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Epidermal growth factor receptor transactivation by intracellular prostaglandin E_2-activated prostaglandin E_2 receptors. Role in retinoic acid receptor-β up-regulation

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ABSTRACT

The pharmacological modulation of renoprotective factor vascular endothelial growth factor-A (VEGF-A) in the proximal tubule has therapeutic interest. In human proximal tubular HK-2 cells, treatment with all-trans retinoic acid or prostaglandin E_2 (PGE_2) triggers the production of VEGF-A. The pathway involves an initial increase in intracellular PGE_2, followed by activation of EP receptors (PGE_2 receptors, most likely an intracellular subset) and increase in retinoic acid receptor-β (RARβ) expression. RARβ then up-regulates transcription factor hypoxia-inducible factor-1α (HIF-1α), which increases the transcription and production of VEGF-A. Here we studied the role in this pathway of epidermal growth factor receptor (EGFR) transactivation by EP receptors. We found that EGFR inhibitor AG1478 prevented the increase in VEGF-A production induced by PGE_2- and all-trans retinoic acid. This effect was due to the inhibition of the transcriptional up-regulation of RARβ, which resulted in loss of the RARβ-dependent transcriptional up-regulation of HIF-1α. PGE_2 and all-trans retinoic acid also increased EGFR phosphorylation and this effect was sensitive to antagonists of EP receptors. The role of intracellular PGE_2 was indicated by two facts; i) PGE_2-induced EGFR phosphorylation was substantially prevented by inhibitor of prostaglandin uptake transporter bromocresol green and ii) all-trans retinoic acid treatment, which enhanced intracellular but not extracellular PGE_2, had lower effect on EGFR phosphorylation upon pre-treatment with cyclooxygenase inhibitor diclofenac. Thus, EGFR transactivation by intracellular PGE_2-activated EP receptors results in the sequential activation of RARβ and HIF-1α leading to increased production of VEGF-A and it may be a target for the therapeutic modulation of HIF-1α/VEGF-A.

1. Introduction

Critical mediators of cellular adaptation to hypoxia are hypoxia-inducible factors (HIFs), HIF-1 and HIF-2 being the most prominent and best characterized. HIF is a transcription factor that consists of an oxygen-sensitive α-subunit, HIF-α and a constitutively expressed β-subunit, HIF-β. In a hypoxic environment HIF-α is stabilized and translocates to the nucleus, and, together with the β subunit and transcriptional coactivators, it binds to hypoxia-responsive elements (HRE) in target genes [1]. HIF-1α supports tissue survival in hypoxia by regulating the expression of gene products that are involved in cellular energy metabolism, angiogenesis, erythropoiesis and other biological processes [2]. These products include enzymes involved in glucose uptake and metabolism, carbonic anhydrase IX, erythropoietin and vascular endothelial growth factor (VEGF) [3]. HIF-1α expression is also increased in normoxia: growth and coagulation factors, hormones, stress factors and inflammatory mediators such as prostaglandin E_2 (PGE_2), cytokines, interleukin-1β, tumor necrosis factor-α, nitric oxide and reactive oxygen species increase the expression of HIF-1α [4,5]. They increase HIF-1α transcription and/or translation [4,6] but not HIF-1α protein stability.

All-trans retinoic acid (ATRA) is the carboxylic acid form of vitamin A and its major metabolite. The actions of ATRA are generally mediated by binding to retinoic acid receptors (RARs), which are members of the nuclear receptor superfamily. RARs are key regulators of multiple physiological processes, most significantly embryonic development and organ homeostasis. At the cellular level, RARs contribute to the regulation of gene networks that control cell growth, differentiation, survival and death [7]. Three RAR subtypes (RARα, RARβ and RARγ) have been identified. They act as ligand-regulated transcription factors by dimerizing with retinoid X receptors (RXR) and binding to retinoic

Abbreviations: $iPGE_2$, intracellular prostaglandin E_2; EPR, EP receptors; RARβ, retinoic acid receptor-β; HIF-1α, factor hypoxia-inducible factor-1α; VEGF-A, factor vascular endothelial growth factor-A; EGFR, epidermal growth factor receptor; ATRA, all-trans retinoic acid; RARE, retinoic acid receptor response element; BG, bromocresol green

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acid response elements (RARE), which are located in the regulatory regions of their target genes [8]. RARβ itself is a RARs target [9]: its promoter region contains a RARE (β-RARE) responsible for the up-regulation of RARβ induced by ATRA in many different cell types [10].

We have recently shown in human renal proximal tubular HK-2 cells that the up-regulation of RARβ by cyclooxygenase (COX)-dependent production of PGE_2 plays a critical role in the increase in HIF-1α expression induced by ATRA, interleukin-1β, PGE_2 analogue 16,16-dimethyl-PGE_2 (16,16-PGE_2) or hypoxia [11,12]. Interestingly, the transcriptional inhibitor actinomycin D prevented the increase in RARβ and HIF-α expression induced in normoxia but not in hypoxia [13].

It is accepted that, once prostanoids are formed, they are quickly released to the outside of cells by simple diffusion and act as autocrine or paracrine mediators in the vicinity of their sites of production to maintain local homeostasis [14–17]. Therefore, it has generally been assumed that PGE_2 exerts its actions via plasma membrane spanning G-protein coupled EP receptors (EPR: EP1, EP2, EP3 and EP4), each EP activating a distinct G protein-coupled signaling pathway [14,15]. However, the up-regulation of RARβ in HK-2 cells by HIF-1α-increasing agents was most likely due to intracellular EPR activated by intracellular PGE_2 ($iPGE_2$) [12]. These data are in good agreement with the location of functional EPR at the nuclear membranes of a variety of cell types and tissues [18–20]. In fact, in HK-2 cells the four EPR are located not only in the cell membrane but also inside the cell [11]. We have also provided data on the regulation of RARβ expression by $iPGE_2$ in other cell lines, which indicates that it is not a phenomenon restricted to HK-2 cells [12]. It is important to take into account that the classical downstream plasma membrane EPR-linked signaling cascades pathways activated by PGE_2 may not necessarily be the same ones linked to $iPGE_2$-activated intracellular EPR. For instance: it has been proposed that $iPGE_2$, acting through intracellular EP4 receptors, maintains epidermal growth factor receptor (EGFR) in an activated state in cardiac myocytes [21]. This activation of EGFR through a molecule that does not, itself, bind EGFR is called transactivation.

The elucidation of the mechanism through which $iPGE_2$-activated EPR increase RARβ expression has important implications. For instance, the up-regulation of the RARβ gene by ATRA presumably plays a critical role in amplifying the ATRA response [10]. In our more specific context, we have shown that the increase in the expression of HIF-1α through this pathway enhances the production of VEGF-A, a putative renoprotective factor [12]. In the present work we have investigated the role of EGFR in the $iPGE_2$-dependent pathway leading to the increase in VEGF-A production under normoxic conditions. We have found that the transactivation of EGFR by $iPGE_2$-activated EPR (upon treatment with 16,16-PGE_2 or ATRA) plays a crucial role in the transcriptional up-regulation of RARβ and that the increase in RARβ expression led, in turn, to the transcriptional up-regulation of HIF-1α and, eventually, to the increase in the production of VEGF-A.

2. Material and Methods

2.1. Reagents

16,16-PGE_2, bromocresol green, diclofenac, AH6809 and GW627368X were purchased from Cayman Chemical (Ann Arbor, MI). AG1478, actinomycin D, YC1 and ATRA were purchased from Sigma (St. Louis, MO). RARβ antagonist LE135 was a generous gift from Prof. Hiroyuki Kagechika (Tokyo Medical and Dental University, Japan). Primary antibodies against RARβ and phospho-EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against HIF-1α was purchased from BD Biosciences (Palo Alto, CA) and anti-β-actin antibody was from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture and treatments

Human kidney HK-2 cells, human prostate cancer cells (PC3) and human renal cell carcinoma (A498) were purchased from American Type Culture Collection (Rockville, MD). HK-2 were maintained in DMEM/F12 supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin/amphoterycin B and 1% glycine (Invitrogen, Carlsbad, CA) and 1% Insulin–Transferrine–Selenium (Sigma, St. Louis, MO). PC3 were maintained in RPMI and A498 cells were maintained in MEM Eagle, and supplemented with 10% FBS and 1% penicillin/streptomycin/amphoterycin B.

The culture was performed in a humidified 5% CO_2 environment at 37 °C. In all experiments, cells were plated at 70–90% confluence and 24 h later they were treated with 1 μM 16,16-PGE_2 and/or 10 μM ATRA, for different periods of time. When appropriated, the following agents were added 1 h before: 50 μM bromocresol green (inhibitor of prostaglandin uptake transporter), 0.3 mM diclofenac (COX inhibitor), 10 μM AH6809 (antagonist of EP1-3 receptors) or GW627368X (antagonist of EP4 receptor), 1 μg/ml actinomycin D (transcription inhibitor), 2 μM LE135 (RARβ antagonist), 10 μM YC1 (HIF-1α inhibitor), 1 μM AG1478 (inhibitor of EGFR activation) were added 1 h before the treatment.

2.3. Real-time quantitative RT-PCR

Total cell RNA from HK-2 cells was isolated with TriReagent (Sigma, St. Louis, MO). One microgram of total RNA, extracted by using the TaqMan® Gene Expression Master Mix (Applied Biosystems), was reverse transcribed in a total reaction volume of 25 μl by means of incubation at 42 °C for 30 min using random hexamer primers. For quantitative PCR, mRNAs were amplified and quantified by TaqMan probes (Applied Biosystems) (Table 1). As template, cDNA from the target and housekeeping gene GAPDH were prepared in separate tubes for each primer master mixture reaction. The comparative threshold cycle (CT) method was used to calculate the relative expression [22].

2.4. Protein isolation and Western-blotting

HK-2 cells were stimulated and washed twice with ice-cold PBS and then harvested, scraped into ice-cold PBS, and then pelleted by centrifugation at 500 ×g, for 5 min, at 4 °C. Cells were kept on ice for 30 min in a solution containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and protease inhibitors. Thereafter, the cells were pelleted by centrifugation at 4000 ×g, for 5 min, at 4 °C. Proteins from cell lysates were denatured by heating. Then, they were resolved by 10% SDS-PAGE, and blotted onto a nitrocellulose membrane (BioTrace/NT), for 1 h, in 50 mM Tris–HCl, 380 mM glycine, 0.1% SDS, and 20% methanol. Blots were incubated overnight at 4 °C with rabbit anti-RARβ (1:1000), mouse anti-HIF-1α (1:1000) and rabbit anti-phospho-EGFR (1:500) antibodies. After treatment for 1 h at room temperature with the corresponding secondary antiserum (1:4000), the signals were detected with enhanced chemiluminescence reagent using β-actin antibody (1: 25000) as loading control.

2.5. Determination of PGE_2 formation

PGE_2 was determined in culture medium and cell lysates using a commercially available enzyme immunoabsorbent assay (EIA) kit

Table 1
TaqMan® probes table.

Gene	Applied Biosystems ref.
HIF-1α	Hs00936368m1
RARβ	Hs00977140m1
GAPDH	Hs03929097g1