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The G protein estrogen receptor (GPER) is regulated by endothelin-1 mediated signaling in cancer cells

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ABSTRACT

Endothelin-1 (ET-1) is a potent endogenous vasoconstrictor involved in many diseases, including certain cardiovascular disorders and cancer. As previous studies have shown that the G protein estrogen receptor (GPER) may regulate ET-1 dependent effects on the vascular system, we evaluated whether GPER could contribute to the effects elicited by ET-1 in breast cancer and hepatocarcinoma cells. Here, we demonstrate that ET-1 increases GPER expression through endothelin receptor A (ET_AR) and endothelin receptor B (ET_BR) along with the activation of PI3K/ERK/c-Fos/AP1 transduction pathway. In addition, we show that GPER is involved in important biological responses observed upon ET-1 exposure, as the migration of the aforementioned tumor cells and the formation of tube-like structures in human umbilical vein endothelial cells (HUVECs). Our data suggest that GPER may contribute to ET-1 action toward the progression of some types of tumor.

1. Introduction

The endothelin (ET) family includes three small peptides named ET-1, ET-2 and ET-3 which act in both autocrine and paracrine manner *via* the G protein-coupled receptor namely ET_AR and ET_BR [1]. ETs by binding to the cognate receptors activate diverse transduction pathways like the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), the mitogen-activated protein kinase/extracellular regulated protein kinase (MAPK/ERK), the phosphokinase C (PKC) and the epidermal growth factor receptor (EGFR) [2]. ET-1 is the predominant family's isoform and a potent endogenous vasoconstrictor involved in different pathophysiological responses [1]. ET-1 is mainly produced by somatic cells like endothelial cells, vascular smooth muscle cells, macrophages, fibroblasts and various types of cancer cells [1,2]. Numerous studies have shown that ET-1 is not merely a vasoconstrictor but a multifunctional peptide exerting a cytokine-like activity in diverse conditions like inflammation, pain and cancer [1]. As it concerns cancer, ET-1 elicits pleiotropic effects on tumor cells and the host microenvironment toward cell proliferation, apoptosis, migration, epithelial mesenchymal transition, chemo-resistance and neovascularization [3]. ET-1 was found overexpressed in a variety of tumors like prostatic, ovarian, lung, colorectal and hepatic malignancies [2] in particular, its expression was associated with aggressive features of breast tumors [4]. It was also reported that increased levels of ET-1 and cognate receptors positively correlate with vascular endothelial growth factor (VEGF) signaling as well as microvessel density [5]. Indeed, ET-1 can induce tumor angiogenesis up-regulating VEGF and triggering stimulatory effects in endothelial cells [3].

It has been recently reported that ET-1-dependent vasoconstriction is inhibited by G protein estrogen receptor (GPER) agonists as G-1, raloxifene, fulvestrant and genistein [6–8]. Accordingly, G-1 failed to repress ET-1 mediated vasoconstriction in GPER deficient mice [9]. In this context, it should be mentioned that GPER is expressed throughout the cardiovascular system of humans and animals of both sexes and mediates beneficial effects of estrogens on vascular and myocardial function [7]. GPER activates a network of transduction pathways like EGFR, MAPK, PI3K, intracellular cyclic AMP (cAMP) and calcium mobilization, leading to a characteristic gene signature involved in relevant pathophysiological responses including cancer [10]. The function of GPER in tumorigenesis is still a subject of intense debate. Previous studies have revealed that GPER may induce cell-cycle arrest and the inhibition of cancer cell growth [11,12]. Moreover, further studies have shown that high GPER expression is favorable for the survival of breast and ovarian cancer patients [13,14]. On the contrary, GPER has been reported to trigger diverse pathways toward the expression of genes stimulating tumor cell migration and proliferation both *in vitro* and *in vivo* [10,15]. In patients with endometrial and ovarian tumors, the expression of GPER was also associated with aggressive features and lower survival rates [16,17]. Likewise, increased tumor size and metastasis in human breast

Abbreviations: ET-1, endothelin-1; ET_AR, endothelin receptor A; ET_BR, endothelin receptor B; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; CTGF, connective tissue growth factor; Egr-1, early related gene; HIF-1, hypoxia inducible factor-1; VEGF, vascular endothelial growth factor.

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malignancies correlated with high levels of GPER expression [18]. In addition, in patients treated with tamoxifen GPER was found increased and negatively correlated with relapse-free survival [19]. The overexpression of GPER and its localization to the plasma membrane were suggested to be critical toward the progression of breast cancer, whereas the absence of GPER in the plasma membrane predicted excellent long-term prognosis in breast cancer patients treated with tamoxifen [20]. On the basis of these observations, an increasing interest is currently addressed toward a better understanding of the mechanisms involved in the regulation of GPER expression and function. In this regard, our previous studies have demonstrated that GPER is upregulated by epidermal growth factor (EGF) [10], insulin growth factor (IGF)-I [21,22], insulin [23] and a main factor contributing to tumor aggressiveness like hypoxia [24]. Moreover, estrogenic GPER signaling was shown to induce hypoxia-inducible factor (HIF)1α-dependent expression of VEGF, which then triggered angiogenesis in different breast cancer models [25,26].

As previous investigations evaluated the functional interaction between GPER and ET-1 signaling in the cardiovascular system, here we have ascertained whether GPER might be involved in the biological responses prompted by ET-1 in cancer cells. We show that ET-1 regulates the expression of GPER which contributes to certain effects mediated by ET-1, like cell migration and angiogenesis. Therefore, our data further extend the current knowledge on the molecular mechanisms by which ET-1 can contribute to worse malignant features, highlighting the potential benefits of combinatorial anti-cancer therapies targeting these transduction pathways.

2. Materials and methods

2.1. Reagents

Endothelin-1 (ET-1), BQ123, BQ788 and 17β-estradiol (E2) were purchased from Sigma-Aldrich Corp. (Milan, Italy). Wortmannin (WM) and PD98059 (PD) were bought from Calbiochem (Milan, Italy). (3aS,4R,9bR)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone (G15) were obtained from Tocris Bioscience (Bristol, UK). Human VEGF was from Peprotech (Rocky Hill, New Jersey, USA). All compounds were solubilized in dimethylsulfoxide (DMSO), except ET-1, which was dissolved in 5% acetic acid and VEGF which were solubilized in water.

2.2. Cell cultures

SkBr3 breast cancer, HepG2 hepatocarcinoma and HUVEC human umbilical vein endothelial cells, were obtained by ATCC (Manassas, USA) and used less than six months after revival. SkBr3 were maintained in RPMI-1640 without phenol red (Invitrogen, Milan, Italy) and HepG2 cells were maintained in DMEM medium (Invitrogen, Milan, Italy), with a supplement of 10% fetal bovine serum (FBS) and 100 μg/ml of penicillin/streptomycin (Gibco, Life Technologies, Milan, Italy). HUVECs were seeded on collagen coated flasks (Sigma-Aldrich Srl, Milan, Italy) and cultured in Endothelial Growth Medium (EGM) (Lonza, Milan, Italy), supplemented with 5% FBS (Lonza, Milan, Italy). All cell lines were grown in a 37 °C incubator with 5% CO_2. Cells were switched to medium without serum and phenol red the day before the experiments.

2.3. Plasmids and transfections

The plasmid DN/c-Fos, which encodes a c-Fos mutant that heterodimerizes with c-Fos dimerization partners but does not allow DNA biding, was a kind gift from Dr. C. Vinson (NIH, Bethesda, MD, USA). Short hairpin constructs against human GPER (shGPER) and CTGF (shCTGF) were obtained and used as previously described [22]. In brief, they were generated in the lentiviral expression vector pLKO.1 purchased by Euroclone (Milan, Italy). The targeting strand generated from the shGPER construct is 5′-CGCTCCCTGCAAGCAGTC TTT-3′. The targeting strand generated from the shCTGF construct is 5′-TAGTACAGCGATTCAAAGATG-3′. The lentiviral shRNA direct against Egr-1 (shEgr1) and the scramble hairpin (shRNA) were purchased from Open Biosystem (Celbio, Italy). Transfection assays were performed using the X-tremeGene9 Transfection Reagent (Roche Diagnostics, Milan, Italy) according to the manufacturer's instructions. Briefly, 50% confluent cells were serum deprived and transfected for 36 h with shGPER and 24 h with shCTGF or shEgr-1 or DN/c-Fos, and then treated as indicated.

2.4. Reverse transcription and real-time PCR

Total RNA was extracted using the TRIzol commercial kit (Invitrogen, Milan, Italy) according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis through agarose gel stained with ethidium bromide. Total cDNA was synthesized from RNA by reverse transcription using the murine leukemia virus reverse transcriptase (Invitrogen, Milan, Italy) following the protocol provided by the manufacturer. The expression of selected genes was quantified by real-time PCR using Step One (TM) sequence detection system (Applied Biosystems Inc., Milano, Italy), following the manufacturer's instructions. Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc., Milano, Italy). Assays were performed in triplicate, and the mean values were used to calculate expression levels, using the relative standard curve method. The primers used were 5′-ACACACCTGGGTGGACACAA-3′ (GPER forward), 5′-GGAGCCAGAAGCCACATCTG-3′ (GPER reverse), 5′-CGAGCCCTTTGATGACTTCCTG-3′ (c-Fos forward), 5′-GGAGCGGGCTGT CTCAGA-3′ (c-Fos reverse), 5′-ACCTGTGGGATGGGCATCT-3′ (CTGF forward), 5′-CAGGCGGCTCTGCTTCTCTA-3′ (CTGF reverse), 5′-AAGATC CACTTGCGGCAGAA-3′ (Egr-1 forward), 5′-GCCGAAGAGGCCACAACA-3′ (Egr-1 reverse), 5′-GCTGATTTGTGAACCCATTC-3′ (HIF-1α forward), 5′-CTGTACTGTCCTGTGGTGAC-3′ (HIF-1α reverse), 5′-GAGCTTCAGGACAT TGCTGT-3′ (VEGF forward), 5′-AGGAAGGTCAACCACTCACA-3′ (VEGF reverse), 5′-GGCGTCCCCCAACTTCTTA-3′ (18S forward) and 5′-GGGCATCA CAGACCTGTTATT-3′ (18S reverse). Assays were performed in triplicate and the results were normalized for 18S expression and then calculated as fold induction of RNA expression.

2.5. Western blotting

Cells were grown in 10-cm dishes, transfected and treated as indicated and then lysed in RIPA buffer (Sigma-Aldrich Corp., Milan, Italy) containing a mixture of protease inhibitors (1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 2 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Protein concentrations were determinated according to the Bradford method (Sigma-Aldrich Corp., Milan, Italy). Equal amount of whole protein extracts (10–50 μg of protein) was electrophoresed through a reducing SDS/10% (w/v) polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences Milan, Italy). Membranes were blocked and probed with primary antibodies for HIF-1α (R&D Systems, Inc. Celbio, Milan, Italy), GPER (N-15), CTGF (L-20), c-Fos (H-125), Egr-1 (C-19), phosphorylated ERK ½ (E-4), ERK2 (C-14), phosphorylated Akt 1/2/3 (ser 473), Akt (H-136) and β-actin (C2) purchased from Santa Cruz Biotechnology (DBA, Milan, Italy). The levels of protein and phosphoproteins were detected with appropriate secondary HRP-conjugated antibodies and the ECL (enhanced chemiluminescence) System (GE Healthcare, Milan, Italy). All experiments were performed in triplicate and blots shown are representative.

2.6. Chromatin immunoprecipitation (ChIP)

Cells grown in 10-cm plates were shifted for 24 h to medium lacking serum and then treated with vehicle or ET-1 100 nM for 2 h. Chip assay was performed as previously described [22]. The immune cleared