ENDOTHELIN-1-INDUCED PROSTAGLANDIN E2-EP2, EP4-SIGNALING REGULATES VASCULAR ENDOTHELIAL GROWTH FACTOR PRODUCTION AND OVARIAN CARCINOMA CELL INVASION¹

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Summary

Cyclooxygenase (COX)-1 and COX-2-derived prostaglandins are implicated in the development and progression of several malignancies. We have recently demonstrated that treatment of ovarian carcinoma cells with endothelin-1 (ET-1) induces expression of both COX-1 and COX-2 which contribute to vascular endothelial growth factor (VEGF) production. In this study, we show that, in HEY and OVCA 433 ovarian carcinoma cells, ET-1 through the binding with ET_A receptor (ET_AR) induces prostaglandin E2 (PGE_2) production, as the more represented PG types, and increases the expression of PGE_2 receptor type 2 (EP2) and type 4 (EP4). The use of pharmacological EP agonists and antagonists indicates that ET-1 and PGE_2 stimulate VEGF production principally through EP2 and EP4 receptors. At the mechanistic level, we prove that the induction of PGE_2 and VEGF by ET-1 involves Src-mediated epidermal growth factor receptor transactivation. Finally, we demonstrate that ET_AR-mediated activation of PGE_2-dependent signaling participates in the regulation of the invasive behaviour of ovarian carcinoma cells by activating tumor-associated matrix-metalloproteinase. These results implicate EP2 and EP4 receptors in the induction of VEGF expression and cell invasiveness by ET-1, and provide a mechanism by which ET_AR/ET-1 can promote and interact with PGE_2-dependent machinery to amplifying its proangiogenic and invasive phenotype in ovarian carcinoma cells. Pharmacological blockade of ET_AR can therefore represent an additional strategy to control PGE_2 signaling, which has been associated with ovarian carcinoma progression.
INTRODUCTION

Cyclooxygenase (COX)\(^3\) is the rate-limiting enzyme for the conversion of arachidonic acid to prostaglandins (PG), which include PGE\(_2\), PGD\(_2\), PGF\(_2\alpha\), PGI\(_2\), and tromboxane A\(_2\) (1). Among these, PGE\(_2\), the most common prostanoid, is involved in tumor progression by inducing angiogenesis (2, 3), invasion and metastasis in several solid tumors (4). PGE\(_2\) participates in these complex mechanisms by stimulating vascular endothelial growth factor (VEGF) secretion (4, 5), cell migration and matrix-metalloproteinase-2 (MMP-2) expression and activation (6, 7). PGE\(_2\) exerts its autocrine/paracrine effects on target cells by coupling to four subtypes of G-protein-coupled receptors (GPCR) which have been classified as EP1, EP2, EP3, and EP4 (4). These receptors are often co-expressed in the same cell and utilize alternate and, in some cases, opposing intracellular signaling pathways (8). Among these, EP2 and EP4 are the principal receptors implicated in mediating tumor progression through their ability to induce proangiogenic factor and/or tumor cell invasiveness (6-7, 9, 10). The study of EP receptor signaling pathways is becoming of clinical relevance especially in tumors, such as ovarian carcinoma, where the overexpression of COX-2, the principal enzyme implicated in PGE\(_2\) synthesis, has been identified as an independent prognostic factor associated with reduced survival and poor response to standard combination chemotherapy (11-13).

In ovarian cancer, endothelin-1 (ET-1) plays a key role in the development and progression of this tumor, promoting tumor cell proliferation (14,15), apoptosis protection (16), invasiveness (17), and neovascularization (18-21). ET-1 and its selective receptor ET\(_A\) (ET\(_A\)R) are overexpressed in primary and metastatic ovarian carcinoma compared with normal ovarian tissue (22). ET-1/ET\(_A\)R interaction results in activation of a pertussis toxin-insensitive G protein that stimulates phospholipase C activity and increases intracellular Ca\(^{++}\) levels, activation of protein kinase C (PKC), mitogen-activated protein kinase (MAPK), paxillin and p125 focal adhesion kinase

\(^3\) The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; EP, E prostanoid; GPCR, G-protein-coupled receptor; ET-1, endothelin-1; ET\(_A\)R, endothelin A receptor; MAPK, mitogen-activated protein kinase; EGFR, epidermal
Among downstream events after ETA R activation, ET-1 causes also the epidermal growth factor receptor (EGFR) transactivation, which is partly responsible for MAPK phosphorylation. Recently, we demonstrated that ET-1 through ETA R induces COX-1 and COX-2 expression and that both enzymes contribute to PGE2 and VEGF production in ovarian carcinoma cells. ETA R blockade by the selective receptor antagonist, ABT-627, has been shown to inhibit the growth of ovarian carcinoma xenografts concomitantly with a reduction of microvessel density, MMP-2, VEGF and COX-2 enzyme expression.

To analyze the contribution of the COX pathway in the mechanism by which ET-1 participates in ovarian cancer progression, we investigated the effect of ET-1 on prostaglandin type secretion and EP receptor expression and their roles in ET-1-induced VEGF production and cell invasiveness in the human ovarian carcinoma cells. We demonstrate that in HEY and OVCA 433 cells 1) ET-1 through ETA R increases the production of PGE2, PGF2α and PGD2, and the expression of EP2 and EP4 receptor transcripts, 2) ET-1-induced PGE2, VEGF and cell invasion are mediated through the binding of PGE2 to EP2 and EP4 receptors, 3) ET-1-induced Src-mediated EGFR-transactivation is involved in PGE2 and VEGF production and cell invasiveness. Thus, for the first time, we defined the prostaglandin secretion pattern and the PGE2 receptor subtype expression in ovarian carcinoma cells providing evidence for the involvement of EP2 and EP4 in ET-1-mediated PGE2-dependent pathways. Moreover, the present findings indicate that blockade of ETA R signaling may result in targeting EP receptor activation and related signaling cascades, which contribute to ET-1-mediated ovarian cancer cell invasion.

growth factor receptor; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
MATERIALS AND METHODS

Cells and cell cultured conditions

Human ovarian carcinoma cell lines, HEY and OVCA 433, previously characterized for ET-1 receptor expression and for ET-1 production (14, 22), were cultured in DMEM containing 10% foetal calf serum (FCS) and 1% penicillin-streptomycin at 37°C under 5% CO2-95% air. The cells were serum-starved by incubation for 24 h in serum-free DMEM. All culture reagents were from Invitrogen (Paisley, Scotland, UK). ET-1 (Peninsula Laboratories, Belmont, CA), EGF (Cell Signaling, Beverly, MA) PGE₂ (Sigma, St. Louis, Missouri, USA), 17-phenyl trinor PGE₂ (EP1 and EP3 agonist), butaprost (EP2 agonist), sulprostone (EP3 agonist), 16,16-dimethyl PGE₂ (EP2-4 agonist) (Cayman, Chemical, Ann Arbor, MI), were incubated with the cells for the indicated time. Pretreatment of cells with BQ 123 (Peninsula Laboratories), SC19220 (EP1 antagonist; Cayman Chemical), AH23848 (EP4 antagonist; Sigma), AG1478 and PP2 (Calbiochem-Novabiochem Corporation, San Diego, CA) was performed for the indicated times prior to the addition of ET-1 or PGE₂.

Western Blot Analysis

For detection of VEGF, EGFR, phospho-EGFR, whole cell lysates were subjected to SDS-PAGE (7.5- 12.5 %) and revealed by Western blotting using Abs anti-VEGF (Sigma), or anti-EGFR, or anti-phospho 845-EGFR (Cell Signalling, Beverly, MA). Blots were developed with the enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, UK). The membranes were reprobed with anti-β-actin to assure the equal amount of protein (Oncogene, CN Biosciences, Inc., Darmstadt, Germany).
**Reverse transcription-PCR**

Total RNA from HEY and OVCA 433 cells was extracted using TRIzol (Invitrogen). RT-PCR was performed using a AccessQuick RT-PCR System (Promega, Madison, WI) according to the manufacturer’s instructions. The primers sets were as follows: EP1, 5’-CCTGTCGTTATCAGTTGTTGTC-3’ and 5’-GGTTGTGCTTAGAAATGGGCTGAGG-3’; EP2, 5’-GCCACGATGCTCATCCTCTTCGCC-3’ and 5’-CTTGTGTTCCTTATGAATCCCGAC-3’; EP3, 5’-GCATAACTGGGGCAAAACCTTTTCTTCGCC-3’ and 5’-CTTAAACAGCAGTAAACCCCAAGGATCC-3’; EP4, 5’-TGGTATGTGGCTGCT-3’ and 5’-GAGGAACCAGTGCGGAGAAT-3’; VEGF, 5’-GGCTCTAGATCGGGCCTCCGAAACCAT-3’ and 5’-GGCTCTAGAGCGCAGAGTCTCCT-3’; GAPDH, 5’- ACCACAGTCATGCCCATCAC-3’ and 5’-ACCACAGTCATGCCCATCAC-3’ and 5’-TCCACCACCCTGTGCTGTA-3’. Thirty-five-cycles of amplification was performed under the following conditions: melting at 95°C for 30 s; annealing at 58°C (EP1-4, GAPDH) and 60°C (VEGF) for 30 s; extension at 72°C for 60 s. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

**ELISA**

The VEGF protein levels in the cell conditioned media were determined in triplicate by ELISA using the Quantikine Human VEGF immunoassay kit (R&D Systems, Minneapolis, MN). The sensitivity of the assay is less than 5.0 pg/ml. Intra-assay variation is 5.4% and inter-assay variation is 7.3%. Levels of PGE2, and PGF2\(\alpha\), released into the medium of treated cells were measured by the PGE2-Correlate-EIA, PGF2\(\alpha\)-Correlate-EIA kit (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer’s instructions. Detection of PGD2 was performed by quantitative determination of 15-deoxy-\(\Delta^{12,14}\)-Prostaglandin J2, one of its ultimate dehydration product (27) using 15-deoxy-\(\Delta^{12,14}\)-Prostaglandin J2- Correlate-EIA (Assay Designs, Inc.). The sensitivity of the assay is 13.4 pg/ml for PGE2, 6.71 pg/ml for PGF2\(\alpha\) and 36.8 pg/ml for PGD2. Intra-assay variation is 5.8% for PGE2, 6.8% for PGF2\(\alpha\), 5.7% for PGD2, and inter-assay variation is 5.1% for PGE2, 5.5% for
PGF₂α, 13.0% for PGD₂. Gelatinase activities in cell conditioned medium were determined using a MMP Gelatinase Activity Assay Kit (Chemicon) according to the manufacturer’s instructions. The sensitivity of the assay is less than 5 ng/ml of MMP in a range of 10-200 ng/ml.

Chemoinvasion assay

Chemoinvasion was assessed using a 48 well-modified Boyden chamber (NeuroProbe, Pleasanton, CA) and 8 µm pore PVP-free polycarbonate Nucleopore filters (Costar, New York, NY) as previously described (28). The filters were coated with an even layer of 0.5 mg/ml Matrigel (Becton Dickinson, Bedford, MA). The lower compartment of chamber was filled with chemoattractants (ET-1 100 nM and PGE₂ 1 µM) and/or inhibitors, and/or antagonists (27 µl/well). Serum starved HEY cells (0.5x10⁶ cells/ml) were harvested and placed in the upper compartment (55 µl/well). Where indicated, the cells were preincubated for 30 min at 37°C with the EP4 antagonist AH23848 or AG1478 or PP2 or BQ 123. After 6 h of incubation at 37°C, the filters were removed, stained with Diff-Quick (Merz-Dade, Dudingen, Switzerland) and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate.

Statistical Analysis

Results are representative of at least three independent experiments each performed in triplicate. All statistical analysis was assessed using a two-tailed Student’s t test.
RESULTS

ET-1 increases prostaglandin levels through ETA R

Ovarian carcinoma cells produce prevalently PGE₂, PGF₂α and PGD₂ types of PG (29). Because ET-1 upregulates COX-1 and -2 expression that are implicated in PG synthesis in ovarian cancer cells (25), we evaluated the role of ET-1/ETA R binding on the production of three major PG metabolites, by measuring their levels in HEY and OVCA 433 cell conditioned media. By ELISA we found that both cell lines released constitutively high levels of PGE₂, PGF₂α, and PGD₂ and that stimulation with ET-1 increased their levels of 2.4-fold and 1.9-fold and 1.73-fold, respectively in HEY cells (Fig. 1 left panel) and 1.84-fold, 1.82-fold, and 1.63-fold in OVCA 433 cells (Fig. 1 right panel). Because the selective ETA R antagonist, BQ 123, blocked the ET-1-induced PG release in both cell lines (Fig. 1) these results show that ET-1 through ETA R is a potent PG inducer and that PGE₂ is the predominantly prostaglandin type induced by ET-1 in ovarian carcinoma cells.

ET-1 induces EP2 and EP4 receptor subtype expression

PGE₂ exerts its effects through interaction with four types of EP receptor (4). To evaluate whether ET-1 could modulate the expression pattern of EP subtype receptors in ovarian carcinoma cells, we performed RT-PCR analysis with specific EP receptor primers. mRNAs of EP2 and EP4 subtype receptors were easily detected in untreated HEY (Fig. 2 right panel) and OVCA 433 (Fig. 2 left panel) cells, while the levels of EP1 and EP3 mRNAs were extremely low. After 6 h of ET-1 stimulation, the mRNA levels of EP2 and EP4 subtype receptors were increased in a dose-dependent manner, (~2.1-fold higher than control) reaching maximum responses at 10 and 100 nM. On the contrary, EP1 and EP3 receptor mRNAs remained unchanged (Fig. 2). A similar induction was observed in ovarian carcinoma cells treated with PGE₂ (Fig. 2). These results demonstrate that ET-1 is endowed with the capacity of upregulating selectively EP2 and EP4 receptor mRNA levels in human ovarian cancer cells.
ET-1 and PGE$_2$ induce VEGF up-regulation via EP2 and EP4 receptor subtypes

To investigate whether the induction of PGE$_2$ by ET-1 may be responsible for VEGF secretion and EP receptor subtype involved in this effect in ovarian carcinoma cells, we examined the role of ET-1, PGE$_2$ and EP receptor-specific agonists on VEGF protein production and gene expression, by ELISA and RT-PCR analysis, respectively. In HEY cells, ET-1, as well as PGE$_2$, induced a significant increase in VEGF expression and secretion. Among EP agonists tested, only butaprost, an EP2 agonist, and 16,16-dimethyl PGE$_2$, an EP2-4 agonist, induced an increase in VEGF secretion and mRNA expression (Fig. 3 A, B) with an extent similar to that induced by ET-1 or PGE$_2$. In contrast, 17-phenyl trinor PGE$_2$, an EP1 and EP3 selective agonist, produced only a weaker VEGF induction, and sulprostone, an EP3 agonist, failed to up-regulate VEGF gene and protein expression (Fig. 3 A, B). In addition, we treated HEY cells with ET-1 or PGE$_2$ alone or in combination with SC19220, antagonist, or AH 23848, an EP4-specific antagonist, and measured VEGF secretion by ELISA (Fig. 3 C). AH 23848 significantly reduced the secretion of VEGF after ET-1 and PGE$_2$ stimulation. On the contrary, SC19220 was unable to significantly inhibit ET-1- and PGE$_2$-induced VEGF production (Fig. 3C). These results indicate that ET-1 and PGE$_2$ induce VEGF secretion and that EP2 and EP4 subtype are the principal prostaglandin receptors involved in this signaling in ovarian carcinoma cells.

ET-1 induces PGE$_2$ and VEGF secretion by Src-mediated transactivation of EGFR

To gain further information on the mechanism by which ET-1 leads to PGE$_2$ and VEGF upregulation, we evaluated whether ET-1-induced EGFR transactivation could participate in these effects. We previously demonstrated that in ovarian cancer cells, ET-1 rapidly activates Src and that this tyrosine kinase could represent a potential intracellular mediator of EGFR transactivation (30). As demonstrated in Fig. 4, ET-1, as well as PGE$_2$ induced a significant increase in the level of EGFR-phosphorylated form (Fig. 4 A). Pretreatment of HEY cells with the specific EGFR kinase inhibitor, AG1478, as well as with the Src-tyrosine kinase inhibitor, PP2, significantly reduced both
ET-1- and PGE2-induced EGFR phosphorylation (Fig. 4 A) indicating that activation of Src is essential for ET-1- and PGE2-induced EGFR transactivation. As shown in Fig. 4B, the interruption of EGFR signalling induced by AG1478, as well as by PP2, resulted in a concomitant reduction of PGE2 secretion, as well as of VEGF expression at protein (Fig. 4C upper panel and D) and mRNA level (Fig. 4C lower panel), indicating that Src-mediated EGFR-transactivation is involved in ET-1-induced PGE2 and VEGF production. Taken together, our results delineate a novel mechanism underlying ET-1-induced up-regulation of PGE2 and VEGF expression involving ET-1-dependent transactivation of EGFR, that require Src function.

**ET-1-induced PGE2 stimulates cell invasion and VEGF production via EP4 receptors**

PGE2 plays a relevant role in modulating the invasive properties of human cancer cells (2,6-7, 9). Thus, we investigated the pathways whereby ETAR-induced EGFR transactivation could impact the invasive behaviour via EP receptor signaling. Remarkably, inhibition of ETAR by BQ 123 completely blocked ET-1-induced cell invasion (Fig. 5A), and MMP activity (Fig. 5B) demonstrating that ET-1-signaling is mediated by ETAR. Pretreatment of HEY cells with AG1478, PP2 and AH23848 resulted in a reduction of ET-1 and PGE2 capacity to induce cell invasion (Fig. 5A) and MMP activity (Fig. 5B), measured by chemoinvasion and MMP gelatinase activity assays, respectively. These data indicate that ET-1 signaling implies ETAR-dependent EGFR transactivation, via Src, leading to cell invasiveness, MMP activity and VEGF production through the activation of PGE2-induced EP4 receptor pathway.
DISCUSSION

Epidemiological and clinical studies have clearly defined that COX-1 and COX-2 enzymes play a key role in the progression of ovarian carcinoma (11-13, 25, 29, 31). In this tumor PGF$_2$\(\alpha\), PGD$_2$ and PGE$_2$ are the bioactive lipids abundantly synthesized by COX enzymes. Among these, PGE$_2$ has been implicated in tumor angiogenesis (2-5), invasive and migratory capacity (6, 7). The study of PGE$_2$ signaling pathways is becoming essential from the biological as well as from the therapeutic point of view. We previously demonstrated that in ovarian cancer cells ET-1 induces COX-1 and COX-2 enzymes (25). Recently, much attention has been focused on the identification of ET-1 receptor pathways involved in ovarian cancer progression to characterize potential therapeutic targets in cancer prevention and treatment (23). The present study demonstrates that ET-1 through the binding with ET$_A$R significantly increases PGE$_2$, PGF$_2$\(\alpha\) and PGD$_2$ secretion and EP2 and EP4 receptor subtype expression, and that ET-1-induced PGE$_2$ pathways actively participate in promoting VEGF production, MMP activity and cell invasion. Furthermore, we prove that the ET-1-induced effects involve an actively cross-talk between ET$_A$R and EGFR signaling pathways.

Cross talk between cell surface receptor which has been early recognized as the mechanism capable of expanding the cellular communication signaling network is now receiving further interest. Receptor cross talk can in fact occur also among distinct family of receptors such as tyrosine kinase receptor (RTK) and GPCR (32-33). In this context, we have previously shown that in ovarian cancer cells EGFR transactivation following ET$_A$R is in part responsible for MAPK activation (24), by a ligand-dependent mechanism involving a non receptor tyrosine kinase such as c-Src (30). In the present study, we described that Src-mediated transactivation of EGFR by ET-1 is also responsible for PGE$_2$ and VEGF upregulation underlying the key role played by the cross talk between GPCR and RTK in tumor biology. In this regard, ET$_A$R-induced EGFR transactivation may serve as a prototype of inter-receptor signaling since multiple apparently independent pathways are in fact coactivated by this network (23). Here, we provide evidence that ET$_A$R-mediated EGFR transactivation is responsible for ET-1-induced MMP activity and ovarian cancer cell invasion.
resulting into tumor progression. Our findings are consistent with a growing body of evidence suggesting that transactivation of EGFR by GPCRs is a recurrent theme in cell signaling to promote migration and invasion (32, 33). Interestingly, we observed that in ovarian carcinoma cells, EGFR transactivation by ETAR stimulates the nuclear translocation of β-catenin leading to the activation of the transcriptional complex β-catenin TCF/LEF that is involved in epithelial to mesenchimal transition and in the development of an invasive phenotype (Rosanò et al. unpublished results). These interconnected signalings allow ETAR to expand the cellular communication network and to amplify its tumor promoting actions.

The natural history of most tumors is invariably characterized by the acquisition of migratory, invasive and angiogenic phenotype (34). In this regard, we have demonstrated that in ovarian cancer cells, ET-1/ETAR pathway contributes to the disruption of host tumor-interaction by impairing the gap junctional intercellular communication system (30) and promoting tumor invasiveness by upregulating protease in terms of expression, and activity (26). Because in ovarian carcinoma cells all the invasive effectors are triggered by the ETAR activation, blockade of this receptor by the specific antagonist ABT-627 results in antitumor effect in experimental animal model that is associated with decreased microvessel density, VEGF and COX-2 expression (25, 26). There is increased evidence that PGE2 contributes to tumor progression also by promoting tumor angiogenesis and that this effect is mediated, at least in part, by modulation of VEGF (4,5). We reported previously (19) that ET-1 was expressed in 84% of the ovarian carcinomas with a strong correlation between ET-1 and VEGF expression and vascularization. Moreover, we demonstrated that, in ovarian carcinoma cell lines, ET-1 stimulates VEGF expression through ETAR and that this effect is mediated by hypoxia-inducible factor (HIF)-1 α accumulation and activation (21, 22). Here we demonstrated that, in HEY and OVCA 433 cells, the ET-1-induced PGE2/EP2, EP4 signaling is involved in VEGF production indicating that ET-1-induced VEGF expression may be regulated through a dual mechanism including HIF-1 α activation and PGE2 signaling. The reduction of ET-1-induced VEGF production, cell invasion and MMP activity by the specific EP4 receptor
antagonist strongly suggests that the production of PGE\(_2\) by ET\(_A\)R-mediated EGFR transactivation could activate EP4 receptor-mediated signalling and in turn stimulate angiogenic and migratory action, thus identifying a novel mechanism underlying ET-1/ET\(_A\)R tumor promoting properties. This hypothesis is sustained by several observations correlating PGE\(_2\)-EP4 receptor interaction and tumor phenotype in various tumor types and in other model systems (6, 7,35-38) where EP4 receptors promote tumor progression increasing proangiogenic factor and tumor cell invasiveness. Moreover, previous reports demonstrate that EP2 and EP4 receptor transcripts are inducible by cytokine (39), that PGE\(_2\) may regulate EP4 receptor expression in non-small cell lung cancer cells (6) and EP2/4 receptors in COX-1-transfected cervical carcinoma cells (40). Remarkably, in endometrial cancer cells, elevated EP2 receptor expression may facilitate the PGE\(_2\)-induced release of pro-angiogenic factors (38). The increased EP2 and EP4 receptor expression observed after ET-1 stimulation open the intriguing possibility of the presence of a positive feedback mechanism in which ET-1-induced PGE\(_2\) upregulation could modulate the expression of PG receptors and in turn facilitate PGE\(_2\) activity to enhance and sustain tumorigenesis in ovarian cancer cells.

In conclusion, our data demonstrate that ET-1-induced PGE\(_2\) promotes the expression and secretion of VEGF and induces invasiveness in ovarian carcinoma cells through EP2 and EP4 receptors via the intracellular transactivation of the EGFR. These data suggest that targeting PGE\(_2\) and EP2 and EP4 related signaling cascade through the blockade of ET\(_A\)R in ovarian carcinoma could effectively impair the transcription of target genes associated with angiogenesis and invasiveness. This study therefore provides further support for the incorporation of ET\(_A\)R antagonists into clinical trials for the treatment of ovarian cancer.

It has been reported that enhanced COX-2, PGE\(_2\) and VEGF expression play a significant role in resistance to chemotherapy-induced apoptosis (41, 42). It is now emerging the hypothesis that drug resistance may occur from the activation of non-canonical escaping pathway that can modulate sensitivity to antitumor therapy. Because of the potential for compensation, interruption of a single signaling network of transforming molecules will not be curative in late invasive cancers.
Therefore, a novel approach to ovarian cancer therapy may be the treatment with multiple selective inhibitors to growth factor receptor or to key post-receptor signaling pathways. In this regard, because multiple signaling network are coactivated by ET_{A}R, combination treatment with ET_{A}R antagonist may represent a new therapeutic approach to ovarian cancer treatment, which could result in increased susceptibility of the cells to apoptosis.

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FIGURE LEGENDS

Fig. 1 ET-1 increases PGE₂, PGF₂α and PGD₂ secretion through ET₄R. Serum-starved HEY (left panel) and OVCA 433 (right panel) cells were cultured for 12 h in the absence or in the presence of ET-1 (100 nM) alone or in combination with BQ 123 (1 µM) and culture media were collected. PGE₂, PGF₂α and PGD₂ production was measured by ELISA. Bars, ± SD. a, p<0.005, compared to the control; b, p<0.0001 compared to ET-1.

Fig. 2 ET-1 induces a dose-dependent increase in EP2 and EP4 receptor transcripts. Serum-starved HEY (left panel) and OVCA 433 (right panel) cells were treated with different concentrations of ET-1 and 1 µg/ml PGE₂ for 6 h, and subsequently subjected to mRNA analysis by RT-PCR, using specific primers for EP1 (323 bp), EP2 (655 bp), EP3 (356 bp), and EP4 (434 bp) receptor transcripts. Primers for the amplification of GAPDH (451 bp) gene were used as controls.
Fig. 3 ET-1 and PGE₂ stimulate VEGF expression through EP2 and EP4 receptors. Serum-starved HEY cells were treated with ET-1 (100 nM), PGE₂ (1 µg/ml), 17-phenyl trinor PGE₂ (1 µM), butaprost (20 µM), sulprostone (20 µM), or 16,16-dimethyl PGE₂ (20 µM). After 12 h of stimulation, cell conditioned media (A), or total protein (B, upper panel) were collected and analyzed for VEGF expression by ELISA and Western blot, respectively. Bars, ± SD. *, p<0.005, compared to the control. After 6 h of stimulation total RNA (B, lower panel) was isolated and analyzed by RT-PCR. Proteins and mRNA levels were normalized for loading using β-actin Ab and GAPDH primers, respectively. C) Serum-starved HEY cells were treated with ET-1 (100 nM) or PGE₂ (1 µg/ml) alone or in combination with either SC19220 (10 µM) or AH23848 (50 nM) for 12 h and VEGF secretion was measured in the cell conditioned media by ELISA. Bars, ± SD. a, p<0.001, compared to the control; b, p<0.005, compared to ET-1; c, p<0.001 compared to PGE₂

Fig. 4 ET-1- and PGE₂-induced VEGF upregulation involves Src-mediated EGFR transactivation. A) Serum starved HEY cells were treated with ET-1 (100 nM) or PGE₂ (1µg/ml) for 15 min alone or in combination with either AG1478 (100 nM) or PP2 (50 nM). Whole cell lysates were analyzed by Western blot using specific Ab that recognized the tyrosine 854 phosphorylated form of EGFR (p-EGFR). B) Serum-starved HEY cells were stimulated with ET-1 (100 nM) alone or in combination with either AG1478 (100 nM) or PP2 (50 nM) for 12 h and cell conditioned media were collected and analyzed for PGE₂ secretion by ELISA. Bars, ± SD. *, p<0.0001, compared to the control; **, p<0.005, compared to ET-1. C) Serum-starved HEY cells were stimulated with ET-1 (100 nM) or PGE₂ (1µg/ml) alone or in combination with either AG1478 (100 nM) or PP2 (50 nM). After 12 h whole cell lysates (upper panel) and after 6 h total RNA (lower panel) were analyzed for VEGF production and mRNA expression by Western blot and RT-PCR respectively. Proteins and RNA levels were normalized for loading with β-actin and GAPDH respectively. D) Cell conditioned media from the same cell treated as in (C) for 12 h, were analyzed
for VEGF production by ELISA. Bars, ± SD. a, p<0.001, compared to the control; b, p<0.005, compared to ET-1; c, p<0.005 compared to PGE₂.

Fig. 5 ET-1-and PGE₂-induced EP4 receptor signaling regulates MMP activity and ovarian cancer cell invasion through Src-mediated EGFR transactivation. A) Serum starved HEY cells were treated with ET-1 (100 nM) or with PGE₂ (1μg/ml) alone or in combination with BQ 123 (1μM), AG1478 (100 nM), PP2 (50 nM), AH23848 (50 nM) and after 6 h cell invasion was measured by chemoinvasion assay. Bars, ± SD. a, p<0.0001, compared to the control; b, p<0.005, compared to ET-1; c, p<0.005, compared to PGE₂. B) Serum starved HEY cells were treated as in (A) for 12 h and cell conditioned media were collected. MMP activity was analyzed by a MMP gelatinase kit. Bars, ± SD. a, p<0.0001, compared to the control; b, p<0.005, compared to ET-1; c, p<0.005, compared to PGE₂.
Figure 1
Figure 2
Figure 3
Figure 5
Endothelin-1-induced prostaglandin E2-EP2,EP4-signaling regulates vascular endothelial growth factor production and ovarian carcinoma cell invasion
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