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Circadian clock genes cause activation of the human *PAI-1* gene promoter with 4G/5G allelic preference

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Abstract Increased plasminogen activator inhibitor-1 (PAI-1) activity is associated with greater risk of myocardial infarction. PAI-1 expression is regulated by a 4G/5G promoter polymorphism. The 4G allele is associated with higher PAI-levels and greater circadian variation. Here we show that clock protein heterodimers BMAL/CLOCK cause greater activation (≈2-fold, $P < 0.05$) of the 4G allele. Site-directed mutagenesis studies suggest that clock genes act on two canonical E-boxes to regulate *PAI-1* promoter activity. These results identify a potential novel mechanism whereby allele-specific clock genes – mediated modulation of PAI-1 expression may contribute to circadian variation in cardiac risk.

Keywords: Fibrinolysis; Plasminogen activator inhibitor-1; Circadian biology; Clock genes; Acute coronary syndrome; 4G/5G polymorphism

1. Introduction

Plasminogen activator inhibitor-1 (PAI-1) is the major inhibitor of fibrinolysis [1]. Elevated plasma PAI-1 levels have been shown to be associated with increased risk of recurrent myocardial infarction (MI) [2]. Circulating PAI-1 levels are under genetic control. In this regard, an insertion/deletion (5G/4G) polymorphism at position −675 of the *PAI-1* promoter has been extensively studied [1]. The 4G allele of *PAI-1* is associated with increased plasma levels [3–7]. Circulating PAI-1 levels also shows circadian variation, with increased levels in the early morning, coinciding with the early morning peak in risk of MI. Many circadian rhythms are regulated directly by an endogenous clock system, which is highly conserved in organisms as diverse as *cyanobacteria*, plants, fruit flies, and mammals [8–10]. The mammalian timing system consists of a set of "clock genes" that display circadian rhythms of expression which in turn regulate the expression of output genes. The model that has emerged is one of interlocking transcriptional/translational feedback loops with positive and negative clock genes. The positive genes include *Clock*, *Bmal1*, *Bmal2*, and *Npas2*, and the negative regulators are *Per1-2*, *Cry1-2* and *Dec1-2* [10]. The CLOCK protein forms a heterodimer with either BMAL1 or BMAL2, both of which bind to and activate the promoters of genes under circadian control, via a DNA element termed an E-box (CACGTG) [10]. Interestingly, recent studies have shown that circadian variation in PAI-1 activity is predominantly confined to the 4G allele, whereas 5G homozygotes do not display diurnal differences in PAI-1 expression [5,6]. In this study we have characterised the interaction of clock gene products with the *PAI-1* promoter and show that the degree of activation is allele specific.

2. Materials and methods

Individuals were identified that were homozygous for either the 4G or 5G allele from our human sample DNA bank. Fragments of the human *PAI-1* 5′-flanking region [−796 to +140, relative to the transcription start point (TSP)] were amplified by PCR from each allele using primers 796F 5′-GTACCATTGGGACCAGGGC-3′ and 140R 5′-AGTCCATCCTCTTTTCGTTTG-3′. This fragment contains all of the reported definite and putative hypoxia-responsive element (HRE) motifs in the promoter at positions −675 (CACGTG), −560 (CACGTG), −453 (CACGTT), −197 (CACTGA), and −154 (CACATG) [11] and also includes the whole of the 5′-untranslated region (UTR). Both of the HRE sites at −675 and −560 are canonical E-Boxes and are termed E-box1 and E-box2 respectively. DNA fragments were then subcloned into the luciferase reporter, pGL3-Basic. *PAI-1* promoter-reporter constructs with individual mutated HREs (in 4G background) were kindly provided by Dr T. Fink [11]. Authenticity of all plasmids were confirmed by sequencing. Expression plasmids of human BMAL1, BMAL2, and CLOCK were kindly provided by Dr J. Hogenesch and mouse CRY2 by Dr D. Weaver.

COS-7 cells were maintained in modified DMEM media containing 10% FCS and plated (at 50% confluency) 24-h prior to transfection. Reporter plasmids (100 ng/well) were co-transfected with 200 ng of each expression plasmid or 200 ng of empty vector as control using Jet-PEI transfection reagent. In addition 10 ng/well of pRL-null *Renilla* luciferase plasmid was co-transfected as a control for transfection efficiency. Each experiment was performed in triplicate and repeated at least three times. To simulate a 4G/5G heterozygous background 50 ng of each reporter was mixed and the resulting 100 ng used for the transfections. Reporter activities were measured at 48 h post-transfection using a dual-luciferase reporter assay system (Promega) and the results adjusted for *Renilla* values and then standardised to the empty vector control. The values are expressed as means ± S.E.M. The significance of differences between groups was determined by Student *t*-test for paired values.

3. Results

Co-transfection assays indicated that clock protein heterodimers (BMAL1/CLOCK or BMAL2/CLOCK) can activate the human *PAI-1* promoter-reporter in vitro (Fig. 1). The

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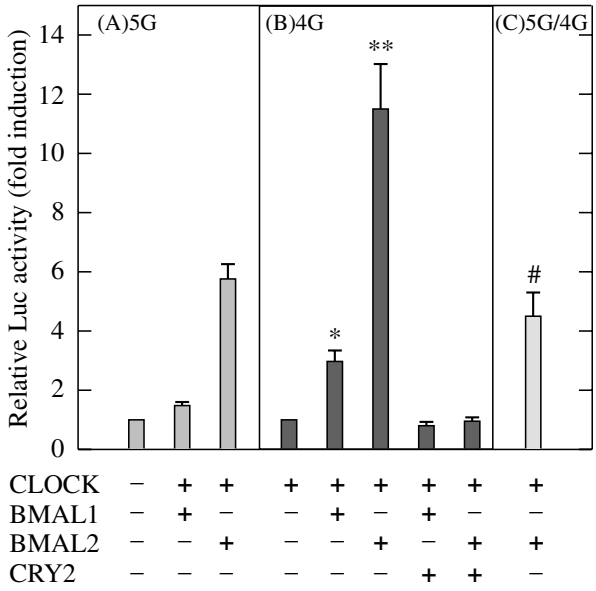

Fig. 1. Transcriptional activation of the human *PAI-1*promoter by clock proteins. Co-transfection analysis with clock protein heterodimers BMAL1/CLOCK and BMAL2/CLOCK using *PAI-1* 5G (A), 4G (B) and 5G/4G (C) allelic reporters. Each experiment was performed in triplicate and each analysis includes the results of at least three independent experiments. Error bars represent S.E.M. $^{*}P < .05$ versus 5G BMAL1/CLOCK ($n = 3$); $^{**}P < .005$ versus 5G BMAL2/CLOCK ($n = 6$); $^{\#}P < .005$ versus 4G BMAL2/CLOCK ($n = 3$).

activation was inhibited by the negative clock factor CRY2. BMAL2 was a stronger activator (approximately three times as potent) than BMAL1 for both alleles ($P < 0.05$). Of particular importance, both BMAL1/CLOCK and BMAL2/CLOCK heterodimers activated the *PAI-1* 4G allele reporter more strongly (by 193 ± 13% and 203 ± 25%, respectively, $P < 0.05$) than the 5G allele (Figs. 1A and B). Simulated 4G/5G heterozygote-reporter activity was similar to the 5G allele reporter (Fig. 1C).

To elucidate the contribution of each putative HRE to the BMAL2/CLOCK induced promoter activity, co-transfection experiments were carried out using site-directed mutated reporters. Mutation of the HRE at position −197 bp had no effect on clock gene activation of the *PAI-1* promoter (Fig. 2). However, mutation of E-box1 (−675 bp) decreased clock gene transactivation by 70%, and mutation of E-box2 (−560 bp) reduced activity by 90%.

4. Discussion

In this study we show that clock protein heterodimers activate the *PAI-1* promoter, and more importantly, that this activation is allele specific which provides a potential explanation for the circadian variation in plasma PAI-1 levels in subjects with the 4G allele and not those with the 5G allele [5,6]. A previous study reported no preference for clock gene activation at the 4G/5G allele, when transfected into bovine endothelial cells [12]. The discrepancy may be due to the difference in the transfection conditions. Schoenhard et al. [12] serum-starved their cells for the latter 24 h (of the 48 h period). Serum-deprivation can cause cellular stress and alters gene expression. Specifically, serum-starvation directly modulates E-box driven activity in endothelial cells [13,14], and this could have affected the results obtained previously [12]. Schoenhard et al. [12] also used reporters with only half (+72 bp) of the 5′-UTR, whereas we have used the full-length (+140 bp). This extra leader sequence contains several putative binding sites for factors such as delta EF1 and Ets-1, which are absent in the +72 bp fragment. Such factors have been shown to mediate both E-box driven gene expression and to specifically activate *PAI-1* expression [15,16]. It is therefore also possible that the different findings in the two studies are due to the different lengths of the 5′-UTR fragment used. In support of this, Kondratov et al. [17] have recently reported that Ets-1 protein can act in concert with clock proteins in the activation of another central clock factor, *Cry-1* in vitro [17]. Collectively, these data suggest the existence of a gene network between Ets-1 and clock proteins that regulate clock-output genes and circadian processes.

We have also characterised the nature of the interaction between clock genes and the *PAI-1* promoter. Previous studies have shown that other E-box containing promoters also have a preference for BMAL2 containing herterodimers [18], although the mechanism for this remains to be elucidated. Our results demonstrate that not only can clock proteins distinguish between E-box-like sequences (CACGTG versus CACGTA) but also between apparent identical palindromic E-boxes (E-box1 and E-box2) in the *PAI-1* promoter. The latter may be due to the immediate 3′ flanking sequence, which is "G" for E-box1 and "A" for E-box2. Hogenesch et al. [18] have demonstrated that clock protein heterodimers have a preference for E-boxes with flanking region specificity for an 3′ "A" and this may explain the greater effect on activation by clock protein heterodimers we observed with disruption with E-box2. Since mutation of either E-box caused a major disruption of activation, our results suggest transcriptional cooperation between the two E-boxes. We propose that E-box2 (and to some extent E-box1) determines the overall level of *PAI-1* gene expression, but E-box1 (coupled with the adjacent 4G/5G polymorphism) drives, at least in part, the circadian expression of PAI-1 activity in humans.

The 5′-sequence to E-box2 (known as the 3-bp spacer, 5′-**AAT**CACGTG) is also essential for TGFβ activation of *PAI-1* gene expression, and mutation of E-box2 abolishes the TGFβ-induced transcription of the human *PAI-1* gene [19]. This might explain the differential binding capacity of clock protein heterodimers to these two canonical E-boxes, and provides a further level of human *PAI-1* regulation. Interestingly, several recent reports have demonstrated that stimuli such as interleukin-1, very low density lipoproteins, and angiotensin II increase PAI-1 expression in vitro preferentially via the 4G allele [3,20,21], suggesting a possible interaction between clock genes and these stimuli.

Further support for the regulation of PAI-1 by clock genes comes from rodent studies. A diurnal expression of *PAI-1* mRNA exists in the mouse heart, which reflects a similar rhythm in plasma PAI-1 activity: these rhythms are abolished in the *Clock* mutant mouse [22,23]. Of interest, inspection of the mouse and rat *PAI-1* proximal promoter reveals a conserved canonical E-box (CACGTG) located at bp −179/−174 and −190/−185, respectively, relative to the TSP.

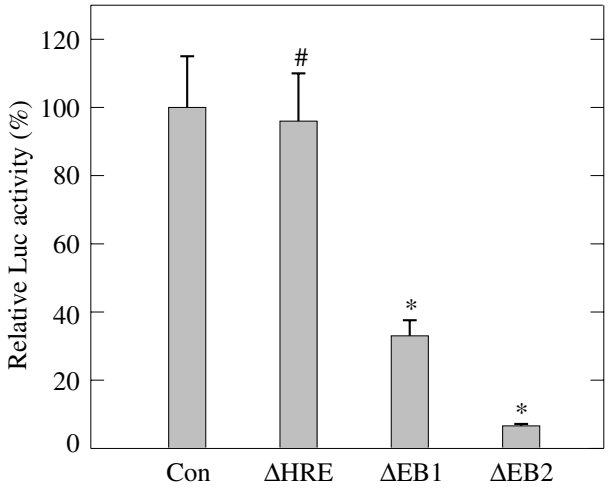

Fig. 2. Palindromic E-boxes confer high-level activation of the human *PAI-1* promoter by BMAL2/CLOCK heterodimers. Co-transfection analysis using different point mutated 4G *PAI-1* promoter-reporter and clock proteins. In the control (Con) experiments using the intact full length *PAI-1* reporter, the Luc activity was set to 100%. ΔHRE, mutated HRE at position −197 bp; ΔEB1, mutated E-box1 at −675 bp; ΔEB2, mutated E-box2 at −560 bp. $^{\#}P > .05$ versus Con; $^{*}P < .005$ versus Con. $N = 3$ independent experiments determined in triplicate. Error bars represent S.E.M.

Our findings have potential pathophysiological relevance. Myocardial infarction shows a significant diurnal variation with a surge in risk in the early morning [24]. Although the overall contribution of the 4G/5G polymorphism to plasma PAI-1 levels has been estimated to be relatively small [7], our results, nonetheless, suggest that increased circadian expression of the 4G allele due to its enhanced interaction with clock proteins could contribute to the surge in MI risk in the early morning [24]. This could in turn explain the reported associations between both plasma PAI-1 level and the 4G/5G polymorphism with risk of MI [4–6,25]. As such this would be one of the first examples of allele-specific clock gene mediated activation of an output gene that could contribute to disease.

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