

# Effect of endothelin-1 on cyclooxygenase-2 expression in human hormone refractory prostate cancer cells

QI SU<sup>1</sup>, RUI-PENG JIA<sup>1</sup>, JIANZHONG LIN<sup>1</sup>, LU-WEI XU<sup>1</sup>,  
ZI-ZHENG WANG<sup>2</sup>, WEN-CHENG LI<sup>1</sup> and SHU-KUI WANG<sup>2</sup>

Departments of <sup>1</sup>Urology, and <sup>2</sup>Central Laboratory,  
Nanjing First Hospital Affiliated with Nanjing Medical University, Nanjing 210006, P.R. China

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**Abstract.** The present study aimed to explore the effects and possible mechanisms of recombinant human endothelin (ET)-1 on cyclooxygenase (COX)-2 expression in human hormone refractory prostate cancer PC3 cells. PC3 cells were treated with 100 nmol/l ET-1 for the indicated times (3, 6, 9, 12 and 24 h) and concentrations (0.1, 1, 10 and 100 nmol/l) for 24 h. Moreover, 100 nmol/l ET-1 was used to treat PC3 cells alone or in combination with endothelin A receptor (ET<sub>A</sub>R) antagonist BQ123 (1  $\mu$ mol/l), endothelin B receptor (ET<sub>B</sub>R) antagonist BQ788 (1  $\mu$ mol/l), MAPK/extracellular signal-regulated kinase kinase (MEK)-selective inhibitor, PD98059 (10  $\mu$ mol/l), p38 mitogen-activated protein kinase (MAPK) antagonist SB203580 (5  $\mu$ mol/l) or epidermal growth factor receptor (EGFR) antagonist AG1478 (0.1  $\mu$ mol/l) for 24 h. COX-2 mRNA and protein expression was detected in the PC3 cells by reverse transcription-polymerase chain reaction and Western blot analysis. ET-1 induced a time- and dose-dependent increase in the mRNA and protein expression of COX-2 in the PC3 cells. BQ123, LY294002, SB203580 and AG1478 prevented the expression of COX-2 in the PC3 cells ( $P < 0.05$ ), while BQ788 did not. ET-1 induced the up-regulation of COX-2 in the PC3 cells. ET<sub>A</sub>R may be involved in the process. Several signaling pathways, including p42/44 MAPK, p38 MAPK and EGFR, are therefore implicated in the regulation of COX-2 expression.

## Introduction

Prostate cancer (Pca) is the most common malignancy affecting males and is the second leading cause of cancer-related death among males in the US (1). One consistent characteristic of this type of cancer is the eventual progression to a hormonal

refractory state. The development of effective novel therapeutic strategies requires an understanding of the mechanisms for the development of such a refractory state. Targeting proliferative and survival pathways provides a rationale for drug design and development for human hormone refractory prostate cancer (HRPC). Prostate cancer cells develop an enhanced redundancy in downstream survival signaling, which is very important for the development and progression of HRPC (2). Cyclooxygenase (COX)-2 is an inducible enzyme stimulated by cytokines, growth factors, oncogenes or tumor promoters during inflammation and malignancy. An increased COX-2 expression is associated with decreased apoptosis, increased tumor invasiveness, immunosuppression and angiogenesis. Furthermore, an increased COX-2 expression correlates with poor differentiation, increased tumor size, increased nodal and distant disease and decreased patient survival in a variety of cancers (3-7). The endothelin (ET) family is composed of three isopeptides, ET-1, -2 and -3, which are potent mitogens for several types of human tumors, including Pca. ET-1 and their receptors are implicated in tumor progression through autocrine and paracrine pathways (8,9).

ET-1 plays an important role in modulating COX-2 expression in various types of normal cells (10-13), but the precise molecular mechanisms controlling these effects remain undefined. Spinella *et al* reported that ET-1 appears to lead to an increased COX-2 expression in human ovarian carcinoma cells (14). However, the role of ET-1 in the regulation of COX-2 in human HRPC cells has yet to be investigated. The present study examined whether activation of the endothelin A receptor (ET<sub>A</sub>R)/endothelin B receptor (ET<sub>B</sub>R) by ET-1 leads to the up-regulation of COX-2 expression. Possible molecular mechanisms in the human PC3 cell line were also investigated.

## Materials and methods

**Cell culture.** The PC3 cell line (American Type Culture Collection, Rockville, MD, USA) was cultured in F12 medium containing 10% fetal bovine serum. The cells were serum-starved for 24 h in serum-free F12 medium. Culture reagents were from Invitrogen (Paisley, Scotland, UK). ET-1 (Merck, Darmstadt, Germany), dissolved in deionized water, was added to the cell medium at the indicated concentration and for the indicated time. BQ123

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*Correspondence to:* Dr Rui-Peng Jia, Department of Urology, Nanjing First Hospital Affiliated with Nanjing Medical University, Nanjing 210006, P.R. China  
E-mail: jiaruipengnj@msn.com

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(ET<sub>A</sub>R antagonist) (1  $\mu$ mol/l), BQ788 (ET<sub>B</sub>R antagonist) (1  $\mu$ mol/l), PD98059 (selective MEK inhibitor) (10  $\mu$ mol/l), p38 SB203580 (p38 MAPK inhibitor) (5  $\mu$ mol/l) and AG1478 [epidermal growth factor receptor (EGFR) antagonist] (0.1  $\mu$ mol/l) (Sigma, St. Louis, MO, USA) were all dissolved in 1% dimethyl sulfoxide (DMSO). After their effects were studied, they were added to the medium for 24 h with or without treatment with ET-1 (100 nmol/l). To remove any possible effect of the solvent DMSO on the cells, the control group also contained 1% DMSO. Experiments were repeated at least three times.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA from PC3 cells was extracted using TRIzol reagent (Invitrogen Life Technologies, Burlington, Ontario, Canada), according to the manufacturer's instructions. The quality of the RNA was verified by agarose gel electrophoresis using ethidium bromide staining. For each PCR, 2  $\mu$ g DNA-free total RNA with oligo (deoxythymidine) primers and reverse transcriptase were used. PCR was performed in 50- $\mu$ l reactions containing 2.5 ng of cDNA, 1  $\mu$ l of each primer pair and 25  $\mu$ l of Premix Taq (Takara, Shiga, Japan). PCR was carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech Inc., Kirkland, Quebec, Canada) to determine the annealing temperature for each set of paired primers. The COX-2 primer pairs used were: 5'-CGAGGTGTATGTATGAGTGTG-3' (forward) and 5'-TCTAGCCAGAGTTTCACCGTA-3' (reverse), with the length of the product being 582 bp. Thirty cycles of amplification were performed under the following conditions: melting at 94°C for 30 sec, annealing at 55.5°C for 30 sec and extension at 72°C for 1 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel. Controls involved omitting reverse transcriptase, cDNA or DNA polymerase and showed no reaction bands. Data were normalized by  $\beta$ -actin RNA.

**Western blot analysis.** The PC3 cells were homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Complete; Roche, Mannheim, Germany). The lysate was then centrifuged at 12,000  $\times$  g for 20 min at 4°C. The total protein concentration of each sample was analyzed using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). Equal amounts (40  $\mu$ g) of protein were resolved by 5 and 10% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Following transfer, membranes were blocked with 5% fat-free milk in Tris-buffered saline plus 0.05% Tween-20 overnight at 4°C. The membranes were then incubated with the primary antibody (goat polyclonal COX-2 antibodies, diluted 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. After being washed in TBST (Tris-buffered saline Tween-ZO) three times, the membranes were incubated with the peroxidase-linked rabbit anti-goat IgG conjugates (Santa Cruz Biotechnology) for 1 h at room temperature. Finally, they were washed again in TBST, incubated in enhanced chemiluminescence reagents (Pierce) for 2 min, and exposed to X-Omat BT film (Eastman Kodak, Rochester, NY, USA). The level of  $\beta$ -actin

expression was used as the internal control for equal loading. The Western blotting bands were scanned and analyzed with a Bio-Rad image analysis system. For negative controls, the primary antibody was omitted.

**Statistical analysis.** Most of the summarized densitometric data represent the average from at least three experiments. Data were retrieved and processed with the software SPSS13.0. In the experimental section dealing with the ET-1 stimulation of COX-2 expression, one-way ANOVA and the unpaired SNK-q-test were used when the adjacent groups were compared. In the experimental section involving the antagonists for each signaling pathway, one-way ANOVA and the LSD test were used when the control and the remaining groups were compared. Data were expressed as the means  $\pm$  standard error of means (SEM).  $P < 0.05$  was considered statistically significant.

## Results

**ET-1 stimulates COX-2 expression.** The premise of whether ET-1 regulates COX-2 expression in the PC3 cell line was initially investigated. ET-1 markedly induced the time-dependent up-regulation of COX-2 mRNA in the PC3 cells. RT-PCR analysis showed that the COX-2 mRNA levels increased in the ET-1-treated cells compared with the control group by 2-, 2.3-, 2.6-, 3- and 2.9-fold at 3, 6, 9, 12 and 24 h, respectively (Fig. 1A). Moreover, ET-1 treatment evoked a time-dependent increase in COX-2 protein levels. Western blot analysis showed a low expression of COX-2 protein in the untreated PC3 cells, but a 1.5-fold increase after 3 h, and a 1.9-, 2.1-, 2.5- and 2.3-fold increase after 6, 9, 12 and 24 h of ET-1 stimulation, respectively (Fig. 1C and E). ET-1 also increased COX-2 mRNA and protein levels in a dose-dependent manner. Treatment of PC3 cells with 0.1 and 1 nM ET-1 for 24 h showed 1.5 and 2-fold increases in the COX-2 protein expression, respectively, which reached maximum responses at 100 nM ET-1 (Fig. 1D). COX-2 mRNA levels increased 1.5-fold at 0.1 nM ET-1 and then increased gradually. The highest level of COX-2 mRNA (2.4-fold compared with the control) was also detected at 100 nM ET-1 (Fig. 1B and F).

**ET-1-induced COX-2 up-regulation is mediated through ET<sub>A</sub>R.** To investigate which receptor subtype mediates the ET-1-induced up-regulation of COX-2 expression, selective ET<sub>A</sub>R and ET<sub>B</sub>R antagonists, BQ123 and BQ788, respectively, were used in the presence or absence of 100 nM ET-1. As shown in Fig. 2B and C, BQ123 was able to completely block ET-1-induced COX-2 mRNA expression (46% for ET-1,  $P < 0.05$ ; 118% for control,  $P > 0.05$ ), whereas BQ788 did not (96% for ET-1,  $P > 0.05$ ; 248% for control,  $P < 0.05$ ). This result was similar to that of the COX-2 protein expression (Fig. 2A). Taken together, these findings indicate that ET-1 acts through ET<sub>A</sub>R to stimulate COX-2 expression in PC3 cells.

**Signaling pathways are involved in ET-1-stimulated COX-2 expression.** To investigate the signaling pathways involved in ET-1-induced COX-2 expression, 100 nmol/l ET-1 were added with PD98059 (selective MEK inhibitor), SB20358 (p38 MAPK antagonist), or AG1478 (specific EGFR antagonist) into the medium for 24 h. The cell extracts were then

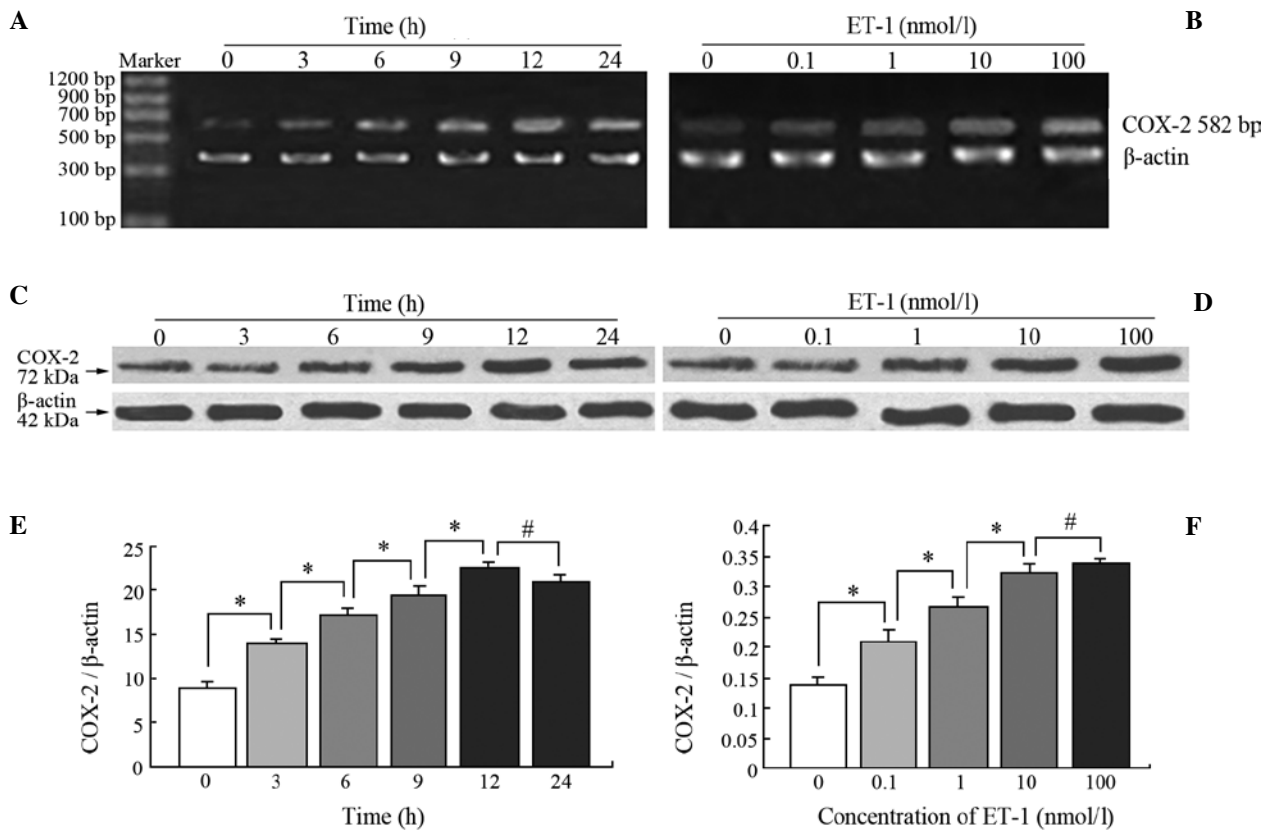


Figure 1. Endothelin (ET)-1 induces a time- and dose-dependent increase in the mRNA and protein expression of cyclooxygenase (COX)-2 in PC3 cells. (A, C and E) Serum-starved PC3 cells were treated with 100 nM ET-1 for the indicated times. (B, D and F) PC3 cells were treated with different concentrations of ET-1 for 24 h. (A and B) COX-2 mRNA induction was analyzed by RT-PCR. Primers for the amplification of  $\beta$ -actin gene were used as controls. (C and D) Kinetics of ET-1 induced a COX-2 protein expression. PC3 cell extracts were analyzed by Western blotting using COX-2 antibodies. Proteins were normalized for loading with  $\beta$ -actin. Histogram represents the densitometric analysis of (E) Western blotting and (F) RT-PCR. Data represent the means of the results from three experiments, each performed in triplicate. Bars,  $\pm$  SEM. \* $P < 0.05$ ; # $P > 0.05$ .

analyzed for COX-2 expression by Western blotting and RT-PCR. PD98059 (10  $\mu$ M) and SB203580 (5  $\mu$ M), which did not affect the COX-2 protein basal levels, markedly inhibited ET-1-stimulated COX-2 protein expression (Fig. 3A and C). Among downstream events after ET<sub>A</sub>R activation, ET-1 resulted in the transactivation of EGFR. Thus, the effect of AG1478 on ET<sub>A</sub>R-mediated effects was examined. Treatment of PC3 cells with AG1478 (0.1  $\mu$ M) markedly inhibited ET-1-induced COX-2 protein production (Fig. 3A and C), indicating an involvement of EGFR in this mechanism. The trend of COX-2 mRNA expression was similar to that of the protein expression (Fig. 3B; data not shown). These findings indicate that ET-1 acts through ET<sub>A</sub>R to induce COX-2 production in PC3 cells and suggest that the transactivation of EGFR, the activation of p38 MAPK-dependent and p42/44 MAPK-dependent pathways are involved in these mechanisms.

## Discussion

Chemical carcinogenesis experiments and epidemiological and clinical studies have collectively identified COX-2 as an important molecule involved in the onset and progression of a variety of malignancies (15). The development of selective inhibitors of COX-2 clearly adds a novel potential pharmacological target to cancer prevention and treatment. Subsequently, studies aimed at identifying the metabolic

pathways involved in COX-2 induction are significant from a biological as well as a clinical point of view.

The ET-1/ET<sub>A</sub>R autocrine pathway plays a key role in the development and progression of prostatic, ovarian and cervical carcinomas (16). Research has demonstrated that ET-1 increases the expression of COX-2 in several cell types, such as human pulmonary epithelial cells, human ovarian carcinoma cells, endothelial cells, mesangial cells and macrophages (14,17-20). As with previous observations, we found that ET-1 markedly induced a time- and dose-dependent up-regulation of the COX-2 mRNA and protein expression in PC3 cells. Notably, although COX-2 expression reached a maximum response following treatment with 100 nM ET-1 for 24 h, no obvious differences were noted between the cell groups treated with 10 and 100 nM ET-1. Thus, we determined that when ET-1 achieves a quantitative concentration, the combination of ET-1 ligands and their receptors reaches a saturation point, and the dose-dependent relationship no longer exists.

ET-1 is known to activate the p42/44 MAPK pathway through ET<sub>A</sub>R in ovarian carcinoma cell lines (21). Moreover, the inhibition of human ovarian tumor growth in nude mice after treatment with the potent ET<sub>A</sub>R-selective antagonist ABT-627 is associated with a reduced COX-2 and vascular endothelial growth factor expression (15). Therefore, we analyzed whether these pathways are involved in ET-1-induced COX-2 expression in PC3 cells and found that the addition of

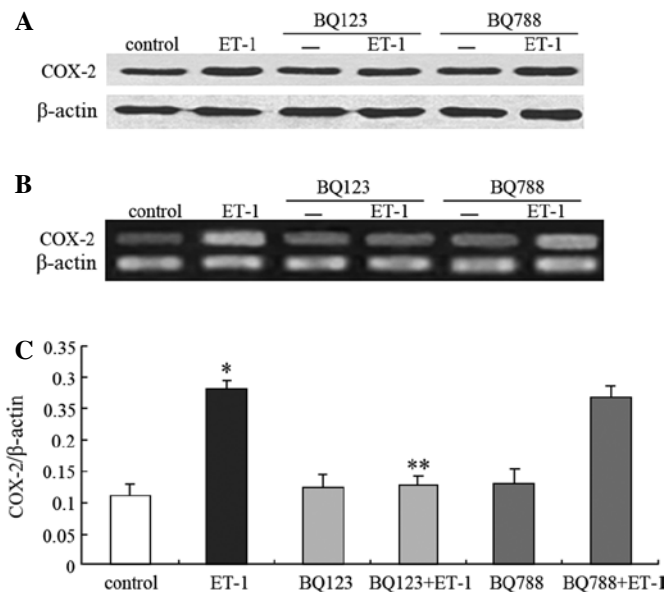


Figure 2. Endothelin (ET)-1 induction of cyclooxygenase (COX)-2 production is mediated through the endothelin A receptor ( $ET_A$ R). Serum-starved PC3 cells were treated with ET-1 (100 nM) alone or in combination with either BQ123 (1  $\mu$ M) or BQ788 (1  $\mu$ M). To remove any possible effect of the solvent DMSO on the cells, the control and ET-1 groups also contained 1% DMSO. (A) Whole cell extracts were analyzed for COX-2 expression by Western blotting. Proteins were normalized for loading with  $\beta$ -actin. (B) COX-2 mRNA was analyzed by RT-PCR. (C) Histogram represents the densitometric analysis of RT-PCR. Data represent the means of the results from three experiments, each performed in triplicate. Bars,  $\pm$  SEM. \* $P$ <0.01, compared with the control; \*\* $P$ <0.01, compared with ET-1.

a specific  $ET_A$ R antagonist, BQ123, blocked the ET-1-induced COX-2 expression. This finding showed that  $ET_A$ R is a key factor in the up-regulation of COX-2 by ET-1. Spinella *et al* (22) and Rosanò *et al* (23) previously demonstrated that using the highly specific antagonist ABT-627, the *in vivo* blockade of the  $ET_A$ R autocrine pathway is associated with an obvious reduction in microvessel density, VEGF expression, matrix metalloproteinase-2, connexin 43 phosphorylation and increased tumor apoptosis. This reduction indicates that the anti-tumoral activity of this small molecule may also be due to the inhibition of COX-2 activity (22,23).

Several signaling pathways, including p38 and p42/44 MAPK, have been implicated in the regulation of COX-2 expression (24). ET-1 regulates COX-2 expression through p38 and p42/44 MAPK in vascular smooth muscle cells (13) and through p42/44 MAPK in osteoblast-like cells (11). The activation of p38 MAPK was found to be involved in ET-1-stimulated COX-2 expression in cultured feline esophageal smooth muscle cells (25). Chen *et al* reported that ET-1 treatment results in an increase in the phosphorylation of both p38 and p42/44 MAPKs in peripheral lung microvascular smooth muscle cells (13). Our study demonstrated that the MEK pathway inhibitor, PD98059, as well as the p38 MAPK inhibitor, SB203580, block the ET-1-induced COX-2 expression, indicating that ET-1-mediated effects are likely to be dependent on the MAPK pathway. Moreover, the ET-1-induced COX-2 expression requires the ligand-independent

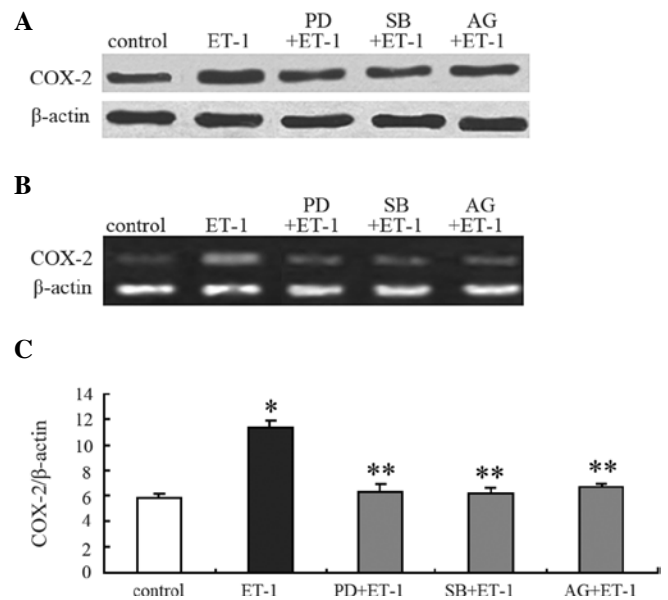


Figure 3. Endothelin (ET)-1 induction of cyclooxygenase (COX)-2 production is mediated through the endothelin A receptor ( $ET_A$ R) and involves mitogen-activated protein kinase (MAPK) pathways. PC3 cells were incubated for 24 h in serum-free medium with ET-1 (100 nM) alone or in combination with either the selective MEK inhibitor PD98059 (PD) (10  $\mu$ M), or p38 MAPK inhibitor SB203580 (SB) (5  $\mu$ M), or with the epidermal growth factor receptor (EGFR) inhibitor AG1478 (AG) (0.1  $\mu$ M). To remove any possible effect of the solvent DMSO on the cells, the control and ET-1 groups also contained 1% DMSO. (A) COX-2 mRNA expression was analyzed by RT-PCR. (B) COX-2 protein expression was analyzed by Western blotting. (C) Histogram represents the densitometric analysis of Western blotting. Data expressed are the means of the results from three experiments, each performed in triplicate. Bars,  $\pm$  SEM. \* $P$ <0.01, compared with the control; \*\* $P$ <0.01, compared with ET-1.

activation of EGFR, as demonstrated by the inhibitory effect exerted by the EGFR tyrosine kinase inhibitor, AG1478, indicating that ET-1-induced effects are also mediated by EGFR transactivation. Consistent with this finding, Guo *et al* (24) showed that, similar to ET-1, gastrin, another G protein-coupled receptor 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.

Our study for the first time demonstrated that the transactivation of the EGFR, p38 MAPK-dependent and p42/44 MAPK-dependent pathways are involved in the  $ET_A$ R-mediated regulation of COX-2 expression in PC3 cells. Although much research has been conducted *in vitro*, the exact roles and the mechanisms of ET-1 and COX-2 *in vivo* remain to be elucidated. However, blocking the activity of ET-1 and COX-2 may have relevant implications in the prevention and treatment of Pca in the future.

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