THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 45, Issue of November 5, pp. 46700–46705, 2004
Printed in U.S.A.


# Endothelin-1-induced Prostaglandin E2-EP2, EP4 Signaling Regulates Vascular Endothelial Growth Factor Production and Ovarian Carcinoma Cell Invasion*

Received for publication, July 28, 2004, and in revised form, September 2, 2004
Published, JBC Papers in Press, September 3, 2004, DOI 10.1074/jbc.M408584200

**Francesca Spinella‡§, Laura Rosanò‡, Valeriana Di Castro‡, Pier Giorgio Natali¶, and Anna Bagnato‡‖**

*From the Laboratories of ‡Molecular Pathology and Ultrastructure and ¶Immunology, Regina Elena Cancer Institute, 00158 Rome, Italy*

**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 contributes 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 behavior 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 amplify 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.**

Cyclooxygenase (COX)[1] 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, in which 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 $ET_A$ ($ET_AR$) are overexpressed in primary and metastatic ovarian carcinoma as compared with normal ovarian tissue (22). ET-1/$ET_AR$ interaction results in activation of a pertussis toxin-insensitive G protein that stimulates phospholipase C activity and increases intracellular $Ca^{2+}$ levels as well as activation of protein kinase C, mitogen-activated protein kinase (MAPK), paxillin, and p125 focal adhesion kinase (23). Among downstream events after $ET_AR$ activation, ET-1 also causes the epidermal growth factor receptor (EGFR) transactivation, which is partly responsible for MAPK phosphorylation (24). Recently, we demonstrated that ET-1 through $ET_AR$ induces COX-1 and COX-2 expression and that both enzymes contribute to $PGE_2$ and VEGF production in ovarian carcinoma cells (25). $ET_AR$ 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 and MMP-2, VEGF, and COX-2 expression (25, 26).

To analyze the contribution of the COX pathway in the

* This work was supported by grants from the Associazione Italiana Ricerca sul Cancro, the Ministero della Salute, and from MIUR-Consiglio Nazionale delle Ricerche. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of fellowship from Fondazione Italiana Ricerca sul Cancro.

‖ To whom correspondence should be addressed: Laboratory of Molecular Pathology and Ultrastructure, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy. Tel.: 39-06-52662565; Fax: 39-06-52662505; E-mail: bagnato@ifo.it.

[1] The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; EP, E prostanoid; GPCR, G-protein-coupled recptor; ET-1, endothelin-1; $ET_AR$, endothelin A receptor; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; EGFR, EGF receptor; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PP2, protein phosphatase 2; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription.

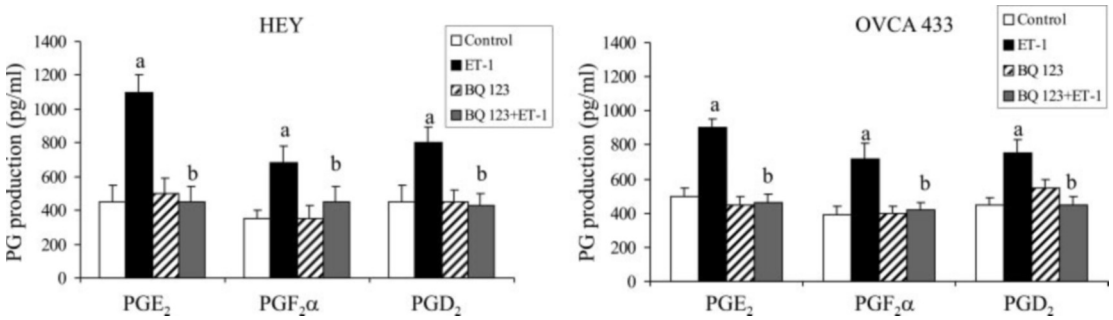


FIG. 1. **ET-1 increases $PGE_2$, $PGF_2\alpha$, and $PGD_2$ secretion through $ET_AR$.** Serum-starved HEY (*left panel*) and OVCA 433 (*right panel*) cells were cultured for 12 h in the absence or in the presence of ET-1 (100 nM) alone or in combination with BQ 123 (1 $\mu$M), and culture medium was collected. $PGE_2$, $PGF_2\alpha$, and $PGD_2$ production was measured by ELISA. *Bars*, ± S.D. *a*, $p < 0.005$, as compared with the control; *b*, $p < 0.0001$ as compared with ET-1.

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 $ET_AR$ increases the production of $PGE_2$, $PGF_2\alpha$, and $PGD_2$ and the expression of EP2 and EP4 receptor transcripts, 2) ET-1-induced $PGE_2$, VEGF, and cell invasion are mediated through the binding of $PGE_2$ to EP2 and EP4 receptors, 3) ET-1-induced Src-mediated EGFR transactivation is involved in $PGE_2$ and VEGF production and cell invasiveness. Thus, for the first time, we defined the prostaglandin secretion pattern and the $PGE_2$ receptor subtype expression in ovarian carcinoma cells, providing evidence for the involvement of EP2 and EP4 in ET-1-mediated $PGE_2$-dependent pathways. Moreover, the present findings indicate that blockade of $ET_AR$ signaling may result in targeting EP receptor activation and related signaling cascades, which contribute to ET-1-mediated ovarian cancer cell invasion.

## 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 Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1% penicillin-streptomycin at 37 °C under 5% $CO_2$, 95% air. The cells were serum-starved by incubation for 24 h in serum-free Dulbecco's modified Eagle's medium. All culture reagents were from Invitrogen. ET-1 (Peninsula Laboratories, Belmont, CA), EGF (Cell Signaling, Beverly, MA), $PGE_2$ (Sigma), 17-phenyl-trinor-$PGE_2$ (EP1 and EP3 agonist), butaprost (EP2 agonist), sulprostone (EP3 agonist), and 16,16-dimethyl-$PGE_2$ (EP2–4 agonist) (Cayman, Chemical, Ann Arbor, MI) were incubated with the cells for the indicated times. Pretreatment of cells with BQ 123 (Peninsula Laboratories), SC19220 (EP1 antagonist; Cayman Chemical), AH23848 (EP4 antagonist; Sigma), and AG1478 and PP2 (Calbiochem-Novabiochem) was performed for the indicated times prior to the addition of ET-1 or $PGE_2$.

*Western Blot Analysis*—For the detection of VEGF, EGFR, and phospho-EGFR, whole cell lysates were subjected to SDS-PAGE (7.5–12.5%) and revealed by Western blotting using antibodies anti-VEGF (Sigma) or anti-EGFR or anti-phospho 845-EGFR (Cell Signaling). Blots were developed with the enhanced chemiluminescence detection system (ECL; Amersham Biosciences). The membranes were reprobed with anti-$\beta$-actin to assure the equal amount of protein (Oncogene, CN Biosciences, Inc., Darmastadt, Germany).

*Reverse Transcription-PCR*—Total RNA from HEY and OVCA 433 cells was extracted using TRIzol (Invitrogen). RT-PCR was performed using an AccessQuick RT-PCR system (Promega, Madison, WI) according to the manufacturer's instructions. The primers sets were as follows: EP1, 5′-CCTGTCGGTATCATGGTGGTGTC-3′ and 5′-GGTTGTGCTTAGAAGTGGCTGAGG-3′; EP2, 5′-GCCACGATGCTCATCCTCTTCGCC-3′ and 5′-CTTGTGTTCTTAATGAAATCCGAC-3′; EP3, 5′-GCATAACTGGGGCAAACCTTTTCTTCGCC-3′ and 5′-CTTAACAGCAGGTAAACCCAAGGATCC-3′; EP4, 5′-TGGTATGTGGGCTGGCTG-3′ and 5′-GAGGACGGTGGCGAGAAT-3′; VEGF, 5′-GGCTCTAGATCGGGCCTCCGAAACCAT-3′ and 5′-GGCTCTAGAGCGCAGAGTCTCCTCTTC-3′; GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. Thirty-five cycles of amplification was performed under the following conditions: melting at 95 °C for 30 s; annealing at 58 (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 medium 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 $PGE_2$ and $PGF_2\alpha$ released into the medium of treated cells were measured by the $PGE_2$ Correlate-EIA and $PGF_2\alpha$ Correlate-EIA kit (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's instructions. The detection of $PGD_2$ was performed by the quantitative determination of 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$, one of its ultimate dehydration products (27), using 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ Correlate-EIA (Assay Designs, Inc.). The sensitivity of the assay is 13.4 pg/ml for $PGE_2$, 6.71 pg/ml for $PGF_2\alpha$, and 36.8 pg/ml for $PGD_2$. Intra-assay variation is 5.8% for $PGE_2$, 6.8% for $PGF_2\alpha$, and 5.7% for $PGD_2$, and inter-assay variation is 5.1% for $PGE_2$, 5.5% for $PGF_2\alpha$, and 13.0% for $PGD_2$. Gelatinase activities in cell-conditioned medium were determined using an MMP gelatinase activity assay kit (Chemicon) according to the manufacturer's instructions. The sensitivity of the assay is less than 5 ng/ml 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-$\mu$m pore polyvinyl pyrrolidone-free polycarbonate Nucleopore filters (Costar, New York, NY) as described previously (28). The filters were coated with an even layer of 0.5 mg/ml Matrigel (BD Biosciences). The lower compartment of chamber was filled with chemoattractants (100 nM ET-1 and 1 $\mu$M $PGE_2$) and/or inhibitors and/or antagonists (27 $\mu$l/well). Serum-starved HEY cells ($0.5 \times 10^6$ cells/ml) were harvested and placed in the upper compartment (55 $\mu$l/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 and then 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 $ET_AR$*—Ovarian carcinoma cells produce prevalently $PGE_2$, $PGF_2\alpha$, and $PGD_2$ types of PG (29). Because ET-1 up-regulates COX-1 and COX-2 expression, which is implicated in PG synthesis in ovarian cancer cells (25), we evaluated the role of ET-1/$ET_AR$ binding on the production of three major PG metabolites by measuring their levels in HEY and OVCA 433 cell-conditioned medium. By ELISA, we found that both cell lines released constitutively high levels of $PGE_2$, $PGF_2\alpha$, and $PGD_2$ and that stimulation with ET-1 increased their levels 2.4-, 1.9-, and 1.73-fold, respectively, in HEY cells (Fig. 1, *left panel*) and 1.84-, 1.82-, and 1.63-fold in OVCA 433 cells (Fig. 1, *right panel*). Because the selective $ET_AR$ antagonist, BQ 123,

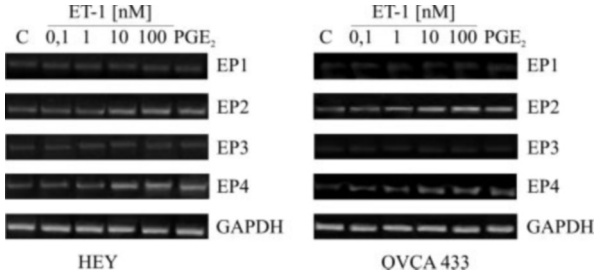


FIG. 2. **ET-1 induces a dose-dependent increase in EP2 and EP4 receptor transcripts.** Serum-starved HEY (*left panel*) and OVCA 433 (*right panel*) cells were treated with different concentrations of ET-1 and 1 $\mu$g/ml $PGE_2$ for 6 h and subsequently subjected to mRNA analysis by RT-PCR, using specific primers for EP1 (323 bp), EP2 (655 bp), EP3 (356 bp), and EP4 (434 bp) receptor transcripts. Primers for the amplification of the GAPDH (451 bp) gene were used to normalize the loading mRNA amount. *C*, control.

blocked the ET-1-induced PG release in both cell lines (Fig. 1), these results show that ET-1 through $ET_AR$ is a potent PG inducer and that $PGE_2$ is the predominant prostaglandin type induced by ET-1 in ovarian carcinoma cells.

*ET-1 Induces EP2 and EP4 Receptor Subtype Expression*—$PGE_2$ exerts its effects through interactions 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, whereas 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 (2.1) in a dose-dependent manner, reaching maximum responses (~2.1-fold higher than control) at 10 and 100 nM ET-1. On the contrary, EP1 and EP3 receptor mRNAs remained unchanged (Fig. 2). A similar induction was observed in ovarian carcinoma cells treated with $PGE_2$ (Fig. 2). These results demonstrate that ET-1 is endowed with the capacity of selectively up-regulating 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 to identify the 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* and *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* and *B*). In addition, we treated HEY cells with ET-1 or $PGE_2$ alone or in combination with SC19220, antagonist, or AH23848, an EP4-specific antagonist, and measured VEGF secretion by ELISA (Fig. 3*C*). AH23848 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. 3*C*). These results indicate that ET-1 and $PGE_2$ induce VEGF secretion and that the EP2 and EP4 subtypes 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 up-regulation, 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 $PGE_2$-induced EGFR phosphorylation (Fig. 4*A*), indicating that activation of Src is essential for ET-1- and $PGE_2$-induced EGFR transactivation. As shown in Fig. 4*B*, the interruption of EGFR signaling induced by AG1478, as well as by PP2, resulted in a concomitant reduction of $PGE_2$ secretion, as well as of VEGF expression at protein (Fig. 4*C*, *upper panel*, and *D*) and mRNA levels (Fig. 4*C*, *lower panel*), indicating that Src-mediated EGFR transactivation is involved in ET-1-induced $PGE_2$ and VEGF production. Taken together, our results delineate a novel mechanism underlying ET-1-induced up-regulation of $PGE_2$ and VEGF expression involving the ET-1-dependent transactivation of EGFR, which requires Src function.

*ET-1-induced $PGE_2$ Stimulates Cell Invasion and VEGF Production via EP4 Receptors*—$PGE_2$ plays a relevant role in modulating the invasive properties of human cancer cells (2,6–7, 9). Thus, we investigated the pathways whereby $ET_AR$-induced EGFR transactivation could impact the invasive behavior via EP receptor signaling. Remarkably, inhibition of $ET_AR$ by BQ 123 completely blocked ET-1-induced cell invasion (Fig. 5*A*) and MMP activity (Fig. 5*B*), demonstrating that ET-1 signaling is mediated by $ET_AR$. Pretreatment of HEY cells with AG1478, PP2, and AH23848 resulted in a reduction of the ET-1 and $PGE_2$ capacity to induce cell invasion (Fig. 5*A*) and MMP activity (Fig. 5*B*), measured by chemoinvasion and MMP gelatinase activity assays, respectively. These data indicate that ET-1 signaling implies $ET_AR$-dependent EGFR transactivation, via Src, leading to cell invasiveness, MMP activity, and VEGF production through the activation of the $PGE_2$-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 capacities (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 $ET_AR$ 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 active cross-talk between $ET_AR$ and EGFR signaling pathways.

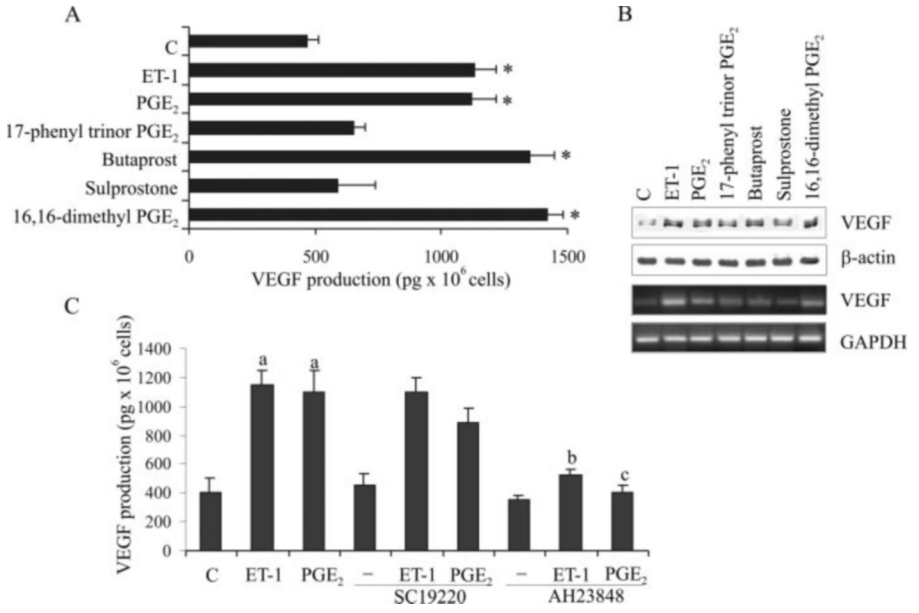


FIG. 3. **ET-1 and $PGE_2$ stimulate VEGF expression through EP2 and EP4 receptors.** Serum-starved HEY cells were treated with ET-1 (100 nM), $PGE_2$ (1 $\mu$g/ml), 17-phenyl-trinor-$PGE_2$ (1 $\mu$M), butaprost (20 $\mu$M), sulprostone (20 $\mu$M), or 16,16-dimethyl-$PGE_2$ (20 $\mu$M). After 12 h of stimulation, cell-conditioned medium (*A*) or total protein (*B*, *upper panel*) were collected and analyzed for VEGF expression by ELISA and Western blot, respectively. *Bars*, ± S.D. *, $p < 0.005$, as compared with 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 $\beta$-actin antibody and GAPDH primers, respectively. *C*, serum-starved HEY cells were treated with ET-1 (100 nM) or $PGE_2$ (1 $\mu$g/ml) alone or in combination with either SC19220 (10 $\mu$M) or AH23848 (50 nM) for 12 h, and VEGF secretion was measured in the cell-conditioned medium by ELISA. *Bars*, ± S.D. *a*, $p < 0.001$, as compared with the control; *b*, $p < 0.005$, as compared with ET-1; *c*, $p < 0.001$ as compared with $PGE_2$.

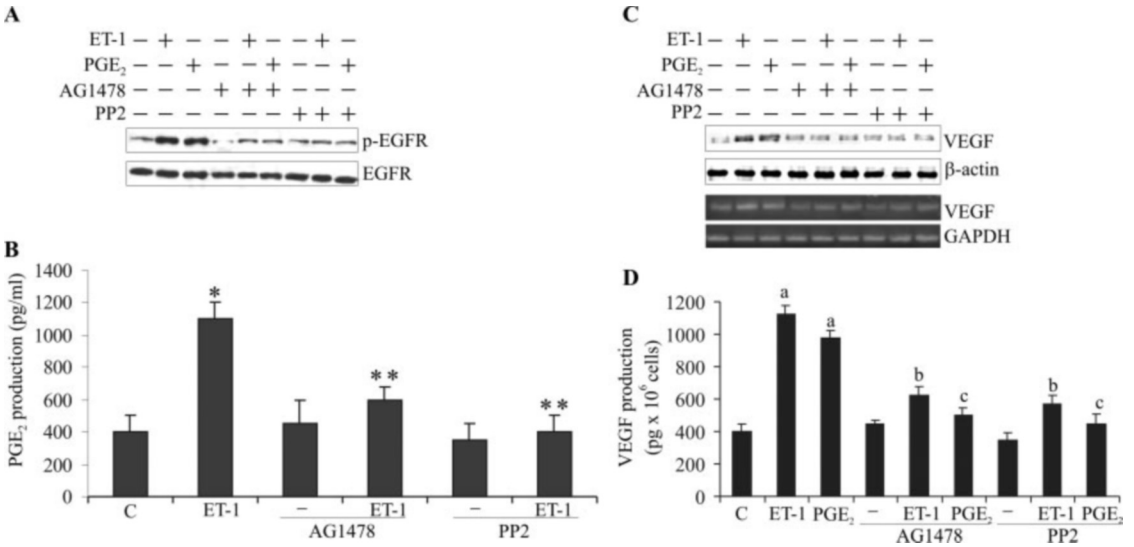


FIG. 4. **ET-1- and $PGE_2$-induced VEGF up-regulation involves Src-mediated EGFR transactivation.** *A*, serum-starved HEY cells were treated with ET-1 (100 nM) or $PGE_2$ (1 $\mu$g/ml) for 15 min alone or in combination with either AG1478 (100 nM) or PP2 (50 nM). Whole cell lysates were analyzed by Western blot using a specific antibody that recognized the tyrosine 845-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 medium was collected and analyzed for $PGE_2$ secretion by ELISA. *Bars*, ± S.D. *, $p < 0.0001$, as compared with the control (*C*); **, $p < 0.005$, as compared with ET-1. *C*, serum-starved HEY cells were stimulated with ET-1 (100 nM) or $PGE_2$ (1 $\mu$g/ml) alone or in combination with either AG1478 (100 nM) or PP2 (50 nM). After 12 h, whole cell lysates (*upper panel*) were analyzed for VEGF production by Western blot, and after 6 h, total RNA (*lower panel*) was analyzed for VEGF mRNA expression by RT-PCR. Proteins and RNA levels were normalized for loading with $\beta$-actin and GAPDH, respectively. *D*, cell-conditioned medium from the same cell treated as in *panel C* for 12 h was analyzed for VEGF production by ELISA. *Bars*, ± S.D. *a*, $p < 0.001$, as compared with the control; *b*, $p < 0.005$, as compared with ET-1; *c*, $p < 0.005$ as compared with $PGE_2$.

Cross-talk between cell surface receptors, which has been recognized early as the mechanism capable of expanding the cellular communication signaling network, is now receiving further interest. Receptor cross-talk can, in fact, also occur among distinct families of receptors such as tyrosine kinase receptor and GPCR (32, 33). In this context, we have previously shown that in ovarian cancer cells, EGFR transactivation following $ET_AR$ 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 up-regulation underlying the key role played by the cross-talk between GPCR and tyrosine kinase receptor in tumor biology. In this regard, $ET_AR$-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 $ET_AR$-mediated EGFR transactivation is responsible for ET-1-induced MMP activity and ovarian cancer cell invasion, resulting in 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 $ET_AR$ stimulates the nuclear translocation of $\beta$-catenin, leading to the activation of the transcriptional complex $\beta$-catenin T-cell factor/lymphoid enhancer factor (TCF/LEF) that is involved in epithelial to mesenchimal

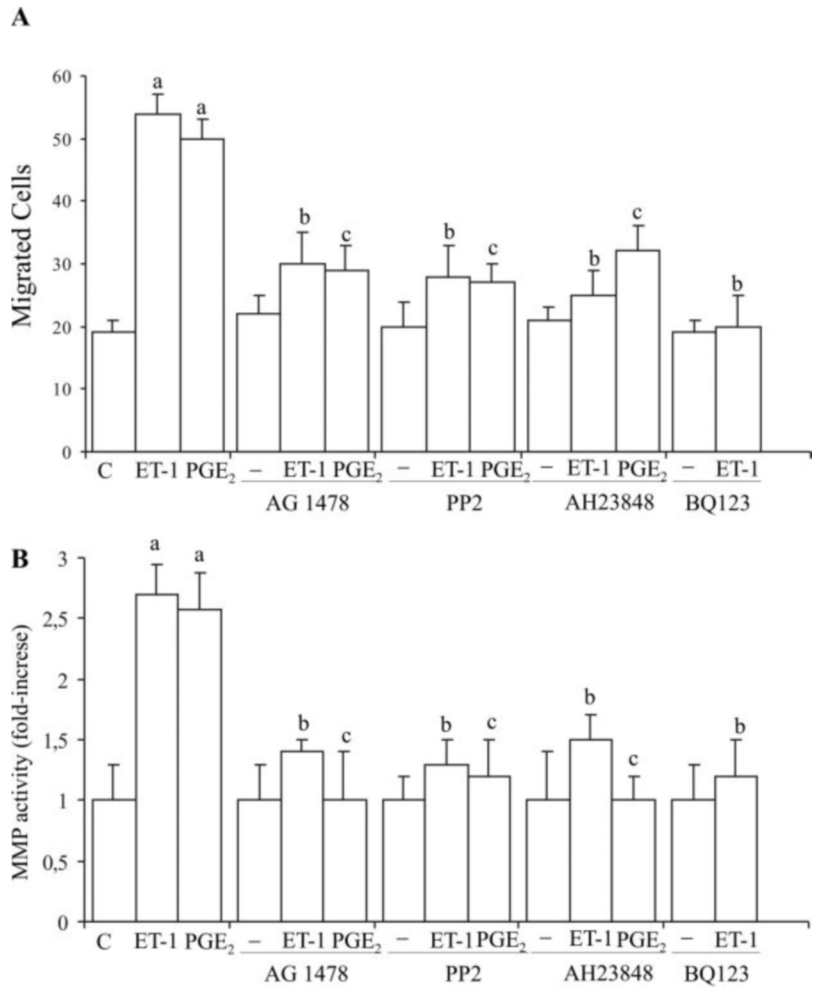


FIG. 5. **ET-1- and $PGE_2$-induced EP4 receptor signaling regulates MMP activity and ovarian cancer cell invasion through Src-mediated EGFR transactivation.** *A*, serum-starved HEY cells were treated with ET-1 (100 nM) or with $PGE_2$ (1 $\mu$g/ml) alone or in combination with BQ 123 (1 $\mu$M), AG1478 (100 nM), PP2 (50 nM), AH23848 (50 nM), and after 6 h, cell invasion was measured by chemoinvasion assay. *Bars*, ± S.D. *a*, $p <$ 0.0001, as compared with the control (*C*); *b*, $p < 0.005$, as compared with ET-1; *c*, $p < 0.005$, as compared with $PGE_2$. *B*, serum-starved HEY cells were treated as in *panel A* for 12 h, and cell-conditioned medium was collected. MMP activity was analyzed by an MMP gelatinase kit. *Bars*, ± S.D. *a*, $p < 0.0001$, as compared with the control; *b*, $p < 0.005$, as compared with ET-1; *c*, $p < 0.005$, as compared with $PGE_2$.

transition and in the development of an invasive phenotype.[2] These interconnected signalings allow $ET_AR$ 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, the ET-1/$ET_AR$ pathway contributes to the disruption of host-tumor interaction by impairing the gap junctional intercellular communication system (30) and promoting tumor invasiveness by up-regulating protease in terms of expression and activity (26). Because in ovarian carcinoma cells all the invasive effectors are triggered by the $ET_AR$ activation, blockade of this receptor by the specific antagonist ABT-627 results in antitumor effect in the experimental animal model that is associated with decreased microvessel density and MMP-Z, VEGF, and COX-2 expression (25, 26). There is increased evidence that $PGE_2$ also contributes to tumor progression by promoting tumor angiogenesis and that this effect is mediated, at least in part, by the 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 $ET_AR$ and that this effect is mediated by hypoxia-inducible factor-1 $\alpha$ accumulation and activation (21, 22). Here, we demonstrated that, in HEY and OVCA 433 cells, the ET-1-induced $PGE_2$/EP2 and EP4 signaling is involved in VEGF production, indicating that ET-1-induced VEGF expression may be regulated through a dual mechanism including hypoxia-inducible factor-1 $\alpha$ activation and $PGE_2$ 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 $ET_AR$-mediated EGFR transactivation could activate EP4 receptor-mediated signaling and in turn stimulate angiogenic and migratory action, thus identifying a novel mechanism underlying ET-1/$ET_AR$ 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) in which 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) and 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 proangiogenic factors (38). The increased EP2 and EP4 receptor expression observed after ET-1 stimulation opens the intriguing possibility of the presence of a positive feedback mechanism in which ET-1-induced $PGE_2$ up-regulation 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

[2] L. Rosanò, F. Spinella, V. Di Castro, P. G. Natali, and A. Bagnato, unpublished results.

invasiveness in ovarian carcinoma cells through EP2 and EP4 receptors via the intracellular transactivation of the EGFR. These data suggest that targeting the $PGE_2$- and EP2- and EP4-related signaling cascade through the blockade of $ET_AR$ 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 $ET_AR$ antagonists into clinical trials for the treatment of ovarian cancer.

It has been reported that enhanced COX-2, $PGE_2$, and VEGF expression plays a significant role in resistance to chemotherapy-induced apoptosis (41, 42). The hypothesis that drug resistance may occur from the activation of a non-canonic escaping pathway that can modulate sensitivity to antitumor therapy is now emerging. 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 networks are coactivated by $ET_AR$, combination treatment with $ET_AR$ antagonist may represent a new therapeutic approach to ovarian cancer treatment, which could result in increased susceptibility of the cells to apoptosis.

*Acknowledgments*—We gratefully acknowledge Giacomo Elia and Samantha Decandia for excellent assistance and Maria Vincenza Sarcone for secretarial assistance.

REFERENCES

1. Williams, C. S., Mann, M., and DuBois, R. N. (1999) *Oncogene* **18,** 7908–7916
2. Dannenberg, A. J., and Subbaramaiah, K. (2003) *Cancer Cell* **4,** 431–436
3. Wang, D., and DuBois, R. N. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 415–416
4. Gupta, R. A., and DuBois, R. N. (2001) *Nat. Rev. Cancer*, **1,** 11–21
5. Fukuda, R., Kelly, B., and Semenza, G. L. (2003) *Cancer Res.* **63,** 2330–2334
6. Dohadwala, M., Batra, R. K., Luo, J., Lin, Y., Krysan, K., Pold, M., Sharma, S., and Dubinett, S. M. (2002) *J. Biol. Chem.* **277,** 50828–50833
7. Sheng, H., Shao, J., Washington, M. K., and DuBois, R. N. (2001) *J. Biol. Chem.* **276,** 18075–18081
8. Ashby, B. (1998) *Biochem. Pharmacol.* **55,** 239–246
9. Buchanan, F. G., Wang, D., Bargiacchi, F., and DuBois, R. N. (2003) *J. Biol. Chem.* **278,** 35451–35457
10. Sonoshita, M., Takaku, K., Sasaki, N., Sugimoto, Y., Ushikubi, F., Narumiya, S., Oshima, M., and Taketo, M. M. (2001) *Nat. Med.* **7,** 1048–1051
11. Denkert, C., Kobel, M., Pest, S., Koch, I., Berger, S., Schwabe, M., Siegert, A., Reles, A., Klosterhalfen, B., and Hauptmann, S. (2002) *Am. J. Pathol.* **160,** 893–903
12. Erkinheimo, T. L., Lassus, H., Finne, P., van Rees, B. P., Leminen, A., Ylikorkala, O., Haglund, C., Butzow, R., and Ristimäki, A. (2004) *Clin. Cancer Res.* **10,** 538–545
13. Ferrandina, G., Ranelletti, F. O., Lauriola, L., Fanfani, F., Legge, F., Mottolese, M., Nicotra, M. R., Natali, P. G., Zakut, V. H., and Scambia, G. (2002) *Gynecol. Oncol.* **85,** 305–310
14. Bagnato, A., Salani, D., Di Castro, V., Wu-Wong, J. R., Tecce, R., Nicotra, M. R., Venuti, A., and Natali, P. G. (1999) *Cancer Res.* **59,** 720–727
15. Bagnato, A., Tecce, R., Di Castro V., and Catt, K. J. (1997) *Cancer Res.* **57,** 1306–1311
16. Del Bufalo, D., Di Castro, V., Biroccio, A., Varmi, M., Salani, D., Rosano, L., Trisciuoglio, D., Spinella, F., and Bagnato, A. (2002) *Mol. Pharmacol.* **61,** 524–532
17. Rosanò, L., Varmi, M., Salani, D., Di Castro, V., Spinella, F., Natali, P. G., and Bagnato, A. (2001) *Cancer Res.* **61,** 8340–8346
18. Salani, D., Di Castro, V., Nicotra, M. R., Rosanò, L., Tecce, R., Venuti, A., Natali, P. G., and Bagnato, A. (2000) *Am. J. Pathol.* **157,** 1537–1547
19. Salani, D., Taraboletti, G., Rosanò, L., Di Castro, V., Borsotti, P., Giavazzi, R., and Bagnato, A. (2000) *Am. J. Pathol.* **157,** 1703–1711
20. Spinella, S., Rosanò, L., Di Castro, V., Natali, P. G., and Bagnato, A. (2002) *J. Biol. Chem.* **277,** 27850–27855
21. Bagnato, A., and Spinella, F. (2003) *Trends Endocrinol. Metab.* **14,** 44–50
22. Bagnato, A., Tecce, R., Moretti, C., Di Castro, V., Spergel, D., and Catt, K. J. (1995) *Clin. Cancer Res.* **1,** 1059–1066
23. Nelson, J. B., Bagnato, A., Battistini, B., and Nisen, P. (2003) *Nat. Rev. Cancer* ***3***, 110–116
24. Vacca, F., Bagnato, A., Catt, K. J., and Tecce, R. (2000) *Cancer Res.*, ***60***, 5310–5317
25. Spinella, F., Rosanò, L., Di Castro, V., Nicotra, M. R., Natali, P. G., and Bagnato, A. (2004) *Clin. Cancer Res.* **10,** 4670–4679
26. Rosanò, L., Spinella, F., Salani, D., Di Castro, V., Venuti, A., Nicotra, M. R., Natali, P. G., and Bagnato, A. (2003) *Cancer Res.* **63,** 2447–2453
27. Azuma, Y., Watanabe, K., Date, M., Daito, M., and Ohura, K. (2004) *J. Pharmacol. Sci.* **94,** 261–270
28. Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., and McEwan, R. N. (1987) *Cancer Res.* **47,** 3239–3245
29. Gupta, R. A., Tejada, L. V., Tong, B. J., Das, S. K., Morrow, J. D., Dey, S. K., and DuBois, R. N. (2003) *Cancer Res.* **63,** 906–911
30. Spinella, F., Rosanò, L., Di Castro, V., Nicotra, M. R., Natali, P. G., and Bagnato, A. (2003) *J. Biol. Chem.* **278,** 41294–41301
31. Denkert, C., Furstenberg, A., Daniel, P. T., Koch, I., Kobel, M., Weichert, W., Siegert, A., and Hauptmann, S. (2003) *Oncogene* **22,** 8653–8661
32. Schäfer, B., Gschwind, A., and Ullrich, A. (2004) *Oncogene* **23,** 991–999
33. Gschwind, A., Prenzel, N., and Ullrich, A. (2002) *Cancer Res.* **62,** 6329–6336
34. Hanahan, D., and Weinberg, R. A. (2000) *Cell* **100,** 57–70
35. Seno, H., Oshima, M., Ishikawa, T., Oshima, H., Takaku, K., Chiba, T., Narumiya, S., and Taketo, M. M. (2002) *Cancer Res.* **62,** 506–511
36. Eibl, G., Bruemmer, D., Okada, Y., Duffy, J. P., Law, R. E., Reber, H. A., and Hines, O. J. (2003) *Biochem. Biophys. Res. Commun.* **306,** 887–897
37. Fujino, H., Xu, W., and Regan, J. W. (2003) *J. Biol. Chem.* **278,** 12151–12156
38. Sales, K. J., Maudsley, S., and Jabbour, H. N. (2004) *Mol. Endocrinol.* **18,** 1533–1545
39. Narko, K., Saukkonen, K., Ketola, I., Butzow, R., Heikinheimo, M., and Ristimaki, A. (2001) *J. Clin. Endocrinol. Metab.* **86,** 1765–1768
40. Sales, K. J., Katz, A. A., Howard, B., Soeters, R. P., Millar, R. P., and Jabbour, H. N. (2002) *Cancer Res.* **62,** 424–432
41. Tran, J., Master, Z., Yu, J. L., Rak, J., Dumont, D. J., and Kerbel, R. S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 4349–4354
42. Subbaramaiah, K., and Dannenberg, A. J. (2003) *Trends Pharmacol. Sci.* **24,** 96–102