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

Jing Wang 1, Lei Yin, and Mitchell A. Lazar 2

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-erbα, a core component of the circadian loop, represses human PAI-1 gene expression through two Rev-erbα binding sites in the PAI-1 promoter. Mutations of these sites, as well as RNA interference targeting endogenous Rev-erbα and its corepressors, led to increased expression of the PAI-1 gene. Furthermore, glycogen synthase kinase 3β (GSK3β) contributes to pAI-1 repression by phosphorylating and stabilizing Rev-erbα 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-erbα that mimics GSK3β phosphorylation dramatically dampened PAI-1 circadian oscillations. Thus, our results suggest that Rev-erbα 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 such as myocardial infarction and stroke not only pose a health threat, but also have implications in the evaluation of cardiovascular risk factors, which means that 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 transcriptional 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-erbα is a key negative feedback regulator of the circadian clock (23). Rev-erbα 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 posttranscriptionally.

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; GSK3β, glycogen synthase kinase 3β; HDAC3, histone deacetylase 3; WT, wild type; SMRT, silencing mediator of retinoid and thyroid hormone receptors; GFP, green fluorescent protein.

* This work was supported in part by National Institutes of Health Grant DK45586 (to M. A. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 Supported in part by a Medical Scientist Training Program grant to the University of Pennsylvania.
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; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; ROR, retinoic acid receptor-related orphan receptor; RORE, ROR response element; GSK3β, glycogen synthase kinase 3β; HDAC3, histone deacetylase 3; WT, wild type; SMRT, silencing mediator of retinoid and thyroid hormone receptors; GFP, green fluorescent protein.
post-transcriptionally (23, 29). Rev-erbα constitutively represses transcription of its target genes, which include Rev-erbα 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-erbα competitively inhibits gene activation by RORα, 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-erbα is a direct repressor of the PAI-1 gene. We show that Rev-erbα potently represses the PAI-1 promoter both by recruiting corepressors and by blocking RORα-mediated activation. Furthermore, repression by Rev-erbα is an important determinant of PAI-1 circadian rhythm, as stabilization of Rev-erbα protein abolishes serum-induced oscillations in PAI-1 expression. Regulation of PAI-1 by Rev-erbα 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-erbα and human RORα1 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 SS5D(SS9D) Rev-erbα 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 μg of PAI-1-luciferase reporter, 0.1–2 μg of PAI-1 expression vector, and 0.1 μg 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.
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-GSK3β, 5'-ggcagagtcgctttgct-3'; Rev-erbo, 5'-ggcagagtcgctttgct-3'; HDAC3, 5'-caagagtcgctttgct-3'; β-galactosidase, 5'-gtcagagtcgctttgct-3'; pSilence-N-CoR, 5'-aagaagagtcgctttgct-3'. Cells in 12-well plates were transfected twice over a 96-h period with 1.6 μg 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× protease inhibitor). The nuclear fraction was resuspended in 500 μl of sonication buffer (0.01% SDS, 10 mM EDTA, 50 mM Tris-HCl, 1× protease inhibitor) and sonicated four times for 10 s each followed by centrifugation at 14,000 × 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 μg of salmon sperm DNA and protein A-Sepharose for 2 h at 4 °C. Immunoprecipitation with the following antibodies was performed at 4 °C overnight: anti-FLAG M2 (Sigma), anti-acetyl histone H4 (Upstate Biotechnology, Lake Placid, NY), anti-N-CoR/SMRT (Affinity Bioreagents, Golden, CO), and anti-Rev-erbo. Immunoprecipitated complexes were collected with protein A-Sepharose beads followed by sequential washes in low salt, high salt, high salt, and Tris-EDTA buffers (34). Precipitates were eluted, and 5 μl of purified DNA was added to reverse cross-links at 65 °C for 6 h. DNA fragments were column-purified (Qiagen, Valencia, CA), and 3 μl 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'-ttcagagtcgctttgct-3' and reverse 5'-ttcagagtcgctttgct-3') and the PAI-1-luciferase primers (forward 5'-ttcagagtcgctttgct-3' and reverse 5'-ttcagagtcgctttgct-3').

Quantitative Reverse Transcription PCR—Total mRNA was prepared using the RNeasy kit (Qiagen). Reverse transcription was performed with 3 μg 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× protease inhibitor. 20 μg 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-DAC3 (Upstate Biotechnology, Lake Placid, NY), anti-GSK3β and GAPDH (Abcam, Cambridge, MA), anti-RORα (Santa Cruz Biotechnology), anti-FLAG M2 (Sigma), and anti-Rev-erbo (34).

RESULTS

Orphan Nuclear Receptor Rev-erboα Represses Activity of Human PAI-1 Promoter—Examination of the human PAI-1 promoter identified two potential Rev-erboα 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-erboα 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
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The PAI-1 Gene Is Induced by the Constitutively Active Nuclear Receptor RORα, and the Induction Is Blocked by Rev-erbα—Rev-erbα is known to cross-talk with RORα, an orphan nuclear receptor that has similar DNA binding specificity to Rev-erbα and acts as a constitutive transcriptional activator (36, 46, 47). Intriguingly, RORα has been implicated in modulating cardiovascular risks, as the RORα-mutant Staggerer mice are prone to lipid abnormalities and atherosclerosis (45). More recently, RORα has been shown to induce the proatherosclerotic 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 RORα might be a positive regulator of the PAI-1 gene. Indeed, RORα markedly induced PAI-1 promoter activity in a RORE-dependent manner (Fig. 2A). Moreover, the induction of PAI-1 transcription by RORα was opposed by increasing amounts of Rev-erbα, which competes for RORE binding with RORα (Fig. 2B). Rev-erbα 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 RORα for binding to the promoter.

FIGURE 3. Endogenous Rev-erbα represses the PAI-1 gene in human liver cells. A, immunoblot showing siRNA knockdown of Rev-erbα and GSK3β in HepG2 cells. Note that GSK3β knockdown also reduced Rev-erbα protein level. GAPDH is the loading control. B, 72-h treatment with therapeutic doses of the GSK3β inhibitor LiCl leads to reduced binding by Rev-erbα to the PAI-1 promoter, which did not change with siRNA treatments. *, p < 0.05 versus control siRNA.

FIGURE 4. GSK3β activity is required for Rev-erbα-mediated PAI-1 repression. A, immunoblot showing siRNA knockdown of Rev-erbα and GSK3β in HepG2 cells. Note that GSK3β knockdown also reduced Rev-erbα protein level. GAPDH is the loading control. B, GSK3β 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 GSK3β inhibitor LiCl leads to PAI-1 derepression. Results were the mean ± S.E. of triplicate experiments; *, p < 0.01. D, PAI-1 derepression assessed by ChIP assay. Lithium treatment led to reduced binding by Rev-erbα 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.
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Endogenous Rev-erbα Represses Native PAI-1 Gene Expression in Human Liver Cells—Having established that Rev-erbα regulates PAI-1 promoter activity in vitro, we next explored the role of endogenous Rev-erbα 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-erbα, N-CoR, or HDAC3 in HepG2 cells (Fig. 3A). Knockdown of Rev-erbα 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-erbα and its effectors, N-CoR-HDAC3 corepressor complex.

Glycogen Synthase Kinase 3β (GSK3β) Activity Is Required For Rev-erbα Repression of the PAI-1 Gene—We have previously reported that GSK3β-dependent phosphorylation of Rev-erbα on serines 55 and 59 stabilizes Rev-erbα protein by protecting it from proteasomal degradation (29). Therefore, we tested whether GSK3β plays a role in PAI-1 gene repression via this mechanism, using siRNA to reduce its expression (Fig. 4A). Indeed, knockdown of endogenous GSK3β led to Rev-erbα protein destabilization (Fig. 4A) and significantly increased endogenous PAI-1 expression (Fig. 4B). Next, we tested the ability of lithium, an inhibitor of GSK3β 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 GSK3β-knockdown (Fig. 4C). To confirm that PAI-1 induction by lithium is due to derepression of the Rev-erbα pathway, we performed ChIP analysis to examine Rev-erbα and corepressor occupancy of the PAI-1 promoter before and after lithium treatment. Treatment with 1 mM lithium chloride abolished Rev-erbα 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-erbα-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-erbα—Given that PAI-1 expression is circadian in vivo, as is Rev-erbα expression, we sought to determine whether Rev-erbα 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-erbα, or a Rev-erbα 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-erbα 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-erbα, which is degraded when GSK3β is inhibited in this manner (29). By contrast, ectopic expression of the more stable S55D/S59D mutant Rev-erbα repressed endogenous PAI-1 expression in a lithium-insensitive manner (Fig. 5B, inset), 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-erbα-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).

FIGURE 5. Transcriptional control of PAI-1 circadian rhythm by Rev-erbα. 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-erbα, or S55D/S59D Rev-erbα. Reverse transcription (RT) PCR (inset) shows comparable mRNA expression of the FLAG-tagged WT and S55D/S59D (SD) Rev-erbα transgenes. GFP, green fluorescent protein. C, 2-h serum shock induces PAI-1 mRNA in control cell lines but not in S55D/S59D Rev-erbα cells. D, circadian oscillation of PAI-1 mRNA is abolished in cells expressing S55D/S59D Rev-erbα. 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 ± S.E. of three experiments.
In contrast, cells expressing S55D/S59D mutant Rev-erbα 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-erbα-expressing cells (Fig. 5D). However, the S55D/S59D Rev-erbα continued to suppress PAI-1 expression and completely abolished its circadian oscillation (Fig. 5D). These results indicate that Rev-erbα 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-erbα as a major regulator of PAI-1 transcription. Rev-erbα recruits the N-CoR-HDAC3 corepressor complex to the PAI-1 promoter as well as antagonizing its activation by the nuclear receptor RORα, making Rev-erbα a potent repressor of PAI-1 basal expression. As a negative component of the circadian core loop, Rev-erbα also regulates PAI-1 circadian rhythm, which is abolished by a stabilized form of Rev-erbα. The PAI-1 promoter is activated by the positive clock components CLOCK-BMAL1, and to our knowledge the present work demonstrates the first negative transcriptional regulation of the PAI-1 gene by a core clock protein.

Rev-erbα 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-erbα 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-erbα also antagonizes PAI-1 transcriptional activation by RORα, 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; RORα potently induces PAI-1 expression, and this is antagonized by Rev-erbα. Interestingly, although RORα and Rev-erbα are both implicated in circadian gene regulation, RORα does not exhibit the robust cyclical mRNA expression that Rev-erbα does (47). Indeed, ectopic expression of degradation-resistant Rev-erbα was sufficient to abolish oscillatory PAI-1 mRNA expression in NIH3T3 fibroblasts, whereas overexpression of the wild-type Rev-erbα did not. These results indicate that PAI-1 is a direct circadian output gene and that Rev-erbα affects PAI-1 rhythm through ROREs in its promoter.

Consistent with the constitutive repression of PAI-1 expression by degradation-resistant Rev-erbα, destabilization of Rev-erbα as a result of GSK3β knockdown markedly derepressed the PAI-1 gene. Moreover, treatment of HepG2 human liver cells with lithium, which inhibits GSK3β activity, abolished Rev-erbα 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 GSK3β-Rev-erbα 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-erbα in determining PAI-1 gene expression and circadian rhythm. Pharmacological regulators of Rev-erbα and its associated corepressor complex, as well as its competitor, RORα, may represent new strategies for reducing PAI-1 expression in vivo and thereby preventing cardiovascular events.

**Acknowledgments—**We thank A. Sehgal and J. D. Alvarez for helpful discussions and technical assistance.

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