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Regulation of the human prostacyclin receptor gene in megakaryocytes: Major roles for C/EBPδ and PU.1

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ABSTRACT

The prostanoid prostacyclin plays a central role in haemostasis and vascular repair. Recent studies investigating the regulation of the human prostacyclin receptor (hIP) gene identified an upstream repressor region (URR) within its regulatory promoter, herein termed the PrmIP. This study aimed to identify the main *trans*-acting factors that bind within the URR to transcriptionally repress PrmIP-directed gene expression in the megakaryoblastic human erythroleukemia (HEL) 92.1.7 cell line. Of the putative *cis*-acting elements examined, disruption of C/EBP and PU.1 elements within the URR substantially increased PrmIP-directed gene expression. Chromatin immunoprecipitation (ChIP) confirmed that C/EBPδ and PU.1, but not C/EBPβ, bind to the URR *in vivo*, while ectopic expression of C/EBPδ substantially reduced hIP mRNA levels and PrmIP-directed gene expression. Phorbol 12-myristate 13-acetate (PMA)-induced megakaryocytic differentiation increased hIP mRNA and PrmIP-directed reporter gene expression and hIP-mediated cAMP generation in HEL cells. Two PMA-responsive regions, termed PRR1 and PRR2, were identified within PrmIP. Disruption of C/EBPδ and PU.1 *cis*-elements within the overlapping PRR1/URR and of Sp1, PU.1 and Oct-1 *cis*-elements within the overlapping PRR2/core PrmIP, revealed that both PRR1 and PRR2 contribute to the PMA- induction of hIP mRNA and gene expression in HEL cells. Furthermore, ChIP analysis established that induction of PrmIP-directed gene expression during megakaryocytic differentiation is largely regulated by PMA-induced dissociation of C/EBPδ and enhanced binding of PU.1 to PRR1 in addition to increased binding of Sp1, PU.1 and Oct-1 to elements within the core promoter/PRR2 *in vivo*. Taken together, these data provide critical insights into the transcriptional regulation of the hIP gene within the vasculature, including during megakaryocytic differentiation.

1. Introduction

Prostacyclin, also referred to as prostaglandin (PG) I_2, is a member of the prostanoid family of bioactive lipids derived from arachidonic acid through the sequential actions of cyclooxygenase (COX)-1/2 and prostacyclin synthase [1,2]. Prostacyclin primarily signals through its cognate receptor referred to as the IP, a G-protein coupled receptor (GPCR), to mediate a diversity of physiologic actions. Within the vasculature, it serves as a potent vasodilator and is the major inhibitory prostanoid in platelet aggregation where its actions generally oppose those of thromboxane (TX) A_2 [3–5]. Hence, alterations or imbalances in the levels of prostacyclin and/or TXA_2 or of their specific synthases or receptors (IP and TP, respectively) have been implicated in a wide range of vascular disorders including thrombosis, stroke, myocardial infarction, atherosclerosis and systemic or pulmonary hypertension [6–8]. Consistent with this, $IP^{-/-}$ null mice display enhanced thrombotic tendency in response to endothelial damage [9,10]. Furthermore, $IP^{-/-}$ mice exhibit diminished pain perception and substantially reduced acute inflammatory responses, highlighting additional roles for prostacyclin in nociception and as a potent pro-inflammatory mediator [9–11]. Prostacyclin also acts as a critical protective agent against coronary artery disease (CAD) [12], mediated at least in part through its inhibition of leukocyte–endothelial cell interaction [13] and its promotion of endothelial cell survival and proliferation, supporting neovascularisation and angiogenesis [4,5,14]. Additionally, a recent study established that transcriptional expression of the human (h) IP is directly up-regulated by estrogen, suggesting that some of the known cardioprotective effects of estrogen within the female vasculature may be mediated through its ability to regulate hIP expression levels within the vasculature [15].

Abbreviations: CAD, coronary artery disease; C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; COX, cyclooxygenase; C-LH, cut-like homeodomain; CVD, cardiovascular disease; DMSO, dimethyl sulfoxide; ERE, estrogen response element; FBS, fetal bovine serum; HEL, human erythroleukemia; hIP, human prostacyclin receptor; IgG, immunoglobulin G; IP, prostacyclin receptor; Nrf, Nuclear respiratory factor; PMA, phorbol 12-myristate 13-acetate; pGL3B, pGL3Basic; pRL-TK, pRL-thymidine kinase; PRR, PMA-responsive region; QT, quantitative reverse-transcriptase; RLU, relative luciferase unit; SEM, standard error of the mean; SNP, single-nucleotide polymorphism; STAT, Signal Transduction And Transcription; TI, transcription initiation; SDM, site-directed mutagenesis; TX, thromboxane; URR, upstream repressor region

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In keeping with the diverse actions of prostacyclin, the hIP is widely expressed throughout the vasculature, including platelets/megakaryocytes, macrophages, vascular endothelial and smooth muscle cells, as well as in the heart, lung, kidney and sensory neurons of the dorsal root ganglion [1,9,10]. The hIP is primarily coupled to Gs-activation of adenylyl cyclase, mediating prostacyclin inhibition of platelet aggregation and vascular tone, but may also regulate a number of other secondary effectors [16,17]. The hIP itself is subject to complex regulation through post-translational lipid modifications including isoprenylation and palmitoylation that greatly influence its primary signalling and functional expression [18–20]. Processing of the newly synthesised hIP is proofed by the unfolded protein response (UPR), while the functionally mature hIP undergoes agonist-induced trafficking through a Rab5a- and Rab11a-dependent mechanism involving a direct interaction between the hIP with Rab11a [21–25]. More recently, it has been established that the hIP can also directly interact with multi-Postsynaptic density-95, Discs large, Zonula occludens-1 (PDZ) domain containing protein PDZ domain-containing protein 1 (PDZK1) and that disruption of this interaction substantially impairs prostacyclin mediated endothelial cell migration and *in vitro* angiogenesis [26].

Hence, there have been significant advances in knowledge of the complex mechanisms of signalling and regulation of the hIP which have, in turn, greatly advanced the understanding of the protective role of prostacyclin and of the hIP within the vasculature including of its role in promoting angiogenesis and/or vascular repair in response to injury [4,5,14]. Furthermore, recent genome-association studies have provided additional evidence of their functional importance within the vasculature. For example, several single nucleotide polymorphisms (SNPs) have been identified within the coding sequence of the hIP that correlate with predisposition to cardiovascular disease (CVD) including enhanced intimal hyperplasia and platelet activation in deep vein thrombosis in individuals carrying the synonymous V53V/S328S and non-synonymous R212C mutations, respectively [27,28]. More recently, 3 additional dysfunctional non-synonymous SNPs were identified within the hIP and the occurrence of major coronary artery obstruction is significantly elevated in CAD subjects carrying any of those dysfunctional hIP variants [29]. While SNPs have also been identified in the 5′ flanking region of the hIP gene (PTGIR) [30], as the regulatory promoter of the hIP gene remains relatively uncharacterised, however, it is currently unknown whether such SNPs may also contribute to population variations in hIP expression levels and/or to predisposition to CVD/CAD.

Through recent studies, we have initiated a series of investigations to obtain a greater understanding of the factors that regulate expression of the hIP gene in cells derived from the human vasculature, including the megakaryoblastic human erythroleukemia (HEL) 92.1.7 cell line as well as in vascular endothelial and aortic smooth muscle cells [15,31]. Through one of those studies, we established that hIP gene expression is under the basal transcriptional regulation of Sp1, PU.1 and Oct-1 through their binding to *cis*-acting elements within the proximal core promoter [31]. In that same study, a major upstream repressor region (URR) was also identified but remained to be characterised. Hence, a central aim of this study was to characterise the URR identified within the hIP regulatory promoter, herein termed the PrmIP, seeking to identify the *cis*-acting elements and *trans*-acting factors that regulate transcriptional repression of the hIP gene in HEL cells. The pluripotent HEL cell line is frequently employed as an early megakaryoblastic model and can be induced to undergo differentiation toward the platelet progenitor megakaryocytic phenotype by cytokines, growth factors and phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) [32]. Hence, given the critical role of prostacyclin-mediated signalling in platelets, the current study also sought to investigate the influence of phorbol ester-induced megakaryocytic differentiation of HEL cells on hIP gene expression. Herein, we have uncovered a novel mechanism of transcriptional repression and PMA-induction of PrmIP-directed gene expression that occurs through the regulated binding of C/EBPδ and PU.1 to the URR and of Sp1, PU.1 and Oct-1 to the core promoter region, respectively. These studies greatly advance the understanding of the mechanisms of regulation of the hIP gene within the vasculature, including during megakaryocytic differentiation of the model platelet progenitor HEL cell line.

2. Materials and methods

2.1. Materials

Dual Luciferase® Reporter Assay System, pGL3Basic (PGL3B) and pRL-Thymidine Kinase (pRL-TK) were from Promega. DMRIE-C®, RPMI 1640 culture media and fetal bovine serum (FBS) were from Invitrogen. *Anti*-C/EBPβ (sc-150 X), *anti*-C/EBPδ (sc-636 X), *anti*-Sp1 (sc-59 X), *anti*-PU.1 (sc-22805 X), *anti*-Oct-1 (sc-232 X), normal rabbit IgG (sc-2027), goat *anti*-rabbit horseradish peroxidase (sc-2004) and mouse *anti*-goat horseradish peroxidase (sc-2354) were from Santa Cruz biotechnology. *Anti*-HDJ-2 antibody (MS225 P1ABX) was from Neomarkers.

2.2. Construction of luciferase-based genetic reporter plasmids

The plasmid pGL3B:PrmIP, encoding PrmIP (nucleotides −2449 to −772, relative to the translational start codon at +1) from the human IP gene in the pGL3B reporter vector, in addition to pGL3B:PrmIP1, pGL3B:PrmIP2, pGL3B:PrmIP3, pGL3B:PrmIP4, pGL3B:PrmIP5, pGL3B:PrmIP6 and pGL3B:PrmIP7 were previously described [31].

2.3. Site-directed mutagenesis

Site-directed mutagenesis (SDM) was carried out using the QuikChange™ method (Agilent). The identities of the PrmIP elements subjected to SDM, with their starting positions in brackets, the nucleotides that were changed in underlined bold, templates used and names of the corresponding plasmids generated, as well as the identity, sequence and corresponding nucleotides of the specific primers used are listed below.

1. p53 (−1472), from ggCATGTct to ggCAT**AC**ct using template pGL3B:PrmIP4 to generate pGL3B:PrmIP4$^{\overline{p53}*}$. Primers Kin 756 (5′-CAGGCTCGAGGGACTGGCAT**AC**CTCTCTCTGGCCAAGC-3′) and complementary Kin 757.
2. PU.1 (−1454), from ccTTCCtc to ccTT**T**Ctc using template pGL3B:PrmIP4 to generate pGL3B:PrmIP4$^{\overline{PU.1}(a)*}$. Primers Kin887 (5′-CTGGCCAAGCCTCCTT**T**CTCAGCTTTCTGGAAG-3′) and complementary Kin888.
3. STAT (−1433), from ctGGAAgg to ct**A**G**C**Agg using template pGL3B:PrmIP4 to generate pGL3B:PrmIP4$^{\overline{STAT}*}$. Primers Kin837 (5′-CTCCTTCCTCAGCTTTCT**A**G**C**AGGAGTGAATTGTGTC-3′) and complementary Kin838.
4. PU.1 (−1375), from gaGGAAtt to gaGG**G**Att using template pGL3B:PrmIP4 to generate pGL3B:PrmIP4$^{\overline{PU.1}(b)*}$, and using template pGL3B:PrmIP4$^{PU.1(a)*}$ to generate pGL3B:PrmIP4$^{PU.1(a,b)*}$. Primers Kin889 (5′-CACTACATCAGAGAGG**G**ATTTCCTGGTCATTTC-3′) and complementary Kin890.
5. Nrf1 (−1356), from tgGTCATtt to tgGT**TG**Ttt using template pGL3B:PrmIP4 to generate pGL3B:PrmIP4$^{\overline{Nrf1}*}$. Primers Kin833 (5′-CAGAGAGGAATTTCCTGGT**TG**TTTCTCAATCCCTGGGC-3′) and complementary Kin834.
6. NFκB (−1359), from gaGGAATTTCCtg to gaGGA**C**TTTCCtg using template pGL3B:PrmIP4 to generate pGL3B:PrmIP4$^{\overline{NFκB}(a)*}$. Primers Kin655 (5′-CACTACATCAGAGAGGA**C**TTTCCTGGTCATTTCTC-3′) and complementary Kin656.
7. NFκB (−1363), from gaGGAATTTCCtg to gaGGAAT**C**TCCtg using template pGL3B:PrmIP4 to generate pGL3B:PrmIP4$^{\overline{NFκB}(b)*}$. Primers