

*Featured Article***Inhibition of Cyclooxygenase-1 and -2 Expression by Targeting the Endothelin A Receptor in Human Ovarian Carcinoma Cells**

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ABSTRACT

Purpose and Experimental Design: New therapies against cancer are based on targeting cyclooxygenase (COX)-2. Activation of the endothelin A receptor (ET_AR) by endothelin (ET)-1 is biologically relevant in several malignancies, including ovarian carcinoma. In this tumor, the ET-1/ET_AR autocrine pathway promotes mitogenesis, apoptosis protection, invasion, and neangiogenesis. Because COX-1 and COX-2 are involved in ovarian carcinoma progression, we investigated whether ET-1 induced COX-1 and COX-2 expression through the ET_AR at the mRNA and protein level in HEY and OVCA 433 ovarian carcinoma cell lines by Northern blot, reverse transcription-PCR, Western blot, and immunohistochemistry; we also investigated the activity of the COX-2 promoter by luciferase assay and the release of prostaglandin (PG) E₂ by ELISA.

Results: ET-1 significantly increases the expression of COX-1 and COX-2, COX-2 promoter activity, and PGE₂ production. These effects depend on ET_AR activation and involve multiple mitogen-activated protein kinase (MAPK) signaling pathways, including p42/44 MAPK, p38 MAPK, and transactivation of the epidermal growth factor receptor. COX-2 inhibitors and, in part, COX-1 inhibitor blocked ET-1-induced PGE₂ and vascular endothelial growth factor release, indicating that both enzymes participate in PGE₂ production to a different extent. Moreover, inhibition of human ovarian tumor growth in nude mice after treatment with the potent ET_AR-selective antagonist ABT-627 is associated with reduced COX-2 and vascular endothelial growth factor expression.

Conclusions: These results indicate that impairing COX-1 and COX-2 and their downstream effect by targeting ET_AR can be therapeutically advantageous in ovarian carcinoma treatment. Pharmacological blockade of the ET_AR is an attractive strategy to control COX-2 induction, which has been associated with ovarian carcinoma progression and chemoresistance.

INTRODUCTION

Ovarian cancer represents the leading cause of death among gynecological malignancies. About 26,250 women are diagnosed yearly, with an overall 5-year survival rate of only 47%. Despite recent advances in surgery and chemotherapy, improvement in long-term survival of these patients has been slight (1). The endothelin (ET) family is composed of three isopeptides, ET-1, ET-2, and ET-3, which are potent mitogens for several human tumors (2–9) including ovarian carcinoma (3, 4). ETs and their receptors have been implicated in tumor progression through autocrine and paracrine pathways. ET-1 is produced by different tumor cells and is biologically active through two distinct subtypes of G protein-coupled receptors (GPCRs), namely, ET A receptor (ET_AR) and ET B receptor, endowed with different affinities for ETs (10). The ET-1/ET_AR autocrine pathway plays a key role in the development and progression of prostatic, ovarian, and cervical carcinoma (11). We have demonstrated previously (4) that ET-1 and ET_AR are overexpressed in primary and metastatic ovarian carcinomas compared with normal ovarian tissues. In this tumor, engagement of ET_AR by ET-1 promotes tumor cell proliferation (3, 12), apoptosis protection (13), invasiveness (14), and neovascularization stimulating vascular endothelial growth factor (VEGF) secretion (15, 16). ET-1 induces VEGF by increasing hypoxia-inducible factor (HIF)-1 α accumulation and activity under both normoxic and hypoxic conditions (17). Among the many growth-promoting factors known to be present in ovarian cancer ascites, ET-1 is found there at significant levels and may play an important role in the development or progression of ovarian cancer (16). ET-1, acting through the ET_AR, consistently induces the activity of multiple metastasis-related proteinases, such as the matrix-metalloproteinases and the urokinase-type plasminogen activator system, that stimulate cellular invasion (14). Loss of gap junction intercellular communication is critical for tumor progression because it allows the tumor cells to escape growth control, invade, and metastasize. In this regard, ET-1 inhibits gap junction intercellular communication by inducing phosphorylation/inactivation of connexin 43 (18). Interestingly, we found that the addition of ET_AR antagonist blocked ET-1-induced migration and invasion of ovarian carcinoma cells (14). These results indicate that the ET-1/ET_AR pathway participates in the progression of ovarian carcinoma and could be targeted for antitumor therapy. Thus, ET_AR blockade by a selective receptor antagonist, ABT-627, has been shown to inhibit the growth of ovarian carcinoma cell xenografts (19).

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Although ET-1 has also been shown to play a role in modulating cyclooxygenase (COX)-2 and COX-2-derived PGE₂ expression in different normal cells (20–23), the molecular mechanisms controlling these effects have not been defined. Moreover, the role of ET-1 in the regulation of COX-1 and COX-2 in tumor cells has not been investigated.

The COX-1 and COX-2 enzymes catalyze the rate-limiting step in the conversion of arachidonic acid to PGs (24) and are involved in tumor progression by inducing proliferation, survival, invasion, and metastasis in several solid tumors (25). It was reported recently (26–29) that expression of COX-2 is elevated in ovarian cancer. Moreover, in ovarian cancer, elevated COX-2 expression has been identified as an independent prognostic factor (30) that is associated with reduced survival (31) and poor response to standard combination chemotherapy (32). High COX-1 expression was also reported in ovarian cancer specimens, in which it is associated with elevated levels of proangiogenic factors, such as VEGF and HIF-1 α (33).

In view of these findings, we investigated whether activation of the ET_AR by ET-1 leads to up-regulation of COX-2 and COX-1 expression and PGE₂ production in two human ovarian carcinoma cell lines in which the ET-1/ET_AR autocrine pathway is biologically active. Ligand binding to the ET_AR results in activation of a pertussis toxin-insensitive G protein that stimulates phospholipase C activity and increases intracellular Ca²⁺ levels, activation of protein kinase C, mitogen-activated protein kinase (MAPK), paxillin, and p125 focal adhesion kinase (12). Among the downstream events after ET_AR activation, ET-1 also causes epidermal growth factor receptor (EGFR) transactivation, which is partly responsible for MAPK phosphorylation (34). Because the activation of ET_AR and EGFR autocrine circuits triggers mitogenic signaling in these tumor cells, we examined whether the ET-1-induced effects may derive from cross-communication between ET_AR and EGFR signaling pathways, which are amplified in ovarian carcinoma. In this study, we report that (a) ET-1 significantly induces COX-2 and COX-1 expression and PGE₂ production through ET_AR activation, and (b) signaling progresses through multiple MAPK pathways including p42/44 and p38 MAPK and EGFR transactivation. Furthermore, we demonstrated that both ET-1-induced COX-1 and COX-2 might contribute, to a different extent, to neovascularization by stimulating VEGF levels. In addition, inhibition of human tumor growth in nude mice after treatment with the nonpeptide orally active ET_AR antagonist ABT-627 (atrasentan) is associated with a reduction of *in vivo* COX-2 expression. Thus, the present findings indicate that pharmacologically blocking ET_AR signaling may result in targeting COX-2 and related signaling cascades that contribute to ET-1-mediated ovarian cancer progression.

MATERIALS AND METHODS

Cells. Human ovarian carcinoma cell lines HEY and OVCA 433, previously characterized for ET-1 receptor expression and ET-1 production (2, 3), were cultured in DMEM containing 10% FCS. The cells were serum starved by incubation for 24 h in serum-free DMEM. All culture reagents were from Invitrogen (Paisley, Scotland, United Kingdom). ET-1 (Peninsula Laboratories, Belmont, CA) was added in the cell

medium at the indicated concentration and for the indicated time. When the effects of the antagonists (BQ 123 and BQ 788; Peninsula Laboratories) were studied, they were added 15 min before agonists and left for the whole experiment. Pretreatment of cells with 10 μ M PD98059, 5 μ M SB203580, and 0.1 μ M AG1478 (Calbiochem-Novabiochem Corp., San Diego, CA) was performed for 1 h, whereas pretreatment with 1 μ M NS-398, 9 nM SC-560, 70 nM SC-58125, 26 μ M indomethacin (Cayman, Chemical, Ann Arbor, MI) was performed for 30 min before the addition of ET-1. Epidermal growth factor (Cell Signaling, Beverly, MA) was used at 10 ng/ml.

Immunoprecipitation and Immunoblotting. Preclarified lysates were immunoprecipitated with anti-EGFR antibody (Cell Signaling) insolubilized on protein A-Sepharose (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). The blots were then incubated for 1 h with anti-phospho-tyrosine monoclonal antibody (PY20; BD Transduction Laboratories, Heidelberg, Germany) or anti-EGFR (Cell Signaling) and developed with an enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech). For detection of COX-2, COX-1, p42/44 MAPK, phospho-p42/44 MAPK, p38 MAPK, phospho-p38 MAPK, and VEGF, whole cell lysates or homogenized HEY tumor specimens were subjected to 7.5–12.5% SDS-PAGE and examined by Western blotting using anti-COX-2 or anti-COX-1 (1:1000; Cayman Chemical) or anti-p44/42 MAPK, anti-phospho-p44/42 MAPK, anti-p38 MAPK, anti-phospho-p38 MAPK (1:1000; Cell Signaling), or anti-VEGF (Sigma, St. Louis, MO). Blots were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech). After being stripped, the membranes were reprobated with β -actin (Oncogene, CN Biosciences, Inc., Darmstadt, Germany).

Northern Blotting. Total RNA from HEY cells was extracted using the TRIzol (Invitrogen) method according to the manufacturer's instructions. RNA samples (30 μ g/lane) were separated by electrophoresis on a 2% denaturing formaldehyde agarose gel and transferred to a nylon membrane. The membranes were UV cross-linked and hybridized in QuickHyb hybridization solution (Stratagene, La Jolla, CA). The cDNA probe (Cayman Chemical) used for analysis of COX-2 mRNA was labeled with [α -³²P]dCTP using a random primer oligolabeling kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. To ensure RNA integrity and confirm equal loading between lanes, the filters were stripped and rehybridized with a probe for 18S rRNA. The relative intensity of signals was quantified using the Scion Image analysis program.

Reverse Transcription-PCR. Reverse transcription-PCR was performed using a Superscript One-Step Reverse Transcription-PCR System (Invitrogen) according to the manufacturer's instructions. Briefly, 1 μ g of RNA was reverse transcribed. The primers sets were as follows: COX-1, 5'-TGCCAGCTCCTGGCCCGCCGCTT-3' and 5'-GTGCATCAACACAGGCGCCTCTTC-3'; and glyceraldehyde-3-phosphate dehydrogenase, 5'-TGAAGTCCGGTGTCAACGGA-3' and 5'-GATGGCATGGACTGTGGTCAT-3'. Thirty-five cycles of amplification were performed under the following conditions: melting at 95°C for 30 s; annealing at 54°C for 45 s; and extension at 72°C for 30 s. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

Immunohistochemistry. Cytospins of HEY cells and cryostat sections of tumor xenografts were fixed in cold acetone for 10 min, and COX-2 expression was detected by streptavidin-biotin immunoperoxidase (Vector Laboratories, Burlingame, CA) using anti-COX-2 (Cayman Chemical). 3-Amino-9-ethylcarbazole was used as chromogenic substrate. Nuclear counterstain was obtained with Mayer's hematoxylin. Negative control staining used reagent absorbed with relevant immunogenic peptide (Cayman Chemical).

ELISA. The VEGF protein levels in the conditioned media were determined in duplicate by ELISA using the Quantikine Human VEGF immunoassay kit (R&D Systems, Minneapolis, MN). The sensitivity of the assay is <5.0 pg/ml. Intra-assay variation is 5.4%, and interassay variation is 7.3%. Levels of PGE₂ released into the medium of treated cells were measured

by ELISA using the PGE₂ High Sensitivity Immunoassay according to the manufacturer's instructions (R&D Systems). The sensitivity of the assay is <8.25 pg/ml. Intra-assay variation is 9.5%, and interassay variation is 10.9%.

Transfection of Reporter Construct and Luciferase Assay. Reporter constructs pHES2 (-1432/+59, -372/+59, -220/+59, -124/+59) containing the 5'-flanking region of the human COX-2 gene (35) were kindly provided by Dr. R. N. DuBois (Vanderbilt University Medical Center, Nashville, TN). For transient transfection, 1×10^5 cells were plated in 6-well plates 48 h before transfection. The cells were cotransfected with 0.5 μ g of COX-2 firefly luciferase plasmid construct and 0.05 μ g of the pCMV- β -galactosidase plasmid (Promega, Madison, WI) using LipfectAMINE reagent (Invitrogen) as de-

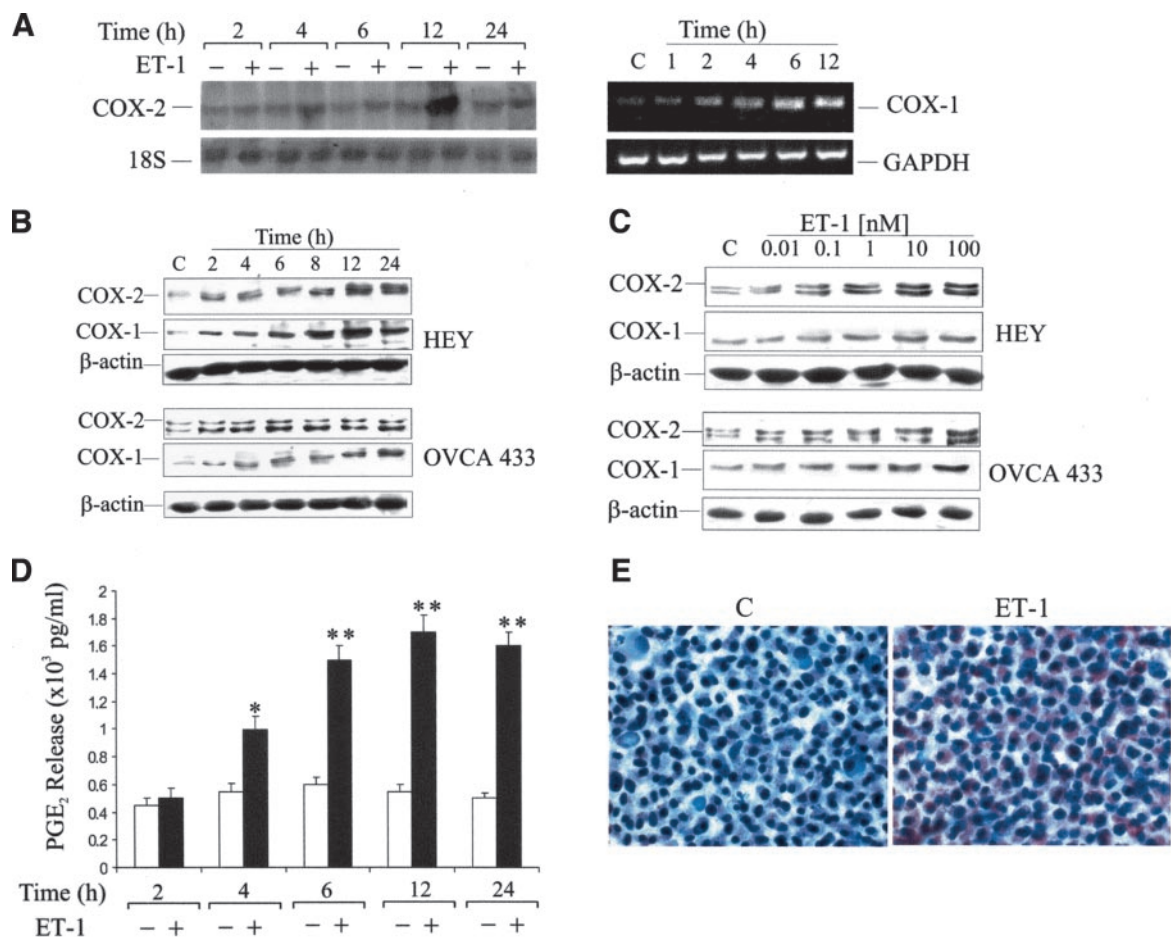


Fig. 1 A, time course of endothelin (ET)-1-induced cyclooxygenase (COX)-2 and COX-1 mRNA up-regulation. Serum-starved HEY cells were treated with 100 nM ET-1 for the indicated times, and steady-state levels of COX-2 mRNA expression were determined by Northern blotting. Probe for 18S rRNA was used as a control for equal loading (left panel). COX-1 mRNA induction was analyzed by reverse transcription-PCR in HEY cells treated with 100 nM ET-1 for the indicated times. Primers for the amplification of *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene were used as controls (right panel). B, kinetics of ET-1-induced COX-2 and COX-1 protein expression. HEY and OVCA 433 cells were incubated with 100 nM ET-1 for the indicated times and analyzed by Western blotting using COX-2 and COX-1 antibodies. C, ET-1 induces a dose-dependent increase in COX-2 and COX-1 expression. HEY and OVCA 433 cells were treated with different concentrations of ET-1 for 24 h, and COX-2 and COX-1 protein levels were analyzed by Western blotting. Anti- β -actin was used as a control for equal loading. D, ET-1 induces a time-dependent prostaglandin E₂ release. HEY cells were treated with 100 nM ET-1 for the indicated times, and prostaglandin E₂ production in the conditioned media was measured by ELISA. Data represent the means of results from three experiments, each performed in triplicate. Bars, SD. *, $P < 0.001$; **, $P < 0.0001$ (compared with control). E, immunohistochemical analysis of COX-2 expression. Serum-starved HEY cells were treated for 24 h in the absence (C) or presence (ET-1) of 100 nM ET-1 cells and immunostained by using monoclonal anti-COX-2. Original magnification, $\times 200$.

scribed in the manufacturer's protocol. The cells were lysed, and their luciferase activities were measured (luciferase assay system; Promega). The results were normalized to β -galactosidase activity. For each experiment, the mean of three independent experiments performed in triplicate is reported.

HEY Xenografts in Nude Mice. Female athymic (nu^{+}/nu^{+}) mice, 4–6 week of age, were purchased from Charles River Laboratories (Milan, Italy). The treatment protocol followed the guidelines for animal experimentation of the Italian Ministry of Health. Mice were given s.c. injection in one flank with 1.5×10^6 viable HEY cells, as determined by trypan blue staining, resuspended in 200 μ l of PBS. After 7 days, when established tumors of approximately 0.2–0.3 cm^3 in volume were detectable, mice were randomized into groups ($n = 10$) to receive different treatments. One group was treated i.p. for 21 days with 2 mg/kg/day ABT-627 (atrasentan; provided by Abbott Laboratories, Abbott Park, IL). Control mice were injected with drug vehicle. On day 40 after tumor injection, tumors were removed and snap frozen in liquid nitrogen for immunohistochemical and Western blot analyses.

Statistical Analysis. All statistical analysis was assessed using a two-tailed Student's *t* test and by two-way ANOVA as appropriate.

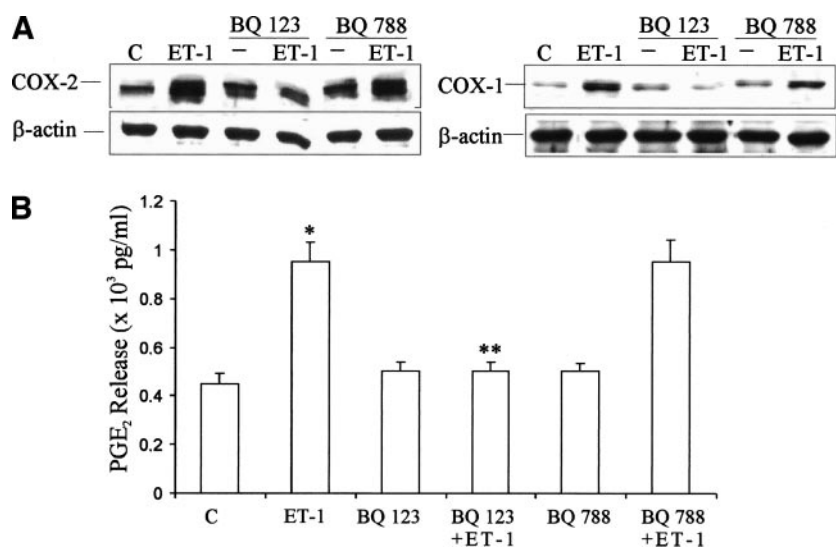
RESULTS

ET-1 Stimulates COX-1 and COX-2 Expression. We first investigated whether ET-1 can regulate COX-1 and COX-2 expression in two ovarian carcinoma cell lines, HEY and OVCA 433, that express functional ET_A R and secrete high levels of ET-1 (3, 4). ET-1 significantly induced time-dependent up-regulation of COX-1 and COX-2 mRNA in HEY cells. Northern blot analysis indicated that the steady-state COX-2 mRNA levels increased in ET-1-treated cells compared with control by 1-, 3.8-, 4-, 5.6-, and 2.8-fold at 2, 4, 6, 12, and 24 h, respectively, as quantified by the Scion Image analysis program (Fig. 1A). Reverse transcription-PCR analysis showed that COX-1 mRNA levels were enhanced for increasing time periods

(1–12 h) in ET-1-treated cells (Fig. 1A). Moreover, ET-1 treatment evoked a time-dependent increase in COX-2 and COX-1 protein levels. Western blots exhibited low expression of both proteins in untreated HEY and OVCA 433 cells but showed a 2-fold increase in COX-2 and COX-1 protein by 2 h and a 4.5- and 5-fold increase in COX-2 and COX-1 protein, respectively, after 24 h of ET-1 stimulation (Fig. 1B). ET-1 also increased COX-1 and COX-2 protein levels in a dose-dependent fashion. Treatment of HEY and OVCA 433 cells with 0.1 and 1 nM ET-1 for 24 h revealed 3.2- and 4.8-fold increases in COX-2 protein expression, respectively, reaching maximum responses (6.8-fold) at 10 and 100 nM ET-1. COX-1 protein levels increased 1.2-fold at 0.01 nM ET-1, reaching maximum levels (5-fold) at 100 nM ET-1 (Fig. 1C). To examine whether the increase of COX-1 and COX-2 expression is associated with elevated PG synthesis, the level of PGE_2 released from HEY cells was measured using ELISA. ET-1 increased PGE_2 levels in the conditioned medium of HEY cells in a time-dependent manner (Fig. 1D). To assess whether ET-1 up-regulates COX-2 cytoplasmic expression, HEY cells were analyzed by immunohistochemistry using anti-COX-2. Although a faint cytoplasmic stain was detected in untreated cells (Fig. 1E), cells treated with ET-1 for 24 h displayed a significantly stronger and homogeneous immunoreactivity.

ET-1-Induced COX-1 and COX-2 Up-Regulation and PGE_2 Production Are Mediated through ET_A R. To investigate which receptor subtype mediates ET-1-induced up-regulation of COX-1 and COX-2 expression, a selective ET_A receptor antagonist, BQ 123, and a selective ET B receptor antagonist, BQ 788, were used in the presence or absence of 100 nM ET-1. BQ 123 was able to completely block ET-1-induced COX-1 and COX-2 expression, whereas BQ 788 did not (Fig. 2A). To determine whether ET_A R blockade was responsible for functional COX activity, PGE_2 production was measured in HEY cell conditioned medium. As shown in Fig. 2B, BQ 123 completely prevented the PGE_2 production induced by treatment with 100 nM ET-1, whereas BQ 788 did not exert any

Fig. 2 A, endothelin (ET)-1-induction of cyclooxygenase (COX)-2 and COX-1 is mediated through ET_A receptor. Serum-starved HEY cells were treated with ET-1 (100 nM) alone or in combination with either BQ123 (1 μ M) or BQ 788 (1 μ M). Whole cell extracts were analyzed for COX-2 and COX-1 expression by Western blot. Proteins were normalized for loading with β -actin. B, ET-1 induces prostaglandin E_2 production via ET_A receptor. Conditioned media of HEY cells incubated for 24 h, as indicated in A, were collected for prostaglandin E_2 detection by ELISA. Data represent the means of results from three experiments, each performed in triplicate. Bars, SD. *, $P < 0.001$ compared with control; **, $P < 0.005$ compared with ET-1.



effect. Taken together, these findings indicate that ET-1 acts through the ET_AR to stimulate COX-1 and COX-2 expression and PGE₂ production in ovarian carcinoma cells.

ET-1 Stimulates COX-2 Promoter Activity. COX-2 expression is controlled at transcriptional and posttranscriptional levels (35–37). To determine whether ET-1 regulates COX-2 promoter activity, HEY cells were transfected with different-sized fragments of the human COX-2 promoter coupled with a luciferase reporter gene, and luciferase activity was measured and normalized to β-galactosidase activity. ET-1 (100 nM) induced a 3.1 ± 0.5 -fold increase in luciferase activity in cells transiently expressing phPES2(-1432/+59) compared with untreated control cultures. A 2.0-fold induction was observed when using a shorter fragment of the COX-2 promoter phPES2(-327/+59). ET-1 did not stimulate an increase in luciferase activity in cells containing the shortest COX-2 promoter constructs phPES2(-220/+59) and phPES2(-124/+59) (Fig. 3A). To study the time course of ET-1-induced COX-2 promoter, phPES2(-1432/+59)-transfected HEY cells were exposed to ET-1 for different times, and luciferase activity was measured (Fig. 3B). After 4 h, the ET-1 treatment doubled (a 1.9 ± 0.2 -fold increase) the luciferase activity of full-length

COX-2 promoter-transfected cells with respect to the control and induced the maximum effect (3.0 ± 0.35 -fold increase) at 12 h. Thereafter, this activity decreased, reaching a 1.5 ± 0.2 -fold increase over the control. phPES2(-1432/+59) was also used to analyze the ET-1 receptor involved in ET-1-induced COX-2 promoter activity. When BQ 123 was added in combination with ET-1, induction of luciferase activity was fully prevented (from a 3.5 ± 0.2 -fold increase to a 1.1 ± 0.2 -fold increase; Fig. 3C). A 3.5 ± 0.2 -fold induction was observed when ET-1 was added to cell medium in combination with BQ 788, demonstrating that ET_AR-mediated signaling pathways are linked to regulation of the COX-2 promoter in ovarian cancer cells (Fig. 3C).

Signaling Pathways Involved in ET-1-Stimulated COX-2 Expression. MAPK signal transduction pathways constitute one of the major mechanisms by which extracellular stimuli are converted into specific nuclear responses. ET-1 is known to activate the p42/44 MAPK pathway through the ET_AR in ovarian carcinoma cell lines (12). Moreover, ET-1 regulates COX-2 expression through p42/44 and p38 MAPK in vascular smooth muscle cells (23) and through p42/44 MAPK in osteoblast-like cells (21). Therefore, we analyzed whether these path-

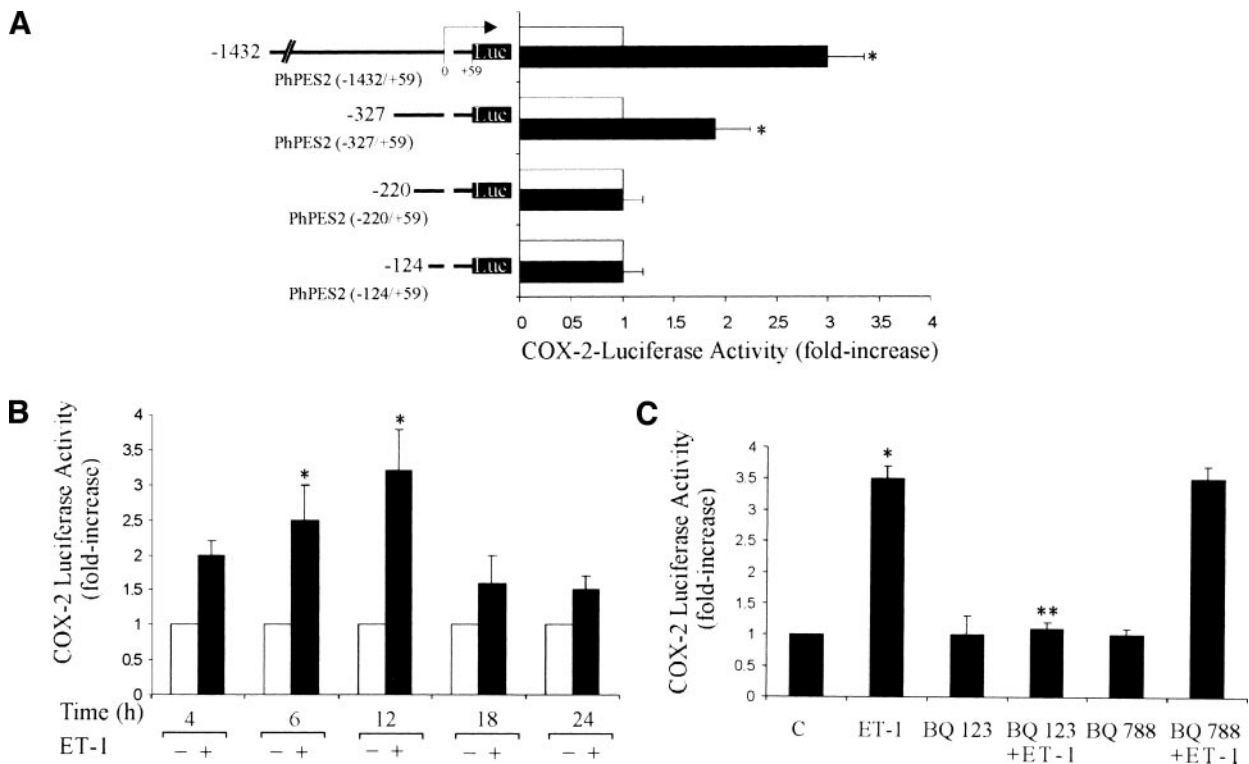


Fig. 3 A, endothelin (ET)-1 stimulates COX-2 promoter activity. Effect of ET-1 on four different-sized fragments of the human COX-2 promoter coupled with a luciferase reporter gene. *Left*, the constructs of four different-sized fragments of the human COX-2 promoter coupled with a luciferase reporter gene are shown. *Right*, COX-2 promoter activity stimulated by ET-1 for 12 h (■) was examined using the luciferase assay system in four independent experiments and expressed as fold increase relative to control (□) after normalizing the differences in transfection efficiency by the activity of the pCMV-β-galactosidase plasmid. *, $P < 0.006$ compared with control. B, time course of ET-1-induced phPES2(-1432/+59) COX-2 promoter activity. HEY cells were incubated with 100 nM ET-1 (■) for the indicated times, and COX-2 promoter activity was measured and expressed as fold increase with respect to the control (□), *, $P < 0.005$. C, ET-1 induces COX-2 promoter activity through ET A receptor. phPES2(-1432/+59)-transfected HEY cells were incubated for 12 h with or without ET-1 (100 nM) alone or in combination with either BQ123 (1 μM) or BQ 788 (1 μM), and COX-2 promoter activity was measured and expressed as fold increase compared with control. Data expressed are the means of results from four experiments, each performed in triplicate. *Bars*, SD. *, $P < 0.004$ compared with control; **, $P < 0.0005$ compared with ET-1.

ways were involved in ET-1-induced COX-2 expression in HEY ovarian cancer cells. We found that ET-1 treatment stimulated the activation of MAPK (Fig. 4). Western blots of HEY cell extracts showed that ET-1 (100 nM) induced a time-dependent increase in phosphorylated p42/44 MAPK that reached a peak

after 10 min, before returning to baseline levels by 30 min (Fig. 4A). ET-1 also increased the phosphorylation of p38 MAPK. Time course experiments revealed that phosphorylated p38 MAPK expression peaked at 10 min and returned to the baseline after 15 min of exposure to 100 nM ET-1 (Fig. 4B). The

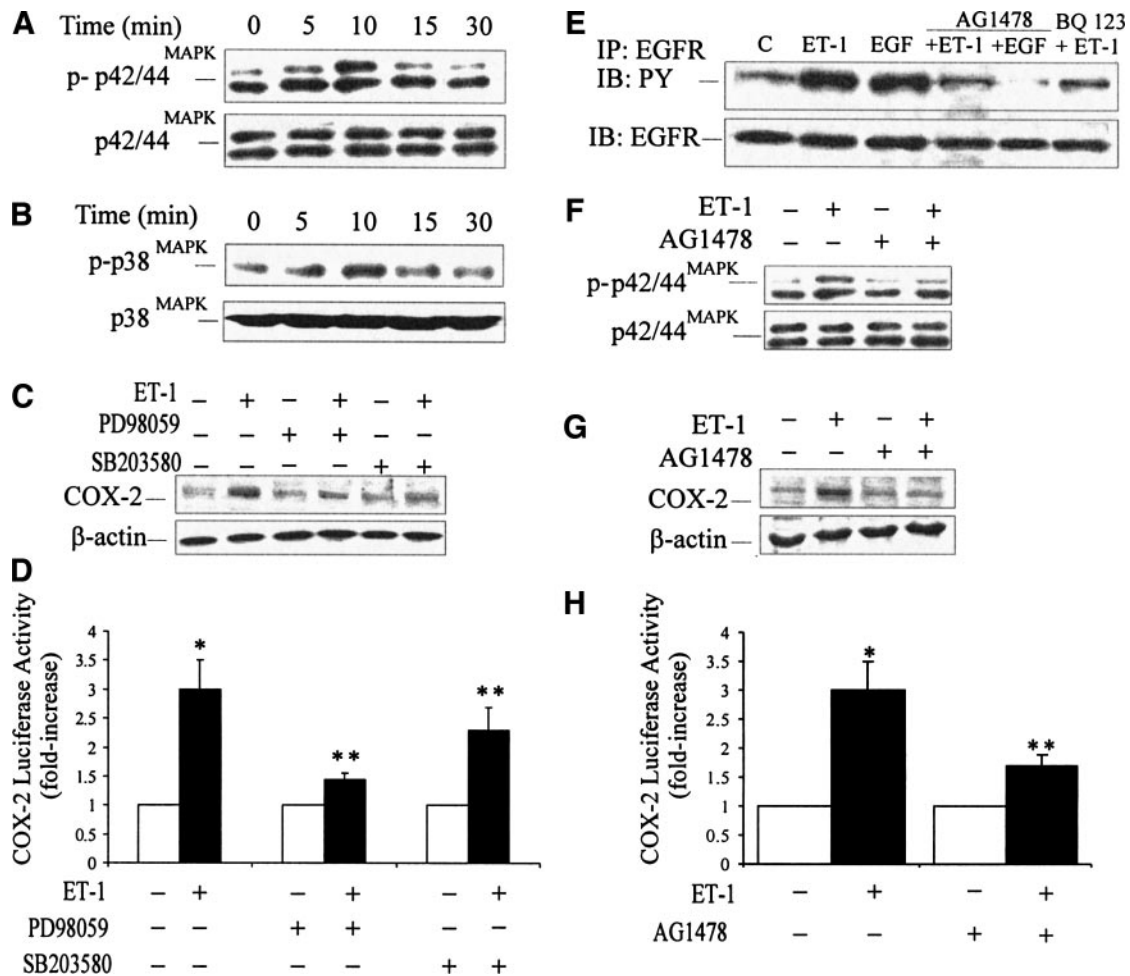


Fig. 4 Endothelin (ET)-1 induces COX-2 expression through mitogen-activated protein kinase (MAPK) pathways and epidermal growth factor receptor (EGFR) transactivation. **A**, time course of ET-1-induced p42/44 MAPK phosphorylation. Serum-starved HEY cells were treated with 100 nM ET-1 for different times and examined by Western blotting using an anti-phospho-p42/44 MAPK antibody. Proteins were normalized for loading against p42/44 MAPK. **B**, time course of ET-1-induced p38 MAPK phosphorylation. Serum-starved HEY cells treated as described in **A** were examined by Western blotting using selective antibody for the phosphorylated (*p-p38*) form of the p38 MAPK. Proteins were normalized for loading against p38 MAPK. **C**, p42/44 and p38 MAPK inhibitors block ET-1-induced COX-2 expression. Serum-starved HEY cells were pretreated with the selective MEK inhibitor PD98059 (10 μ M) and the selective p38 MAPK inhibitor SB203580 (5 μ M) and then stimulated with 100 nM ET-1 for 10 min. Cell lysates were examined for COX-2 protein expression by Western blotting. Proteins were normalized for loading against β -actin. **D**, ET-1-induced COX-2 promoter activity is mediated through MAPK. phPES2(-1432/+59)-transfected HEY cells were pretreated with PD98059 or SB203580 and incubated for 12 h with ET-1 (100 nM; ■). ET-1-induced COX-2 promoter activity was indicated as fold increase with respect to the control (□). *, $P < 0.005$ compared with control; **, $P < 0.0004$ compared with ET-1. **E**, ET-1 induces EGFR transactivation. Serum-starved HEY cells were stimulated with ET-1 (100 nM) or epidermal growth factor (10 ng/ml) alone or in combination with either AG1478 (0.1 μ M) or BQ 123 (1 μ M), and cell lysates were immunoprecipitated with anti-EGFR and immunoblotted with anti-phospho-tyrosine. The same filter was stripped and reprobed with anti-EGFR. **F**, EGFR tyrosine kinase inhibitor decreases ET-1-induced p42/44 MAPK. Serum-starved HEY cells were preincubated with EGFR tyrosine phosphorylation inhibitor AG1478 (0.1 μ M) and then treated with ET-1 (100 nM) for 10 min. p42/44 MAPK phosphorylation was examined by Western blotting using anti-phospho-p42/44 MAPK. Proteins were normalized for loading against p42/44 MAPK. **G**, EGFR tyrosine kinase inhibitor reduces ET-1-induced COX-2 protein expression. Serum-starved HEY cells were pretreated with AG1478 (0.1 μ M) and then treated with 100 nM ET-1 for 24 h, and COX-2 protein expression was examined by Western blotting. Proteins were normalized for loading with β -actin. **H**, AG1478 reduces ET-1-induced COX-2 promoter activity. phPES2(-1432/+59)-transfected HEY cells were pretreated with AG1478 (0.1 μ M) and incubated for 12 h with ET-1 (100 nM; ■). ET-1-induced COX-2 promoter activity was indicated as fold increase with respect to the control (□). *, $P < 0.005$ compared with control; **, $P < 0.0003$ compared with ET-1.

selective inhibitors of MAPK/extracellular signal-regulated kinase [MEK (PD98059)] and p38 MAPK (SB203580) significantly inhibited ET-1-stimulated COX-2 protein expression (Fig. 4C) and promoter activity (Fig. 4D). Furthermore, in HEY cells, ET-1 (100 nM for 5 min) significantly increased tyrosine phosphorylation of EGFR to an extent similar to that induced by epidermal growth factor (Fig. 4E). Treatment of HEY cells with the EGFR tyrosine kinase inhibitor, AG1478 (0.1 μ M), significantly inhibited epidermal growth factor- and ET-1-induced EGFR activation (Fig. 4E). BQ 123 was able to reduce the EGFR transactivation induced by ET-1 (Fig. 4E). The p42/44 MAPK phosphorylation induced by ET-1 was significantly but incompletely reduced by AG1478, indicating that dual signaling pathways converge on p42/44 MAPK activation, one of which is dependent on EGFR kinase activity, and the other is independent of EGFR transactivation (Fig. 4F). As shown in Fig. 4, G and H, AG1478 significantly inhibited the ET-1-induced COX-2 protein levels and COX-2 promoter activity. Taken together, these data indicate that transactivation of the EGFR, p38 MAPK, and p42/44 MAPK-dependent pathways are involved in ET_AR-mediated regulation of COX-2 expression.

COX-1 and -2 Inhibitors Decrease ET-1-Induced PGE₂ and VEGF Production. Selective or nonselective COX inhibitors were used to discriminate between COX-2- and COX-1-derived PGE₂ production after ET-1 stimulation. COX-2 inhibitors NS-398 (1 μ M) and SC-58125 (70 nM), as well as the non-COX isotype-selective inhibitor indomethacin (26 μ M), significantly blocked PGE₂ production after 24 h of ET-1 (100 nM) treatment, whereas COX-1 inhibitor SC-560 (9 nM) partially decreased ET-1-induced PGE₂ production (Fig. 5A). These data indicate that both enzymes participate, to a different extent, in PGE₂ production in these COX-1/COX-2-positive ovarian carcinoma cell lines. Several lines of evidence indicate that COX-derived PGE₂ also plays an important role in tumor angiogenesis (38). Therefore, we evaluated COX-2- and COX-1-mediated VEGF production in ET-1-stimulated ovarian cancer cells by ELISA. Pretreatment of HEY cells with both COX-2 inhibitors, NS-398 (1 μ M) and SC-58125 (70 nM), indomethacin (26 μ M), and COX-1 inhibitor (SC-560; 9 nM) resulted in a significant decrease of ET-1-induced VEGF production compared with untreated cells (Fig. 5B), suggesting that both COX enzymes participate in the angiogenic effect of ET-1.

ET_AR Antagonist-Induced Inhibition of Tumor Growth in HEY Xenografts Is Associated with Decreased COX-2 Expression. To evaluate whether *in vivo* COX-2 is a downstream signal during ET_AR/ET-1-induced tumor growth and progression, we examined the effect of a potent ET_AR antagonist, ABT-627, on COX-2 expression in murine ovarian carcinoma xenografts. The treatment with 2 mg/kg/day dose of ABT-627 produced a 65% inhibition of HEY tumor growth on day 40 after tumor injection ($P < 0.001$ compared with control) and was generally well tolerated, with no detectable signs of acute or delayed toxicity (19). Immunohistochemical evaluation of the expression of COX-2 on HEY tumors at day 40 after tumor cell injection revealed a significant and homogeneous decrease of cytoplasmic COX-2 staining in HEY tumor treated with ABT-627 (Fig. 6A) compared with tumors of untreated animals. Western blot analysis of COX-2 protein expression

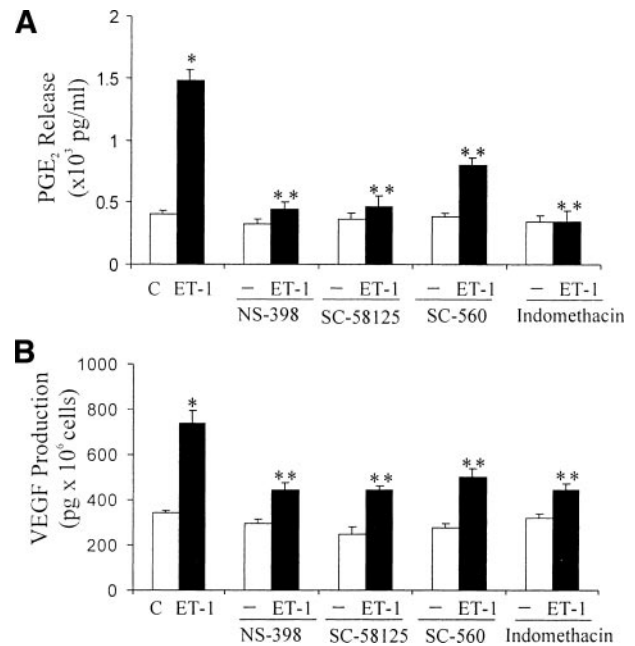


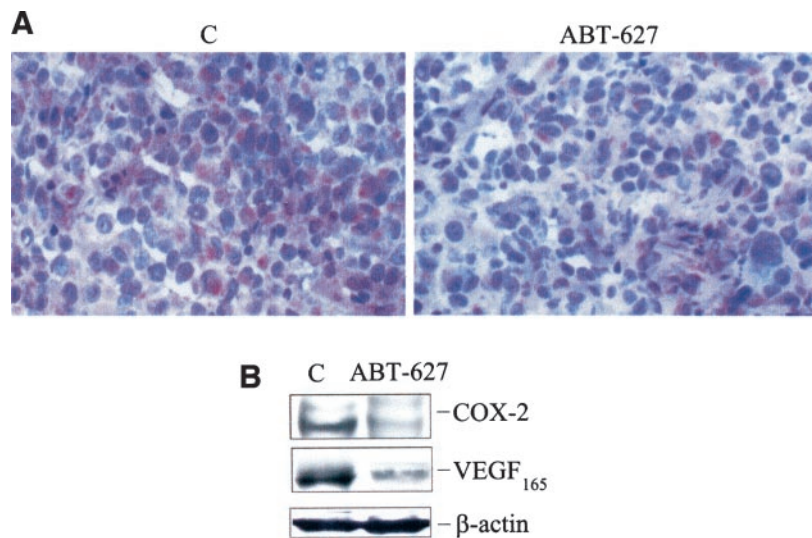
Fig. 5 Cyclooxygenase (COX)-1 and COX-2 inhibitors affect endothelin (ET)-1-induced prostaglandin E₂ and vascular endothelial growth factor production. **A**, serum-starved HEY cells were pretreated with COX-2-selective inhibitors NS-398 (1 μ M) and SC-58125 (70 nM), COX-1-selective inhibitor SC-560 (9 nM), or a nonselective COX inhibitor, indomethacin (26 μ M), and cultured in the absence (□) or presence of 100 nM ET-1 (■) for 24 h. Prostaglandin E₂ release was measured by ELISA. Data represent the means of results from three experiments, each performed in triplicate, Bars, SD. *, $P < 0.005$ compared with control; **, $P < 0.0001$ compared with ET-1. **B**, vascular endothelial growth factor production in conditioned media from HEY cells treated as described in **A** was evaluated by ELISA. Data represent the means of results from three experiments, each performed in triplicate, Bars, SD. *, $P < 0.001$ compared with control; **, $P < 0.0002$ compared with ET-1.

performed on HEY tumor xenografts revealed a marked reduction of COX-2 in ABT-627-treated mouse compared with control, which paralleled the ability of ABT-627 to inhibit VEGF expression (Fig. 6B). These data demonstrate that ET_AR antagonist prevents *in vivo* COX-2 expression and subsequent VEGF expression, indicating that blocking of the ET_AR may contribute to tumor growth inhibition by also reducing COX-2-mediated angiogenesis.

DISCUSSION

Chemical carcinogenesis experiments and epidemiological and clinical studies have collectively identified PGs and their rate-limiting enzymes, COX-1 and COX-2, as molecules involved in the onset and progression of a variety of malignancies (39). The development of selective inhibitors of COX-2 clearly adds a novel potential pharmacological target in cancer prevention and treatment. In view of this, studies aimed at identifying metabolic pathways involved in COX induction are of major relevance from a biological as well as a clinical point of view. Different growth factors that bind tyrosine kinase and GPCR have been shown to induce COX-2 expression (36, 37). The

Fig. 6 The endothelin A receptor antagonist, ABT-627, inhibits cyclooxygenase (COX)-2 expression *in vivo*. **A**, comparative immunohistochemical analysis of COX-2 expression in HEY tumor xenografts. Levels of COX-2 staining are significantly higher in control (C) specimens compared with those detected in biopsies of treated animals with the endothelin A receptor antagonist ABT-627 (original magnification, $\times 200$). **B**, Western blot analysis of total cell lysates obtained from freshly excised tumor on day 40 after tumor cell injection. An equal amount of proteins from untreated mice (C) or ABT-627-treated mice was subjected to Western blotting and analyzed for COX-2 and vascular endothelial growth factor protein levels. Proteins were normalized for loading against β -actin.



present study implicates for the first time COX-1 and COX-2 as downstream signals during the ET_AR/ET-1 autocrine pathway, providing evidence that ET-1 drastically increases COX-2 promoter activity, COX-1 and COX-2 expression, and, in parallel, PGE₂ levels. By using selective and nonselective COX inhibitors, we were able to discern the contribution of each COX enzyme to ET-1-induced PGE₂ production. Furthermore, we dissected the signaling pathways mediating COX-2 induction by ET-1 in ovarian tumor cells using different pharmacological inhibitors.

Several signaling pathways, including p42/44 and p38 MAPK, have been implicated in the regulation of COX-2 expression (20, 23, 36). Here we demonstrated that the MEK pathway inhibitor as well as the p38 MAPK inhibitor blocked ET-1-induced COX-2 promoter activity and COX-2 protein expression, indicating that ET-1-mediated effects are likely to be dependent on the MAPK pathway. Moreover, ET-1-induced COX-2 expression and activity require ligand-independent activation of EGFR, as demonstrated by the inhibitory effect exerted by the EGFR tyrosine kinase inhibitor AG1478, indicating that ET-1-induced effects related to COX-2 expression are also mediated by EGFR transactivation.

It should be noted that EGFR transactivation is not exclusively responsible for the mitogenic activity of ET-1 in ovarian carcinoma cells and that cross-talk with other signaling pathways could be relevant for ET-1-induced proliferation. Although tyrphostin AG1478 completely inhibits the activation of EGFR and COX-2 expression and activity, it only partially inhibits p42/44 MAPK phosphorylation in response to ET-1, indicating that an alternative pathway is also involved in ET-1-induced mitogenic signaling and p42/44 MAPK activation. Consistent with this finding, Guo *et al.* (36) demonstrated that like ET-1, gastrin, another GPCR agonist, stimulates COX-2 expression through multiple signaling pathways including EGFR transactivation in intestinal epithelial cells, thus identifying a mechanism involved in the initiation and progression of colorectal cancer.

Over the past few years, numerous studies have investi-

gated the role of EGFR in GPCR signal transduction. Daub *et al.* (40) first identified EGFR as a critical element in ET-1-, lyso-phosphatidic acid-, and thrombin-induced MAPK activation. EGFR transactivation is now recognized as a key mechanism that couples distinct signal transduction pathways to diverse cellular responses (41). Several reports have demonstrated that GPCR-induced EGFR transactivation may be mediated via an intracellular signaling pathway. The cross-signaling mechanism leading to activation of the EGFR kinase may involve protein kinase C or the intracellular tyrosine kinase Src (42). In our previous report (18), we demonstrated that ET-1 is able to activate Src by inducing phosphorylation on Tyr⁴¹⁸. In view of this, it is possible to speculate that this mechanism could be involved in ET-1-induced EGFR transactivation.

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 (38). We reported previously (16) 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 the ET_AR and that this effect is mediated by HIF-1 α accumulation and activation (16, 17). Here we demonstrated that in HEY and OVCA 433 cells, the ET-1-induced PGE₂ release paralleled the increase in VEGF production, indicating that ET-1-induced VEGF expression may be regulated through a dual mechanism including HIF-1 activation and COX expression. The possibility that ET-1-induced up-regulation of VEGF may be regulated through coordinate interdependent mechanisms involving HIF-1 directly and indirectly through COX-2 expression and PGE₂ production remains to be explored in the future.

The ability of COX-2-selective inhibitors to reduce ET-1-mediated PGE₂ formation indicates that COX-2 plays a major role in PG production in these ovarian cancer cells. These results are further supported by the recent data of Denkert *et al.* (43), who demonstrated that COX-2 is the major source of PGE₂ in a different COX-1- and COX-2-positive ovarian carcinoma cell

line. However, the ability of the COX-1-selective inhibitor SC-560 to induce a partial reduction in ET-1-induced PGE₂ and VEGF production suggested that, in HEY cells, the ET-1-induced COX-1 up-regulation represents an active and concomitant mechanism that can, in part, increase PGE₂. These data open the intriguing possibility that ET-1 may use both COX enzymes and their products toward the development of ovarian carcinoma.

Interestingly, we found that the addition of a specific ET_AR antagonist, BQ 123, blocked ET-1-induced COX-1 and COX-2 expression and COX-2 promoter activity. Moreover, BQ 123 was able to block PGE₂ secretion to the same extent as the COX nonselective inhibitor indomethacin, demonstrating the key role of the ET_AR-mediated pathway in the induction of both COX-1 and COX-2-derived PGE₂ production. We have demonstrated previously that *in vivo* blockade of the ET_AR autocrine pathway using the highly specific antagonist ABT-627 is associated with a significant reduction of microvessel density, VEGF expression, and matrix metalloproteinase 2 and connexin 43 phosphorylation and with increased tumor apoptosis (18, 19). In this study, we found that ABT-627 treatment decreases COX-2 expression concomitantly with growth of tumor xenografts, indicating that the antitumoral activity of this small molecule may also be due to the inhibition of COX-2 activity.

In conclusion, the present results implicate ET_AR in the induction of COX-1 and COX-2 expression and their downstream effect in ovarian cancer. Given that the two enzymes are overexpressed in the majority of these tumors (26–33), the possibility of blocking their activity may have relevant implication in the prevention and treatment of this malignancy. Targeting COX and related signaling cascade via ET_AR blockade may be therapeutically advantageous in the treatment of ovarian carcinoma. COX-2 expression has been shown to be a factor indicating poor prognosis (30, 31, 44, 45) and a predictor of chemoresistance in ovarian cancer patients treated with standard chemotherapy (32). Consistent with these data, a functional link between increased levels of COX-2 and cellular resistance against apoptosis in response to a variety of extracellular and intracellular stimuli has recently been established (46, 47). In this regard, combination treatment with COX-2 inhibitors and ET_AR antagonist may be warranted to design newer therapeutic approaches to ovarian cancer, which could result in increased susceptibility of the cells to apoptosis.

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