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Regulated expression of the prostacyclin receptor (IP) gene by androgens within the vasculature: Combined role for androgens and serum cholesterol

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ABSTRACT

The prostanoid prostacyclin plays a key cardioprotective role within the vasculature. There is increasing evidence that androgens may also confer cardioprotection but through unknown mechanisms. This study investigated whether the androgen dihydrotestosterone (DHT) may regulate expression of the prostacyclin/I prostanoid receptor or, in short, the IP in platelet-progenitor megakaryoblastic and vascular endothelial cells. DHT significantly increased IP mRNA and protein expression, IP-induced cAMP generation and promoter (PrmIP)-directed gene expression in all cell types examined. The androgen-responsive region was localised to a *cis*-acting androgen response element (ARE), which lies in close proximity to a functional sterol response element (SRE) within the core promoter. In normal serum conditions, DHT increased IP expression through classic androgen receptor (AR) binding to the functional ARE within the PrmIP. However, under conditions of low-cholesterol, DHT led to further increases in IP expression through an indirect mechanism involving AR-dependent upregulation of SCAP expression and enhanced SREBP1 processing & binding to the SRE within the PrmIP. Chromatin immunoprecipitation assays confirmed DHT-induced AR binding to the ARE *in vivo* in cells cultured in normal serum while, in conditions of low cholesterol, DHT led to increased AR and SREBP1 binding to the functional ARE and SRE *cis*-acting elements, respectively, within the core PrmIP resulting in further increases in IP expression. Collectively, these data establish that the human IP gene is under the transcriptional regulation of DHT, where this regulation is further influenced by serum-cholesterol levels. This may explain, in part, some of the protective actions of androgens within the vasculature.

1. Introduction

The prostanoid prostacyclin, or prostaglandin (PG)I_2, is synthesized from arachidonic acid (AA) by the sequential actions of cyclooxygenase (COX)1/2 and prostacyclin synthase (PGIS) mainly within the vascular endothelium and smooth muscle [1]. Prostacyclin predominantly signals through its cognate I Prostanoid receptor or, in short, the IP, a G protein coupled receptor (GPCR) primarily coupled to Gs-mediated activation of adenylyl cyclase, increasing cellular cAMP levels [2,3]. The protective role of prostacyclin within the vasculature is well documented, where it largely counteracts the actions of the pro-thrombotic prostanoid thromboxane (TX)A_2 inhibiting platelet activation/aggregation while also acting as a potent vasodilator, and promotes re-endothelialisation/blood vessel repair in response to injury [4]. Imbalances in the levels of prostacyclin, or of its synthase or its receptor, the IP, have been implicated in several cardiovascular diseases (CVDs) including atherothrombosis, thrombotic stroke, myocardial infarction and in pulmonary arterial hypertension [5–7]. Furthermore, several single nucleotide polymorphisms occur within the human IP gene, the PTGIR, that correlate with receptor dysfunction including enhanced platelet activation in deep vein thrombosis and increased intimal hyperplasia, accelerating atherothrombotic events [8,9]. The cardioprotective role of the prostacyclin/IP axis is also highlighted in mouse model systems. For example, $IP^{-/-}$ mice display increased incidence of occlusive thrombi and enhanced predisposition towards atherosclerosis and restenosis compared to wild type mice

Abbreviations: AA, arachidonic acid; ActD, actinomycin D; ADT, androgen deprivation therapy; AR, androgen receptor; ARE, androgen response element; ARR, androgen responsive region; bHLH-LZ, basic helix-loop-helix leucine zipper; BIC, bicalutamide; ChIP, chromatin immunoprecipitation; CHO, cholesterol; CHX, cycloheximide; COX, cyclooxygenase; CSS, charcoal stripped serum; CVD, cardiovascular disease; DAPI, 4′-6-diamidino-2-phenylindole; DHT, 5α-dihydrotestosterone; E_2, estrogen; ER, estrogen receptor; ERE, estrogen response element; ENZ, enzalutamide; FBS, fetal bovine serum; GPCR, G-protein coupled receptor; HEL, human erythroleukemia; HF, hydroxyflutamide; hIP, human I prostanoid receptor/prostacyclin receptor; 1°HUVECs, primary human umbilical vein endothelial cells; IP, I prostanoid receptor; LCS, low cholesterol serum; NS, normal serum; PGI_2, prostaglandin I_2; PGIS, prostacyclin synthase; PK, protein kinase; PrmIP, hIP promoter; PTGIR, hIP gene; qRT-PCR, quantitative reverse-transcriptase PCR; SCAP, SREBP cleavage activating protein; SREBP, sterol regulatory element binding protein; SRE, sterol response element.

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[10,11]. Moreover, endothelial progenitor cells (EPCs) from $IP^{-/-}$ mice fail to promote re-endothelialisation/vessel repair in mice challenged with wire-induced vessel injury, highlighting the central role of the prostacyclin-IP axis in limiting neointimal hyperplasia/restenosis following endothelial injury [12].

Given the significant protective roles of the IP in the vasculature, identifying the factors that regulate expression of the human IP receptor gene (the PTGIR) is of significant interest to understanding the etiology and/or predisposition to such vascular diseases [13]. In addition to prostacyclin, the benefits of low serum cholesterol in protecting against CVDs are widely accepted. Indeed, some of the benefits of low serum cholesterol have been partly attributed to its influence on prostacyclin within the vasculature [14]. For example, reductions in LDL-cholesterol increases prostacyclin generation in endothelial cells (ECs) and occurs through the transcriptional upregulation of COX2, but not COX1 or PGIS, by binding of the cholesterol-responsive transcription factor sterol response element binding protein (SREBP) 1 to a sterol response element (SRE) within the COX2 promoter [15]. Moreover, and consistent with this, reduced serum cholesterol can also increase PTGIR/IP expression and function within the vasculature and this occurs through a similar transcriptional mechanism involving direct binding of the cholesterol-responsive SREBP1 to a conserved *cis*-acting SRE within the core promoter region of the PTGIR gene [16].

Marked differences occur in the age-of-onset and risk of developing CVD between men and women, where women will typically exhibit cardiovascular problems approximately 10 years later than men. Such gender-related differences in the rate of occurrence of CVD has been largely attributed to the cardioprotective role of 17β-estradiol, or estrogen, in younger women, effects that are lost in estrogen-deplete females and/or in older women post-menopause [17–20]. In parallel with the protective effects of low LDL cholesterol and prostacyclin, it is now also widely accepted that some of the cardioprotective effects of estrogen (E_2) are also mediated through its ability to regulate expression and signalling by the protective prostacyclin/IP axis. For example, E_2 increases expression of COX1/2 and PGIS [21], resulting in up to 6-fold elevations in systemic prostacyclin levels, while female $IP^{-/-}/LDLR^{-/-}$ double null mice lose the atheroprotective effects of E_2 observed in $LDLR^{-/-}$ single knockout mice [22]. Moreover, E_2 can directly upregulate expression of the IP through a transcriptional mechanism involving direct binding of the estrogen receptor (ER)α to a highly conserved estrogen response element (ERE) within the PTGIR promoter [23].

Similar to the protective effects of estrogens in women, it is now also suggested that androgens may also confer certain cardioprotective effects in men although this is not without controversy [24–27]. Indeed, recent studies show a clinical correlation between low testosterone levels and risk/onset of CVD. For example, there is an increased incidence of CVD in patients where androgen levels are insufficient/deficient, including in men with hypogonadism and in men undergoing androgen deprivation therapy (ADT) as part of their treatment for prostate cancer [28–30]. Consistent with this, a 5 year study in elderly men showed that high serum testosterone was associated with a decreased risk of cardiovascular incidents [31]. Testosterone, or its more active metabolite 5α-dihydrotestosterone (DHT), mainly signals through the androgen receptor (AR), a member of the nuclear receptor family of steroid hormone receptors [32]. Androgen (testosterone/DHT)-binding to the AR induces a conformational change in the AR involving release of several heat shock proteins, AR-phosphorylation, homodimerisation and transcriptional activation of target genes with *cis*-acting androgen response elements (AREs) within their regulatory promoter regions [33]. The role of the AR in conferring protection against atherosclerosis was demonstrated in $AR^{-/-}$ null mice where double $AR^{-/-}/ApoE^{-/-}$ null mice show an increased rate of atherosclerotic events compared to the single knockouts [34]. Furthermore, administration of testosterone reduced the rate of atherosclerosis in both $ApoE^{-/-}$ mice and $AR^{-/-}/ApoE^{-/-}$ mice, suggesting that there may be androgen-independent as well as androgen-dependent mechanisms involved in this atheroprotection [34]. In addition to this, androgens may also influence expression of genes involved in cholesterol (CHO) metabolism, including in the co-ordinate upregulation of genes involved in both fatty acid and CHO synthesis [35]. Noteworthy, the observed androgen-mediated upregulation of genes involved in lipid/CHO metabolism does not occur through the classic direct AR-dependent mechanism, but rather occurs through a novel, indirect AR-independent mechanism of androgen action involving AR-induced activation of the aforementioned cholesterol-responsive SREBP and, in turn, leading to transcriptional upregulation of SREBP-SRE targeted genes [36,37].

However, despite such convincing evidence supporting the role of androgens in cardioprotection in men, the underlying mechanism(s) whereby this occurs remain largely unknown. In view of the critical role of prostacyclin within the vasculature, including in mediating many of the atheroprotective effects of both reduced serum cholesterol and estrogen (E_2), a compelling question arises regarding the possible influence of androgens on the prostacyclin-IP axis. Hence, focussing on the IP, the aim of the current study was to investigate whether the androgen dihydrotestosterone (DHT) regulates expression and function of the IP within the vasculature and to explore the mechanism(s) by which this regulation might occur. Herein, we uncovered a functional ARE located in the core promoter region of the PTGIR, lying within close proximity to the previously discovered cholesterol-responsive SRE [16]. Furthermore, it was established that DHT significantly increased IP expression in cultured vascular endothelial cells and in platelet-progenitor human erythroleukemic (HEL) cells which occurred through the direct binding of the AR to the ARE located within the core promoter. Moreover, it was discovered that in conditions of low cholesterol, DHT can lead to a further increase on IP/PTGIR expression through a co-ordinated mechanism involving binding of the AR and SREBP to the ARE and SRE, respectively, which lie in close proximity to each other within the core PTGIR promoter. Collectively, this study not only greatly advances understanding of the factors determining the transcriptional regulation of the PTGIR, but also helps to delineate the possible mechanisms whereby androgens may offer cardioprotection including in response to serum-cholesterol levels.

2. Materials and methods

2.1. Materials

Dual Luciferase® assay system, pGL3Basic (pGL3B) and pRL-Thymidine Kinase (pRL-TK) were from Promega. The DMRIE-C®, RPMI 1640 media, L-glutamine, fetal bovine serum (FBS), Alexa Fluor 488-conjugated *anti*-rabbit IgG, Superscript reverse transcriptase and TRIzol were from Invitrogen Life Sciences. Dulbecco's Modified Eagle Medium (DMEM) and Endothelial Cell Growth Supplement/Heparin (ECGS/H) were from Lonza. Effectene® was from Qiagen. Low cholesterol serum was from Panbiotech. The Brilliant II Sybr Green QPCR kit and pCRE-luc were from Agilent. *Anti*-AR (sc-816X), *anti*-SREBP1 (sc-8984X), goat *anti*-rabbit horseradish peroxidase (HRP; sc-2004), goat *anti*-mouse HRP (sc-2005) were from Santa Cruz Biotechnology. *Anti*-HDJ2 was from Neomarkers. Medium 199, dextran-coated charcoal, actinomycin D (ActD), cycloheximide (CHX), β-estradiol (E_2), dihydrotestosterone (DHT), ICI 182 780, hydroxyflutamide (HF) and bicalutamide (BIC) were from Sigma. Enzalutamide (ENZ) was from Selleck Biochem. All oligonucleotides were from Sigma-Genosys and *small interfering* (*si*) RNAs were synthesized by Eurofins Scientific.

2.2. Cell culture

Human erythroleukemic (HEL) 92.1.7 cells, obtained from American Type Culture Collection, were cultured in RPMI 1640, 10% fetal bovine serum (FBS). Human endothelial EA.hy926 cells, obtained from the Tissue Culture Facility at UNC Lineberger Comprehensive Cancer Centre, Chapel Hill, NC, were cultured in DMEM, 10% FBS. Primary (1°) human