PAI-1 circadian rhythm, as stabilization of Rev-erba protein abolishes serum-induced oscillations in PAI-1 expression. Regulation

of PAI-1 by Rev-erba therefore represents a novel link between the circadian clock and cardiovascular function.

MATERIALS AND METHODS

Plasmids and Reagents—The PAI-1-luciferase reporter construct was generated by PCR-amplifying the proximal 840-bp human PAI-1 promoter and subcloning it into a short half-life pGL4.15 luc2P/Hygro vector (Promega, Madison, WI). RORE mutants were generated by site-directed mutagenesis using the Quik-Change kit (Stratagene, La Jolla, CA) and confirmed by sequencing analysis. The expression vectors encoding human Rev-erba and human ROR α 1 have been described previously (34, 36). Lithium chloride was purchased from Sigma. Protein A-Sepharose was obtained from Amersham Biosciences.

Mammalian Cell Culture and Transfection—HepG2, HEK-293, and NIH3T3 cells were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were grown at 37 C in 5% CO₂. Stable NIH3T3 cell lines expressing ectopic WT or S55D/S59D Rev-erba have been described previously (29). All transient transfection assays were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. For repression assay, cells were grown in 12-well plates and transfected with 0.2 μ g of PAI-1-luciferase reporter, 0.1–2 μ g

Rev-erba expression vector, and 0.1 μ g of β -galactosidase expression vector. The total amount of expression plasmid transfected per well was kept constant by adding varying amounts of empty vector. At 48-h post-transfection, cells were lysed and their luciferase activity assayed using a reporter assay kit (Promega). Luciferase units were normalized to β -galactosidase expression. Each experiment was performed three times in triplicate.

Serum Shock—The protocol used for serum shock was as described (41, 42). In brief, NIH3T3 fibroblasts were grown to confluence in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen). Cells were then starved in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum for 24 h. On the day of serum shock, 50% horse serum was added for 2 h, and then the medium was changed back to starvation medium. Cells were harvested for protein and RNA extraction at indicated time points.

Regulation of PAI-1 by Rev-erb α

RNA Interference—Vectors expressing hairpin small interfering RNAs (siRNA) under the human U6 or H1 promoter were described previously (29, 43). Control was pEntry β -galactosidase plus pSilence Scramble siRNA. The target sequences were as follows: pEntry-GSK3 β , 5'-ggcaccagagttgatcttg-3'; Rev-erb α , 5'-ggcatgggtgttactgtgtaa-3'; HDAC3, 5'-cagcgattgatgaccagagttaca-3'; β -galactosidase, 5'-gtgcacctggtaaatttat-3'; pSilence-N-CoR, 5'-aagaaggatccagcattcgga-3'. Cells in 12-well plates were transfected twice over a 96-h period with 1.6 μ g of siRNA vector per well. After the second transfection, cells were harvested for RNA analysis or protein analysis.

Chromatin Immunoprecipitation (ChIP) Assay—Cells were grown in 10-cm plates and either transfected or treated with 1 mM lithium for the indicated experiments. After cross-linking in formaldehyde, cells were lysed in hypotonic buffer (50 mM Tris-HCl, 85 mM KCl, 0.5% Nonidet P-40, 1 \times protease inhibitor). The nuclear fraction was resuspended in 500 μ l of sonication buffer (0.01% SDS, 10 mM EDTA, 50 mM Tris-HCl, 1 \times protease inhibitor) and sonicated four times for 12 s each followed by centrifugation at 14,000 \times g for 10 min. Supernatants were collected and diluted in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM Tris-HCl, 167 mM NaCl) followed by preclearing with 2 μ g of salmon sperm DNA and protein A-Sepharose for 2 h at 4 $^{\circ}$ C. Immunoprecipitation with the following antibodies was performed at 4 $^{\circ}$ C overnight: anti-FLAG M2 (Sigma), anti-acetyl histone H4 (Upstate Biotechnology, Lake Placid, NY), anti-N-CoR/SMRT (Affinity Bioreagents, Golden, CO), normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Rev-erb α . Immunoprecipitated complexes were collected with protein A-Sepharose beads followed by sequential washes in low salt, high salt, lithium, and Tris-EDTA buffers (34). Precipitates were eluted, and 5 M NaCl was added to reverse cross-links at 65 $^{\circ}$ C for 6 h. DNA fragments were column-purified (Qiagen, Valencia, CA), and 3 μ l of purified DNA was used in 28–32 cycles of PCR using primers encompassing both RORE regions of the human endogenous PAI-1 promoter (forward 5'-tccacgtttgatggaggtt-3' and reverse 5'-ctctgggagtcgctctgaac-3') and the PAI-1-luciferase primers (forward 5'-tccacgtttgatggaggtt-3' and reverse 5'-tcttccatgggtgctttacc-3').

Quantitative Reverse Transcription PCR—Total mRNA was prepared using the RNeasy kit (Qiagen). Reverse transcription was performed with 3 μ g of total RNA using the ImpromII RT kit (Promega) according to manufacturer's instructions. The cDNA was subject to quantitative reverse transcription PCR using an ABI Prism 7900 HT detection system (Applied Biosystems, Foster City, CA). All primers and probes were purchased from Applied Biosystems. Target gene expression was normalized to housekeeping gene GAPDH or 36B4. The average Ct value from each triplicate was used to calculate fold induction of the gene, with the control group normalized to 1.

Immunoblotting—Cells were lysed in whole-cell lysis buffer (150 mM NaCl, 10 mM Tris pH 7.6, 0.1% SDS, 5 mM EDTA) with 1 \times protease inhibitor. 20 μ g of lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were probed with the following primary antibodies: anti-N-CoR/SMRT (Affinity Bioreagents, Golden, CO), anti-

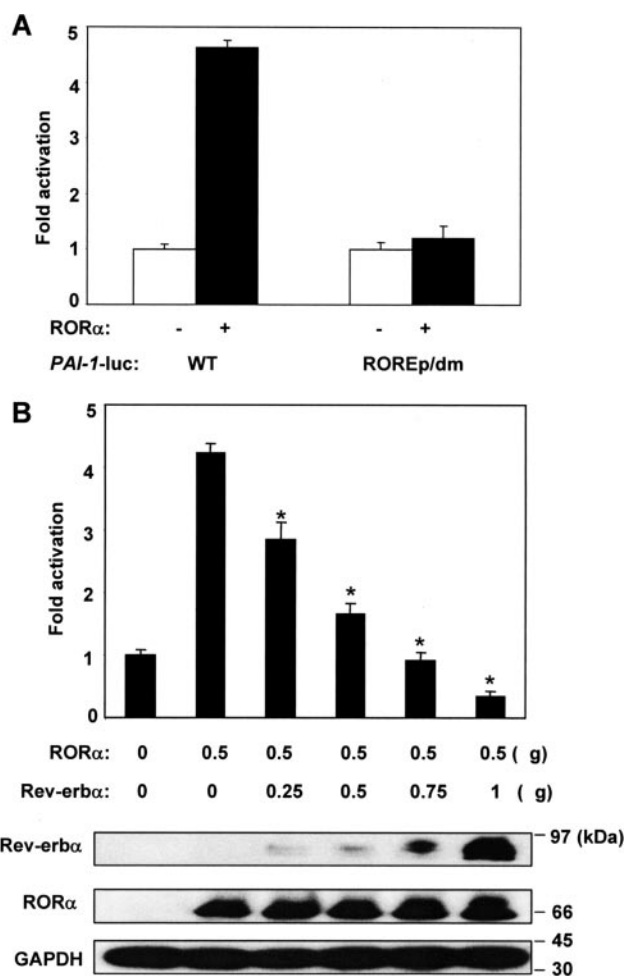


FIGURE 2. Rev-erb α functionally competes with ROR α to regulate PAI-1 gene expression. A, ROR α transactivation of the PAI-1 promoter depends on the presence of RORE sites. *p/dm*, proximal/distal double mutant. B, Rev-erb α inhibits ROR α activation of the PAI-1 promoter in a dose-dependent manner. A constant amount of PAI-1-luciferase (0.2 μ g) and ROR α (0.5 μ g) was transfected into 293T cells, and Rev-erb α in increasing doses (0.25–1 μ g) was co-transfected. The total amount of plasmids was kept constant by adding empty vector. *, $p < 0.05$ versus ROR α alone. Western blotting shows expression levels of transfected Rev-erb α and ROR α as well as endogenous GAPDH protein.

HDAC3 (Upstate Biotechnology, Lake Placid, NY), anti-GSK3 β and GAPDH (Abcam, Cambridge, MA), anti-ROR α (Santa Cruz Biotechnology), anti-FLAG M2 (Sigma), and anti-Rev-erb α (34).

RESULTS

Orphan Nuclear Receptor Rev-erb α Represses Activity of Human PAI-1 Promoter—Examination of the human PAI-1 promoter identified two potential Rev-erb α monomer binding sites (ROREs) at distances of 418 and 265 bp from the transcription start (Fig. 1A). The proximal RORE had been previously shown to act as a binding site for Nur77, another nuclear receptor implicated in PAI-1 transcription regulation (44). To determine whether the PAI-1 promoter is sensitive to Rev-erb α regulation, we cloned the proximal PAI-1 promoter that includes the two putative ROREs into a luciferase reporter vector and transfected it into human 293T cells. The PAI-1 promoter had

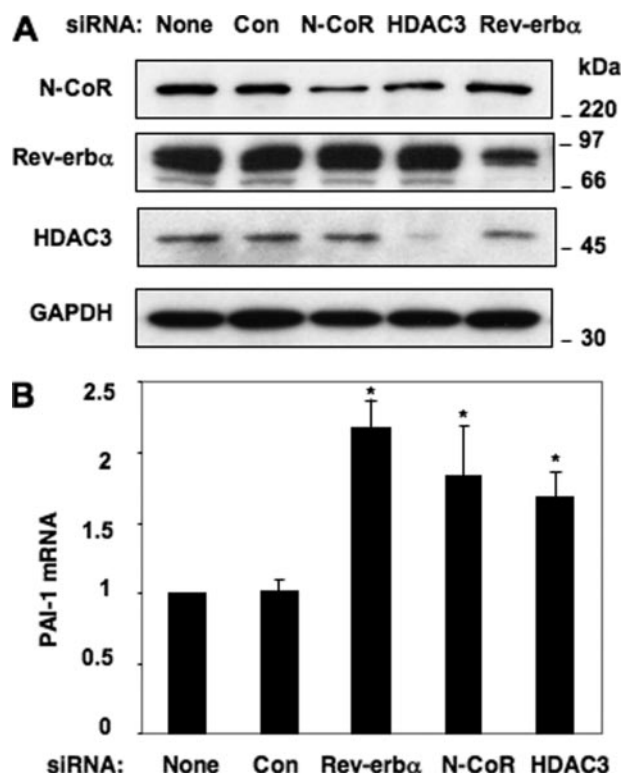


FIGURE 3. Endogenous Rev-erb α represses the PAI-1 gene in human liver cells. *A*, immunoblot for N-CoR, Rev-erb α , HDAC3, and GAPDH (control) after transfecting HepG2 cells with or without siRNA for β -galactosidase (control (Con)), N-CoR, Rev-erb α , or HDAC3. *B*, knockdown of endogenous Rev-erb α or corepressors N-CoR/HDAC3 leads to increased native PAI-1 gene expression in HepG2 cells. PAI-1 mRNA expression was normalized to GAPDH control, which did not change with siRNA treatments. *, $p < 0.05$ versus control siRNA.

strong basal activity in these cells, which was dramatically repressed by ectopic expression of human Rev-erb α (Fig. 1B). The repression was dose-dependent, as increasing amounts of the co-transfected Rev-erb α expression plasmid further reduced luciferase activity (Fig. 1C). Similar results were obtained using human HepG2 liver cells and mouse fibroblast NIH3T3 cells (data not shown), indicating that Rev-erb α -mediated repression of the PAI-1 promoter is a cell-autonomous phenomenon.

We next investigated whether the two putative PAI-1 ROREs were necessary for Rev-erb α -mediated repression by mutating either or both of them and testing their ability to recruit and be repressed by Rev-erb α . Consistent with the requirement for two Rev-erb α monomers to allow corepressor recruitment (31, 33), a GG/CA mutation in either RORE led to dramatic reductions in the ability of Rev-erb α to repress the promoters, and mutation of both sites abolished repression altogether (Fig. 1D). ChIP analysis showed that the wild-type PAI-1 promoter is bound by Rev-erb α and corepressor N-CoR/SMRT, and mutation of both ROREs effectively reduced Rev-erb α binding and abolished N-CoR recruitment, explaining the loss of repression of the mutant promoters (Fig. 1E). Single mutation of the ROREs reduced Rev-erb α recruitment to the PAI-1 promoter by ~50%, consistent with the ability of Rev-erb α to bind the RORE as a monomer. However, N-CoR association with the PAI-1 pro-

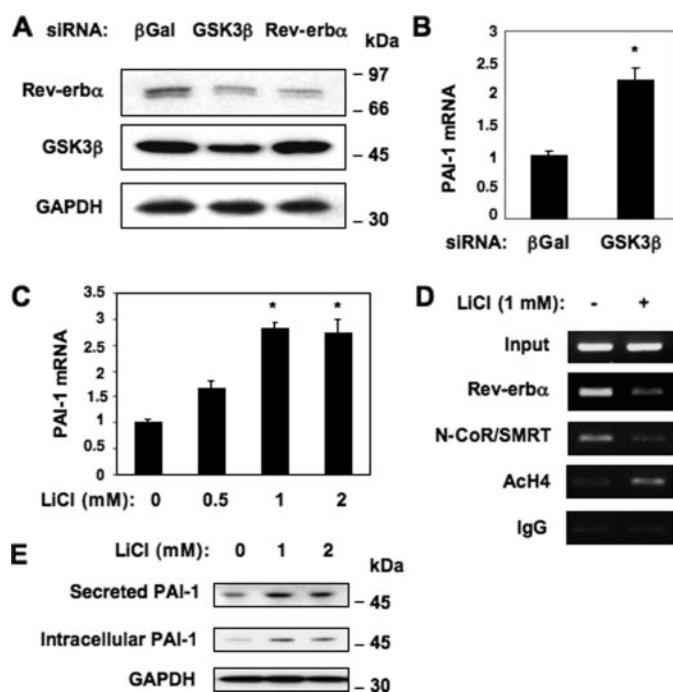


FIGURE 4. GSK3 β activity is required for Rev-erb α -mediated PAI-1 repression. *A*, immunoblot showing siRNA knockdown of Rev-erb α and GSK3 β in HepG2 cells. Note that GSK3 β knockdown also reduced Rev-erb α protein level. GAPDH is the loading control. *B*, GSK3 β knockdown leads to increased endogenous PAI-1 gene expression. PAI-1 expression was normalized to GAPDH control. *C*, 72-h treatment with therapeutic doses of the GSK3 β inhibitor LiCl leads to PAI-1 derepression. Results were the mean \pm S.E. of triplicate experiments; *, $p < 0.01$. *D*, PAI-1 derepression assessed by ChIP assay. Lithium treatment led to reduced binding by Rev-erb α and N-CoR/SMRT and increased histone H4 acetylation at the PAI-1 promoter. *E*, lithium stimulates PAI-1 protein production and secretion by HepG2 cells.

motor was more drastically reduced when either RORE was mutated (Fig. 1E), consistent with the requirement that two Rev-erb α molecules be bound for productive N-CoR recruitment, as well as with the functional data in Fig. 1D.

The PAI-1 Gene Is Induced by the Constitutively Active Nuclear Receptor ROR α , and the Induction Is Blocked by Rev-erb α —Rev-erb α is known to cross-talk with ROR α , an orphan nuclear receptor that has similar DNA binding specificity to Rev-erb α and acts as a constitutive transcriptional activator (36, 46, 47). Intriguingly, ROR α has been implicated in modulating cardiovascular risks, as the ROR α -mutant *Staggerer* mice are prone to lipid abnormalities and atherosclerosis (45). More recently, ROR α has been shown to induce the pro-atherothrombotic fibrinogen- β gene, and *Staggerer* mice have reduced levels of fibrinogen, which could be atheroprotective (37). Because ROR activates target genes via ROREs in their promoters, we reasoned that ROR α might be a positive regulator of the PAI-1 gene. Indeed, ROR α markedly induced PAI-1 promoter activity in a RORE-dependent manner (Fig. 2A). Moreover, the induction of PAI-1 transcription by ROR α was opposed by increasing amounts of Rev-erb α , which competes for RORE binding with ROR α (Fig. 2B). Rev-erb α actually reduced the PAI-1 promoter activity to levels well below the base line (Fig. 2B), indicating that its effects are due to active repression in addition to competition with ROR α for binding to the promoter.

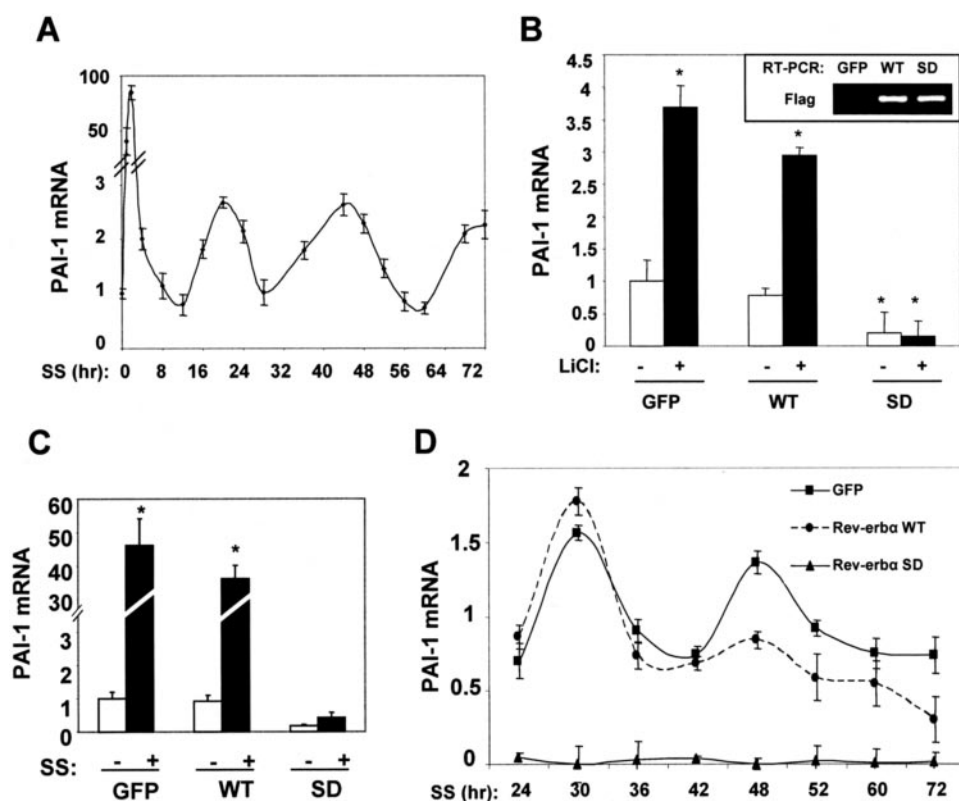


FIGURE 5. Transcriptional control of PAI-1 circadian rhythm by Rev-erba. *A*, serum shock (SS) in NIH3T3 cells synchronizes oscillations of the native *PAI-1* gene. *B*, lithium-mediated induction of *PAI-1* in NIH3T3 stable cells expressing ectopic, WT Rev-erba, or S55D/S59D Rev-erba. Reverse transcription (RT) PCR (inset) shows comparable mRNA expression of the FLAG-tagged WT and S55D/S59D (SD) Rev-erba transgenes. GFP, green fluorescent protein. *C*, 2-h serum shock induces *PAI-1* mRNA in control cell lines but not in S55D/S59D Rev-erba cells. *D*, circadian oscillation of *PAI-1* mRNA is abolished in cells expressing S55D/S59D Rev-erba. Total mRNA was prepared from cells at the indicated time points, and *PAI-1* gene expression was analyzed relative to *GAPDH* by quantitative PCR. Shown are the mean \pm S.E. of three experiments.

Endogenous Rev-erba Represses Native PAI-1 Gene Expression in Human Liver Cells—Having established that Rev-erba regulates *PAI-1* promoter activity *in vitro*, we next explored the role of endogenous Rev-erba and the N-CoR/HDAC3 corepressor complex in the basal expression of *PAI-1* in liver cells. We used small interfering RNA to reduce expression of Rev-erba, N-CoR, or HDAC3 in HepG2 cells (Fig. 3A). Knockdown of Rev-erba significantly increased the expression of *PAI-1* mRNA, as did knockdown of either N-CoR or HDAC3 (Fig. 3B). These data indicate that the *PAI-1* gene is basally repressed in liver cells by Rev-erba and its effector, N-CoR/HDAC3 corepressor complex.

Glycogen Synthase Kinase 3 β (GSK3 β) Activity Is Required For Rev-erba-Repression of the PAI-1 Gene—We have previously reported that GSK3 β -dependent phosphorylation of Rev-erba on serines 55 and 59 stabilizes Rev-erba protein by protecting it from proteasomal degradation (29). Therefore, we tested whether GSK3 β plays a role in *PAI-1* gene repression via this mechanism, using siRNA to reduce its expression (Fig. 4A). Indeed, knockdown of endogenous GSK3 β led to Rev-erba protein destabilization (Fig. 4A) and significantly increased endogenous *PAI-1* expression (Fig. 4B). Next, we tested the ability of lithium, an inhibitor of GSK3 β kinase activity, to block *PAI-1* repression. Treatment of HepG2 cells with therapeutic doses of lithium chloride (1–2 mM) for 72 h resulted in significant induction of *PAI-1* mRNA, similar

to the effects of GSK3 β -knockdown (Fig. 4C). To confirm that *PAI-1* induction by lithium is due to derepression of the Rev-erba pathway, we performed ChIP analysis to examine Rev-erba and corepressor occupancy of the *PAI-1* promoter before and after lithium treatment. Treatment with 1 mM lithium chloride abolished Rev-erba and N-CoR binding to the endogenous *PAI-1* promoter, accompanied by an increase in acetylated histone H4 in the same region (Fig. 4D). These results suggest that lithium antagonizes Rev-erba-mediated repression, leading to increased *PAI-1* gene expression. Derepression of the *PAI-1* gene also led to an increase in *PAI-1* protein levels and secretion (shown in Fig. 4E).

Circadian Rhythm of PAI-1 Gene Expression Is Regulated Transcriptionally by Rev-erba—Given that *PAI-1* expression is circadian *in vivo*, as is Rev-erba expression, we sought to determine whether Rev-erba regulates the oscillatory expression of the *PAI-1* gene. To address this question, we first sought to establish a cell culture system for studying *PAI-1* circadian rhythm. NIH3T3 fibroblasts are a cell type known to sustain circadian

rhythms in culture (41). Indeed, serum shock in wild-type NIH3T3 cells led to an immediate early induction in *PAI-1* mRNA followed by robust cycling over 72 h (Fig. 5A). Next, we studied *PAI-1* expression in NIH3T3 stable cell lines ectopically expressing either control green fluorescent protein (GFP), wild-type Rev-erba, or a Rev-erba mutant with both serines 55 and 59 mutated to aspartate (S55D/S59D), which mimics the phosphorylated state that stabilizes the protein (29). The expression of the transgenes was comparable at the mRNA level between WT and the S55D/S59D cell lines (Fig. 5B, inset). However, the WT and S55D/S59D Rev-erba isoforms differ in their protein stability (29), resulting in differential ability to repress target genes. Lithium treatment induced expression of *PAI-1* in control GFP cells and in cells ectopically expressing wild-type Rev-erba, which is degraded when GSK3 β is inhibited in this manner (29). By contrast, ectopic expression of the more stable S55D/S59D mutant Rev-erba repressed endogenous *PAI-1* expression in a lithium-insensitive manner (Fig. 5B), consistent with the increased stability of the protein in the presence of lithium.

We next compared the ability of these cell lines to induce rhythmic *PAI-1* gene expression in response to serum shock. Both GFP and WT Rev-erba-expressing cells responded to serum shock by potently up-regulating the *PAI-1* gene (Fig. 5C), similar to what was observed in normal NIH3T3 cells (Fig. 5A).

In contrast, cells expressing S55D/S59D mutant Rev-erba not only had lower basal *PAI-1* gene expression but were also insensitive to serum (Fig. 5C). In the 72 h following serum shock, we detected rhythmic expression of *PAI-1* mRNA in both GFP and WT Rev-erba-expressing cells (Fig. 5D). However, the S55D/S59D Rev-erba continued to suppress *PAI-1* expression and completely abolished its circadian oscillation (Fig. 5D). These results indicate that Rev-erba is a direct regulator of the endogenous rhythm of the *PAI-1* gene.

DISCUSSION

The protease inhibitor PAI-1 has gained recognition as an important modulator of cardiovascular disease. Clinical studies have correlated elevated PAI-1 levels to increased risk for coronary thrombosis and stroke, as well as decreased efficacy of thrombolytic therapy in the morning when PAI-1 production is at its circadian peak. Our work identifies the nuclear receptor Rev-erba as a major regulator of *PAI-1* transcription. Rev-erba recruits the N-CoR/HDAC3 corepressor complex to the *PAI-1* promoter as well as antagonizing its activation by the nuclear receptor RORa, making Rev-erba a potent repressor of *PAI-1* basal expression. As a negative component of the circadian core loop, Rev-erba also regulates *PAI-1* circadian rhythm, which is abolished by a stabilized form of Rev-erba. The *PAI-1* promoter is activated by the positive clock components CLOCK/BMAL (12), and to our knowledge the present work demonstrates the first negative transcriptional regulation of the *PAI-1* gene by a core clock protein.

Rev-erba typically binds as a monomer to the hexameric sequence AGGTCA preceded by an A/T-rich flank (the RORE). We took note of two ROREs in the proximal *PAI-1* promoter, located at -418 and -265 bp from the transcriptional start site. Although Rev-erba binds cooperatively as a homodimer to ROREs in a DR2 configuration, it has also been demonstrated to function as a potent repressor from two monomer binding sites (31, 33, 34). Indeed, in the case of the *PAI-1* promoter, mutation of either RORE dramatically abrogated repression. In addition to active repression through corepressor recruitment and histone deacetylation, Rev-erba also antagonizes *PAI-1* transcriptional activation by RORa, a constitutively active nuclear receptor that also recognizes the RORE. Cross-talk between the two nuclear receptors has been observed for a number of target genes, including *Bmal1* and fibrinogen- β (37, 46). Here we have found that this dual regulation also pertains to the *PAI-1* gene; RORa potently induces *PAI-1* expression, and this is antagonized by Rev-erba. Interestingly, although RORa and Rev-erba are both implicated in circadian gene regulation, RORa does not exhibit the robust cyclical mRNA expression that Rev-erba does (47). Indeed, ectopic expression of degradation-resistant Rev-erba was sufficient to abolish oscillatory *PAI-1* mRNA expression in NIH3T3 fibroblasts, whereas overexpression of the wild-type Rev-erba did not. These results indicate that *PAI-1* is a direct circadian output gene and that Rev-erba affects *PAI-1* rhythm through ROREs in its promoter.

Consistent with the constitutive repression of *PAI-1* expression by degradation-resistant Rev-erba, destabilization of Rev-erba as a result of GSK3 β knockdown markedly derepressed the *PAI-1* gene. Moreover, treatment of HepG2 human liver

cells with lithium, which inhibits GSK3 β activity, abolished Rev-erba and corepressor N-CoR recruitment to the *PAI-1* promoter, paralleled by an increase in histone acetylation, and thereby increased *PAI-1* mRNA and protein production. Lithium is commonly prescribed for patients with bipolar disorder, and our finding that therapeutic concentrations induce PAI-1 via inhibition of the GSK3 β -Rev-erba stabilization pathway raises the question of whether such induction occurs *in vivo*. There is a surprising paucity of controlled studies of lithium cardiotoxicity in adult human subjects, but a case-controlled study (48) suggests that lithium use is associated with a significantly increased risk of myocardial infarction, and it is possible that the effect of lithium on PAI-1 could contribute to this risk. Future studies should determine whether lithium treatment in humans results in physiologically significant elevations of PAI-1 and whether this poses a cardiovascular risk for patients receiving long-term lithium therapy.

In summary, our data strongly suggest a role for Rev-erba in determining *PAI-1* gene expression and circadian rhythm. Pharmacological regulators of Rev-erba and its associated corepressor complex, as well as its competitor, RORa, may represent new strategies for reducing PAI-1 expression *in vivo* and thereby preventing cardiovascular events.

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