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Role of Prostaglandin Receptor EP2 in the Regulations of Cancer Cell Proliferation, Invasion, and Inflammation

Jianxiong Jiang and Ray Dingledine

Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia

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ABSTRACT

Population studies, preclinical, and clinical trials suggest a role for cyclooxygenase-2 (COX-2, *PTGS2*) in tumor formation and progression. The downstream prostanoid receptor signaling pathways involved in tumorigenesis are poorly understood, although prostaglandin E2 (PGE_2), a major COX-2 metabolite which is usually upregulated in the involved tissues, presumably plays important roles in tumor biology. Taking advantage of our recently identified novel selective antagonist for the EP2 (*PTGER2*) subtype of PGE_2 receptor, we demonstrated that EP2 receptor activation could promote prostate cancer cell growth and invasion in vitro, accompanied by upregulation of the tumor-promoting inflammatory cytokines, such as IL-1β and IL-6. Our results suggest the involvement of prostaglandin receptor EP2 in cancer cell proliferation and invasion possibly via its inflammatory actions, and indicate that selective blockade of the PGE_2-EP2 signaling pathway via small molecule antagonists might represent a novel therapy for tumorigenesis.

Introduction

As the inducible isozyme of cyclooxygenase (COX), COX-2 is often upregulated in damaged tissue (Nakayama et al., 1998; Steinauer et al., 2000) and thereby contributes to inflammation-related secondary injury (Nogawa et al., 1997; Nakayama et al., 1998; Hawkey, 1999; Iadecola et al., 2001; Minghetti, 2004; Serrano et al., 2011). Over the past decade, COX-2 and its prostanoid products have attracted substantial attention for their possible roles in progression of tumors including those of lung, head and neck, prostate and colon, ovary, breast, and liver (Williams et al., 1999; Soslow et al., 2000; Bae et al., 2001; Grivennikov et al., 2010). Genetic ablation of COX-2 reduces colorectal polyp formation by 86% in a model of human familial adenomatous polyposis, which can be recapitulated by administration of COX inhibitors (Oshima et al., 1996). Epidemiologic studies, preclinical, and clinical trials suggest that taking COX-2 inhibitor drugs regularly might reduce the rates of certain cancers and cancer-related deaths (Pentland et al., 1999; Masferrer et al., 2000; Subbaramaiah and Dannenberg, 2003; Menter et al., 2010), although COX-2 inhibitors are not FDA approved for these indications. Which downstream prostanoid receptor signaling pathway is involved in tumorigenesis has not been fully uncovered; however, upregulation of COX-2 in tumor tissues is accompanied by high levels of prostaglandin E2 (PGE_2) (Gupta and Dubois, 2001; Loh et al., 2002; Murakami and Kudo, 2006). Moreover, administration of PGE_2 can enhance colon carcinogenesis in an azoxymethane (AOM)-induced colon tumor model (Kawamori et al., 2003).

As a major COX-2 product, PGE_2 acts on four G protein-coupled receptors: EP1, EP2, EP3, and EP4. Among these, EP2 and EP4 receptors are positively coupled through Gs to cyclic AMP (cAMP) production (Hirata and Narumiya, 2011). Elevated cAMP in turn initiates multiple downstream events via protein kinase A (PKA) or exchange protein activated by cAMP. For example, PGE_2 via PKA-mediated epidermal growth factor receptor (EGFR) activation can promote cancer cell growth by activating inducible nitric oxide synthase (iNOS)/guanylate cyclase (GC) and mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase 1/2 (ERK1/2) (Donnini et al., 2007). Genetic ablation of the EP2 receptor attenuates tumor growth and prolongs survival in syngeneic mouse tumor models, accompanied by cancer-associated immunodeficiency and defective dendritic-cell differentiation (Yang et al., 2003), decelerates the progression of intestinal polyposis with decreased COX-2 expression in polyp tissues (Sonoshita et al., 2001) and suppresses skin tumor development with reduced blood vessels and increased apoptotic cells in a skin cancer model (Sung et al., 2005). Global ablation of the EP2 gene suppressed hyperplasia of mammary glands that was induced by COX-2 overexpression

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ABBREVIATIONS: ANOVA, analysis of variance; AOM, azoxymethane; cAMP, cyclic AMP; CNS, central nervous system; COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle medium; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GC, guanylate cyclase; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MSD, Meso Scale Discovery; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; PGE_2, prostaglandin E2; PKA, protein kinase A; SID, PubChem substance identifier; TNF-α, tumor necrosis factor-α; TR-FRET, time-resolved fluorescence resonance energy transfer.

(Chang et al., 2005a), and EP2 receptor expression is correlated with vascular endothelial growth factor induction by PGE_2 in mouse mammary tumor cells (Chang et al., 2005b). In addition, EP2 signaling was proposed to regulate tumor angiogenesis in endothelium by enhancing endothelial cell motility and cell survival, to mediate epidermal hypertrophy and tumor aggression in response to UV-irradiation, and to induce skin carcinogenesis (Hawkey, 1999; Steinauer et al., 2000; Majima et al., 2003; Kamiyama et al., 2006; Sung et al., 2006; Brouxhon et al., 2007).

The pathologic functions of EP2 signaling in tumorigenesis described above were derived from studies employing either a selective EP2 agonist (e.g., butaprost) or EP2-deficient mice. Studies based on direct pharmacological inhibition of EP2 receptor are absent, because in contrast to all other prostaglandin receptors no selective antagonist for the EP2 receptor had been available until recently (af Forselles et al., 2011; Jiang et al., 2012). Utilizing our newly discovered EP2 antagonist, we demonstrate here that EP2 receptor activation can promote in vitro cancer cell proliferation, invasion, and the induction of tumor-promoting cytokines. Our results indicate that pharmacological block of PGE_2-EP2 signaling might provide an alternative strategy to suppress tumor formation and progression possibly via an anti-inflammatory mechanism.

Materials and Methods

Cell Culture. Rat C6 glioma (C6G) and human embryonic kidney 293 (HEK) cells stably expressing the human prostanoid receptors were established in the laboratory. Human prostate cancer cell lines DU145, LNCap, and PC3 were purchased from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Grand Island, NY), supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

Chemicals and Drugs. PGE_2 and butaprost were purchased from Cayman Chemical (Ann Arbor, MI). Rolipram was purchased from Sigma-Aldrich (St. Louis, MO). Compound TG4-155 (PubChem SID 17,515,129) was either purchased from ChemDiv (#C281-0155, San Diego, CA) or synthesized by Dr. Thota Ganesh in the laboratory (Jiang et al., 2012).

TR-FRET cAMP Assay. Cytosol cAMP was measured with a homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) method (Cisbio Bioassay) (Jiang et al., 2010). Cells were seeded into 384-well plates in 30 μl complete medium (4,000 cells/well) and grown overnight. The medium was thoroughly withdrawn and 10 μl Hank's buffered salt solution (Mediatech, Manassas, VA) plus 20 μM rolipram was added into the wells to block phosphodiesterase. The cells were incubated at room temperature for 0.5–1 hour and then treated with vehicle or test compound for 5–10 minutes before incubation with increasing concentrations of the EP2 agonist butaprost for 40 minutes. The cells were lysed in 10 μl lysis buffer containing the FRET acceptor cAMP-d2 and 1 minute later another 10 μl lysis buffer with anti-cAMP-cryptate was added. After 60 minutes of incubation at room temperature, the FRET signal was measured by an Envision 2103 Multilabel Plate Reader (PerkinElmer Life Sciences, Waltham, MA) with a laser excitation at 337 nm and dual emissions at 665 nm and 590 nm for d2 and cryptate, respectively. The FRET signal is expressed as: $F665/F590 \times 10^4$.

Schild Regression Analysis. Schild regression was characterized by the equation $\log (dr - 1) = \log X_B - \log K_B$, where dose ratio (dr) = fold shift in EC_{50}, X_B = [antagonist], K_B = equilibrium dissociation constant for the antagonist-receptor complex. A linear regression of $\log (dr - 1)$ on $\log X_B$ with a slope of unity characterizes a competitive antagonism and the K_B value indicates the antagonist concentration required for twofold rightward shift in the dose-response curve. Thus, a smaller K_B value indicates a higher inhibitory potency (Jiang et al., 2012).

Potency of TG4-155 on Prostanoid Receptors. Inhibition by TG4-155 of Gs-coupled prostanoid receptors DP1, EP2, EP4, and IP was assessed by TR-FRET cAMP assay in C6G or HEK cells stably expressing the human receptors. Inhibition by TG4-155 of prostanoid receptors EP1, EP3, FP, and TP was carried out by Cerep (www.cerep.com) and the Schild K_B values were estimated from their cell-based assays.

Off-Target Activity Assays. The effects of TG4-155 on a panel of 40 enzymes, ion channels, and receptors were assessed by either enzyme assays or radioligand binding assays at SRI International (www.sri.com).

Western Blot Analysis. DU145, LNCap, and PC3 cells were grown in 60-mm dishes for 3 days and lysed on ice with 350 μl of RIPA buffer (25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a mixture of protease and phosphatase inhibitors (Roche Applied Science, Penzberg, Upper Bavaria, Germany). The homogenates were centrifuged (15,000 rpm, 15 minutes, 4°C) and protein concentration in the supernate was measured by Bradford assay (Thermo Fisher Scientific, Waltham, MA). Proteins (20 μg) were resolved by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto PVDF membranes (Millipore, Billerica, MA). Membranes were blocked for 2 hours at room temperature with 5% nonfat milk, then incubated overnight at 4°C with primary antibodies: COX-2 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) or EP2 (1:1,000, Cayman Chemical), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:3,000, Santa Cruz Biotechnology). The blots were developed by enhanced chemiluminescence (ECL) (Thermo Fisher Scientific).

Cell Proliferation Assay. In vitro cell proliferation was quantified by Vybrant MTT cell proliferation assay (Molecular Probes, Eugene, OR). In brief, PC3 cells were seeded in 96-well plates (2,000 cells/well) in complete DMEM medium with test compound. After incubation for 48 hours, medium was removed and cells were incubated for 4 hours with 100 μl fresh phenol red-free medium in the presence of 10 μl 12 mM MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Living cells convert MTT to insoluble formazan. After solubilization of formazan in 50 μl DMSO, absorbance of the formazan was measured by a microplate reader (Molecular Devices, Sunnyvale, CA) at 540 nm.

Cell Invasion Assay. Twenty-four-well cell culture inserts (BD Biosciences, San Jose, CA) were coated with 250 μl/ml Matrigel (Invitrogen). PC3 cells (10,000 cells/well) in 0.5 ml DMEM medium were added to the culture inserts. Butaprost with vehicle or test compound in 0.5 ml medium were added to the lower wells. Invasion was measured in triplicate by counting the cells that moved across the membrane coated with Matrigel for 24 hours. Cells were stained with HEMA 3 stain set (Fisher Diagnostics, Middletown, VA) and counted in 5 random fields/well at a magnification of 200 $\times$. Data were reported as the total number of cells counted/well.

Cytokine Measurement. Cytokines secreted from cultured cancer cells were measured using the Meso Scale Discovery (MSD; Rockville, MD) multiplex cytokine/chemokine kit according to the manufacturer's instruction. The MSD plates were read using the MSD SECTOR Imager 2400.

Cytotoxicity Assay. Cytotoxicity of TG4-155 was examined with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Fitchburg, WI) by measuring cellular ATP level, which correlates with cell viability. Briefly, C6G cells were plated in 384-well plates at 2,000 cells/well in 25 μl DMEM plus test compound and incubated for 2 days. CellTiter-Glo reagent (25 μl) was then added to each well. The contents were mixed for 2 minutes on an orbital shaker to induce cell lysis and incubated at room temperature for 10 minutes. Relative viability is proportional to luminescence intensity as measured by a microplate reader (Molecular Devices) with an integration time of 1 second.

Data Mining. Gene expression levels in human cancer cells were obtained from the Agilent mRNA database of the National Cancer

Institute (NCI) CellMiner (http://discover.nci.nih.gov). Data on all mRNA probes were normalized using GeneSpring GX software (Agilent Technologies, Santa Clara, CA) according to Liu et al. (2010). The average was calculated if multiple probes were used for one gene. Data were transformed to linear scale and plotted appropriately.

Statistical Analysis. Data were plotted with Origin software (Northampton, MA). Statistical analyses were performed using GraphPad Prizm software (La Jolla, CA) with one-way analysis of variance (ANOVA) and post hoc Bonferroni test. $P < 0.05$ was considered to be statistically significant. All data are presented as mean ± S.E.M.

Results

Pharmacological Inhibition of Prostaglandin Receptor EP2 in Cancer Cells. PGE_2 is markedly elevated in some tumors (Loh et al., 2002; Murakami and Kudo, 2006). Furthermore, PGE_2-EP2 signaling has been suggested to mediate tumor progression and tumor-associated angiogenesis from studies using EP2 agonists (Chang et al., 2005b; Kamiyama et al., 2006), EP2 deficient mice (Sonoshita et al., 2001; Yang et al., 2003; Chang et al., 2005a; Sung et al., 2005; Kamiyama et al., 2006; Brouxhon et al., 2007), and EP2 overexpressing mice (Sung et al., 2006). However, the effect of direct EP2 inhibition on tumor progression has not been evaluated yet. Taking advantage of our newly-identified EP2 antagonist TG4-155 (PubChem SID 17,515,129) (Fig. 1A), we wanted to study the pharmacological effect of selective EP2 inhibition in the prostate cancer cells. First, we evaluated the selectivity of TG4-155 for EP2 receptor against other prostanoid receptors in cell-based functional assays. In a comparison of Schild K_B values, TG4-155 displayed at least 1000-fold selectivity for the EP2 receptor over human EP3, EP4, and FP receptors; at least 500-fold selectivity against human EP1 and IP receptors; at least 300-fold selectivity against human TP receptor; and approximately 14-fold selectivity against human DP1 receptor (Fig. 1B). These results indicate that of

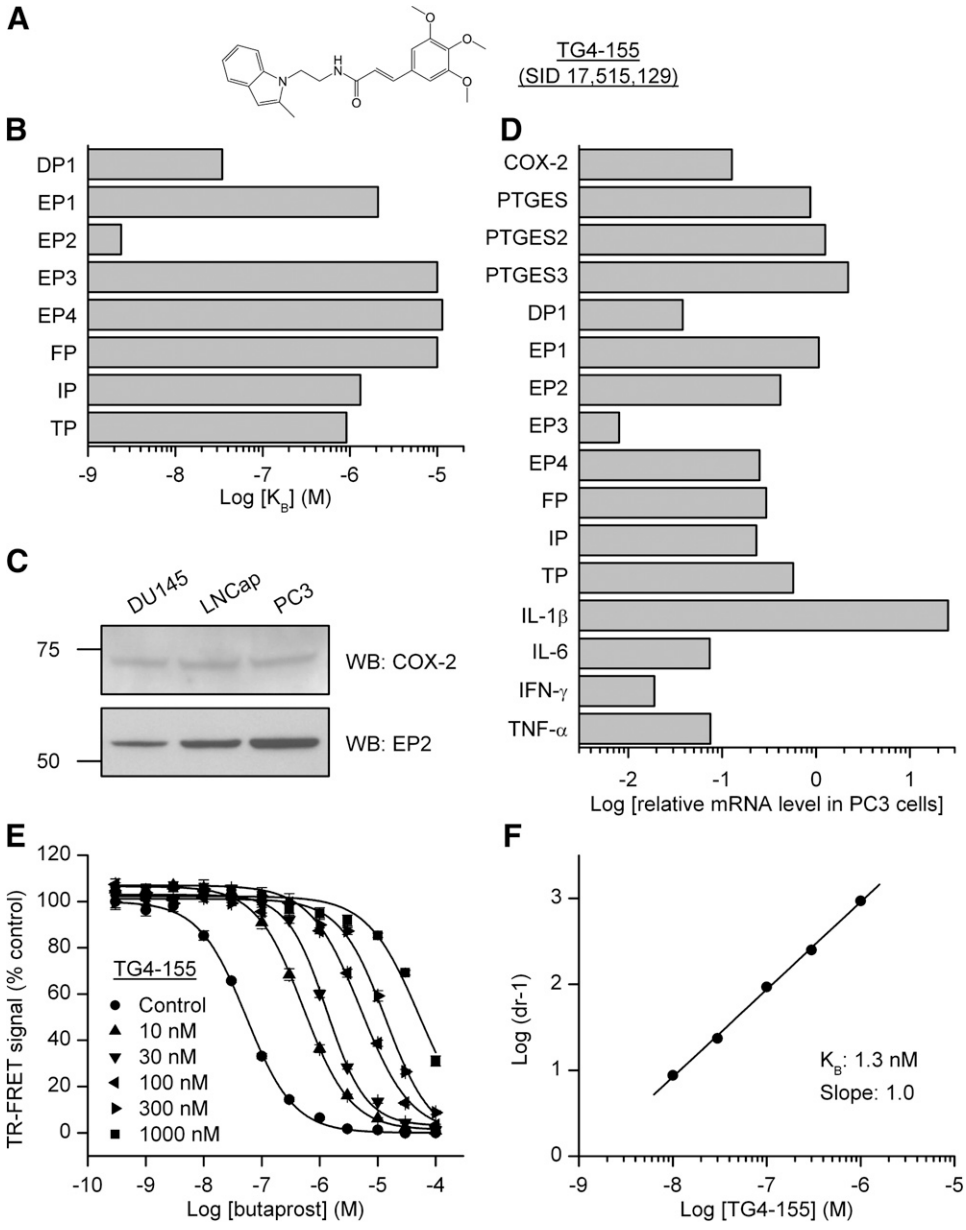

Fig. 1. COX-2 and prostaglandin receptor EP2 signaling in cancer cells. (A) Chemical structure of EP2 antagonist TG4-155 (SID 17,515,129). (B) The Schild K_B values of TG4-155 were compared for inhibition of eight human prostanoid receptors. Schild K_B values: 34.5, 2100, 2.4, 10,000, 11,400, 10,000, 1320, and 910 nM for DP1, EP1, EP2, EP3, EP4, FP, IP, and TP receptors, respectively. (C) The expression of COX-2 and EP2 receptor in three human prostate cancer cell lines, DU145, LNCap, and PC3, was examined by Western blot analysis. A low basal level of COX-2 (72 kDa) was detected in all three types of cancer cells and the PC3 cells have a relatively high level of EP2 (52 kDa) expressed. (D) Data mining from NCI CellMiner (http://discover.nci.nih.gov). Relative mRNA levels in PC3 cells of COX-2; prostaglandin E synthase; prostanoid receptors DP1, EP1, EP2, EP3, EP4, FP, IP, and TP; and cytokines IL-1β, IL-6, IFN-γ, and TNF-α, as measured by Agilent Whole Human Genome Oligo Microarray kit (Agilent-mRNA, Agilent Technologies). (E) Butaprost induced cAMP production in PC3 cells with an EC_{50} = 54 nM, which was substantially blocked by EP2 antagonist TG4-155 in a concentration-dependent manner. Data were normalized as percent maximum response; points represent mean ± S.E.M. (n = 4 independent experiments). (F) Schild regression analysis was performed to evaluate the potency of TG4-155 in PC3 cells. TG4-155 displayed a competitive antagonism mode of action on EP2 receptor shown by Schild plot with a K_B value 1.3 nM and a slope of 1.0.

the eight canonical prostanoid receptors, TG4-155 shows low nanomolar antagonist activity against only EP2 and DP1, the receptor activated by prostaglandin D2 (PGD_2). Interestingly, the EP2 and DP1 genes are oriented head to head in close proximity to each other in both human and mouse genomes. In the mouse genome, the DP1 gene is located on chromosome 14: 44.85–44.86 Mb and the EP2 gene is located on chromosome 14: 44.99–45.00 Mb; in human genome, the DP1 gene is located on chromosome 14: 52.73–52.74 Mb and the EP2 gene is located on chromosome 14: 52.78–52.80 Mb. This information indicates that they might be the result of a recent gene duplication. Indeed, of the eight prostanoid receptors, EP2 and DP1 share the closest sequence homology (Hirata and Narumiya, 2011). Thus it is unsurprising that EP2 and DP1 receptors share ligand-binding properties. In addition, other off-target activity assays showed that TG4-155 had negligible effect on a panel of 40 human enzymes, ion channels, and receptors (IC_{50} values > 10 μM) except that TG4-155 weakly inhibited the serotonin 5-HT2B receptor with $IC_{50} = 2.6$ μM (Table 1). At high concentration (10 μM) TG4-155 had little or no effect on the enzymatic activity of COX-1 (3% inhibition) and of COX-2 (–4% inhibition), and inhibited leukotriene B4 (LTB_4) receptor BLT1 by 15% (Table 1).

TABLE 1

Off-target activity of EP2 antagonist TG4-155

TG4-155 inhibited the serotonin 5-HT2B receptor with $IC_{50} = 2.6$ μM and hERG (human *Ether-à-go-go*-Related Gene) with $IC_{50} = 12$ μM ($n = 2$).

Target	% Inhibition at 10 μM
Acetylcholinesterase (AChE)	−2
Adenosine A2A	11
Adrenergic α1B	3
Adrenergic α1D	−7
Adrenergic α2A	43
Adrenergic α2C	1
Adrenergic β1	7
Androgen AR	13
Cannabinoid CB1	12
Cyclooxygenase-1 (COX-1)	3
Cyclooxygenase-2 (COX-2)	−4
Cytochrome P450 1A2 (CYP1A2)	8
Cytochrome P450 2B6 (CYP2B6)	18
Cytochrome P450 2C9 (CYP2C9)	34
Cytochrome P450 2C19 (CYP2C19)	26
Cytochrome P450 2D6 (CYP2D6)	13
Cytochrome P450 3A4 (CYP3A4)	30
Dopamine D1	2
Dopamine D2L	12
Estrogen ERα	4
Histamine H1	5
Histamine H2	−10
Leukotriene, BLT (LTB4)	15
Monoamine oxidase (MAO-A)	31
Monoamine oxidase (MAO-B)	10
Nicotinic acetylcholine α, bungarotoxin	−3
Nicotinic acetylcholine $\alpha\beta$, cytisine	−13
Opiate κ (OP2, KOP)	2
Opiate μ (OP3, MOP)	12
Phosphodiesterase PDE3	12
Phosphodiesterase PDE4	−2
Potassium channel K_v11.1 (hERG)	43
Progesterone PR-B	27
Serotonin 5-HT1B	10
Serotonin 5-HT2A	15
Serotonin 5-HT2B	82
Serotonin 5-HT4	31
Transporter, dopamine (DAT)	4
Transporter, norepinephrine (NET)	−19
Transporter, serotonin (SERT)	7

Next, we examined the protein levels of COX-2 and EP2 in three human prostate cancer cell lines: DU145, LNCap, and PC3, by Western blot analysis. All three cell types express a low basal level of COX-2; the PC3 cell line has a relatively high EP2 expression (Fig. 1C), thus was selected for further studies. The NCI-60 panel consists of 60 human cancer cell lines derived from nine types of tumors: breast, central nervous system, colon, kidney, leukemia, lung, melanoma, ovarian, and prostate. Among these are two cell lines with prostate origin—DU145 and PC3. The mRNA and microRNA expression profiles in these cancer cell lines have been extensively studied by microarray and the data are available on NCI CellMiner database (http://discover.nci.nih.gov). We examined the mRNA expression data of PGE_2 signaling-related genes and several proinflammatory cytokine genes in PC3 cells generated by the Agilent whole human genome oligo microarray kit (Agilent-mRNA, Agilent Technologies) (Liu et al., 2010). Among all four Gs-coupled prostanoid receptors, EP2 has the highest mRNA level in PC3 cells, approximately 11-fold higher than DP1 (Fig. 1D).

EP2 activation stimulates adenylate cyclase activity resulting in elevated cytoplasmic cAMP level. We used a cell-based time-resolved fluorescence resonance energy transfer (TR-FRET) assay to monitor cAMP accumulation in PC3 cells induced by butaprost, a selective EP2 agonist. The assay is based on generation of a strong FRET signal upon the interaction of two molecules: an anti-cAMP antibody coupled to a FRET donor (cryptate) and cAMP coupled to a FRET acceptor (d2). Endogenous cAMP produced by cells competes with labeled cAMP for binding to the cAMP antibody and thus reduces the FRET signal (Jiang et al., 2010). To evaluate the potency of EP2 antagonist TG4-155 in cancer cells, PC3 cells were incubated first with vehicle or TG4-155 for 5–10 minutes, then with increasing concentrations of butaprost for 40 minutes to activate EP2 receptors. Butaprost induced cAMP production in PC3 cells with an EC_{50} 54 nM, which was blocked by the EP2 antagonist TG4-155 in a concentration-dependent manner (Fig. 1E). Schild regression analysis was performed to evaluate the potency of EP2 antagonist TG4-155 in PC3 cells. TG4-155 displayed a competitive antagonism mode of action on the EP2 receptor as shown by Schild plot with a K_B value of 1.3 nM (Fig. 1F), which is similar to that measured in human EP2 overexpressing cell lines (Fig. 1B) (Jiang et al., 2012). These results indicate a strong COX-2-PGE_2-EP2-cAMP signaling pathway in cancer cells and confirm TG4-155 as a highly potent antagonist at the endogenous EP2 receptor in prostate cancer cells.

EP2 Receptor Activation and Inhibition of Cancer Cell Proliferation and Invasion. Upregulation of COX-2 in tumor tissues typically yields high levels of PGE_2 (Gupta and Dubois, 2001; Loh et al., 2002; Murakami and Kudo, 2006), which can promote cancer cell growth and invasion through PKA-mediated EGFR activation (Donnini et al., 2007). Next, we investigated whether PGE_2 receptor subtype EP2 is involved in the process of cancer cell growth and invasion using the EP2 antagonist TG4-155. First, PC3 cells were treated with the EP2 natural ligand PGE_2 to mimic the elevated COX-2 and PGE_2 levels in the tumor tissues in the presence of vehicle or TG4-155. After 48 hours of cell proliferation were quantified by MTT assay. PGE_2 stimulation

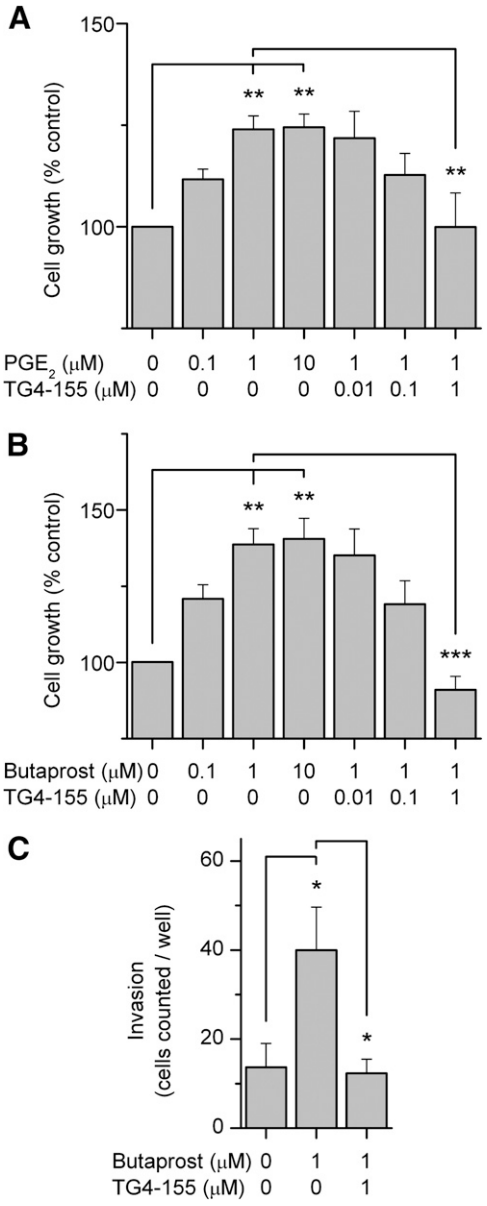

Fig. 2. Effects of EP2 receptor activation and inhibition on cancer cell proliferation and invasion. (A) EP2 activation by its natural ligand, PGE_2, promoted PC3 cell growth, measured by MTT cell proliferation assay; growth was attenuated by treatment with compound TG4-155 in a concentration-dependent manner. (B) EP2 selective agonist butaprost promoted PC3 cell growth, which was also attenuated by TG4-155. (C) TG4-155 blocked the PC3 cell invasion triggered by EP2 activation. Butaprost (1 μM) treatment significantly increased the number of cells crossing the filter coated with Matrigel, which was blocked by cotreatment with EP2 antagonist TG4-155 (1 μM). Bars represent the mean ± S.E.M. (n = 4 independent experiments). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by one-way ANOVA and post hoc Bonferroni test with selected pairs.

significantly enhanced PC3 cell growth in a concentration-dependent manner with a maximal response being obtained at approximately 1 μM (Fig. 2A). This PGE_2-induced cancer cell proliferation was significantly suppressed by TG4-155 also in a concentration-dependent manner (Fig. 2A). These results were recapitulated by using an EP2 selective agonist, butaprost, in a parallel experiment (Fig. 2B) and suggest that among all four PGE_2 receptor subtypes EP2 is prominently involved in cancer cell growth.

To further investigate the effect of EP2 inhibition on cancer cell invasion, PC3 cells were seeded into culture inserts that were precoated with Matrigel. Butaprost (1 μM) as chemoattractant molecule was added into the lower wells in the presence of vehicle or TG4-155 (1 μM). After 24 hours, the cells that moved across the membrane coated with Matrigel were stained and counted. Butaprost (1 μM) significantly promoted PC3 cell invasion, which was completely prevented by cotreatment with the TG4-155 (Fig. 2C). These results together with the cell proliferation data demonstrate that PGE_2 mediates tumor activities at least partially through the EP2 receptor and suggest a potential value of EP2 antagonist in cancer therapy.

EP2 Receptor Activation Induces Tumor-Promoting Cytokines in Cancer Cells. PGE_2 is emerging as the primary inflammatory prostaglandin in a variety of involved tissues via its receptor EP2 subtype, while inflammation is now recognized as a critical component of tumor progression (Aggarwal et al., 2006; Grivennikov et al., 2010). To examine the effect of EP2 receptor activation on inflammation in cancer cells, PC3 cells were treated with butaprost in the

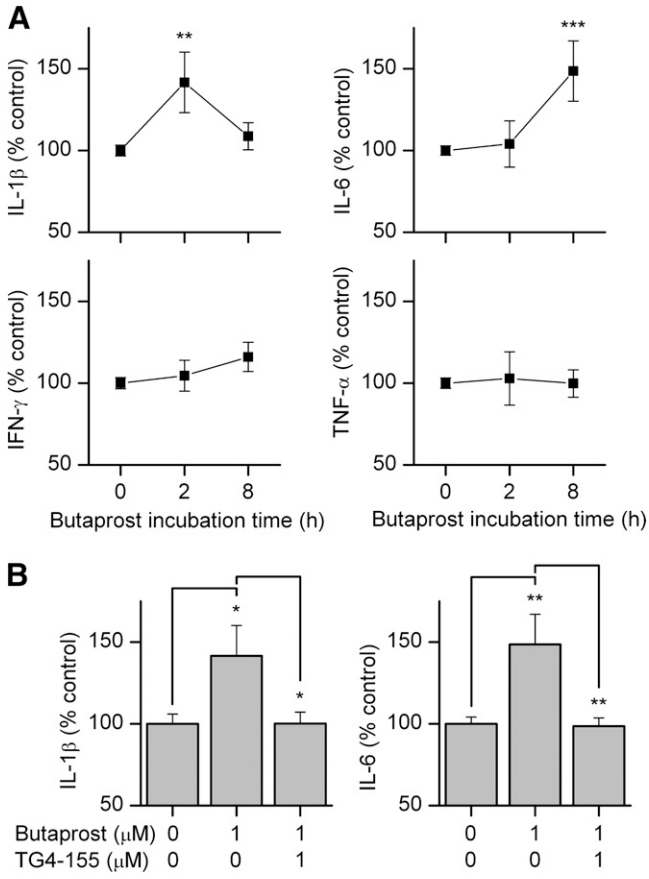

Fig. 3. EP2 receptor activation induces inflammatory cytokines in cancer cells. (A) PC3 cells were incubated with 1 μM butaprost for 0, 2, and 8 hours, and the levels of four proinflammatory cytokines, IL-1β, IL-6, IFN-γ, and TNF-α in the medium were measured. EP2 activation by butaprost significantly elevated IL-1β at 2 hours and IL-6 at 8 hours after butaprost stimulation, but not IFN-γ and TNF-α. (B) Cytokine induction following EP2 activation was mitigated by treatment with EP2 antagonist TG4-155 (1 μM), assessed at 2 hours and 8 hours after treatment of IL-1β and IL-6, respectively. Bars represent the mean ± S.E.M. (n = 5 independent experiments). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by one-way ANOVA and post hoc Bonferroni test with selected pairs.

presence of vehicle or EP2 antagonist TG4-155 and secretion of four proinflammatory cytokines into the culture medium was measured by the Meso Scale Discovery (MSD) multiplex cytokine/chemokine kit. EP2 activation by butaprost significantly elevated tumor-promoting cytokines IL-1β at 2 hours and IL-6 at 8 hours after butaprost stimulation, but expression of the antitumor cytokines IFN-γ and TNF-α was unaffected (Fig. 3A). EP2-dependent cytokine induction was substantially mitigated by TG4-155 (Fig. 3B). Among these four cytokines, IL-1β has a high basal mRNA level in PC3 cells (Fig. 1D).

Interestingly, TG4-155 did not show significant cytotoxicity, indicating that its antitumor action is unlikely caused via a cytotoxic mechanism like doxorubicin (Fig. 4A). Taken together, these results suggest the involvement of a prostaglandin signaling pathway in the inflammatory cascade in cancer cells and that inhibition of prostaglandin receptor EP2 by selective small molecule antagonists might provide a novel strategy to repress cancer cell activities via reducing inflammation (Fig. 4B).

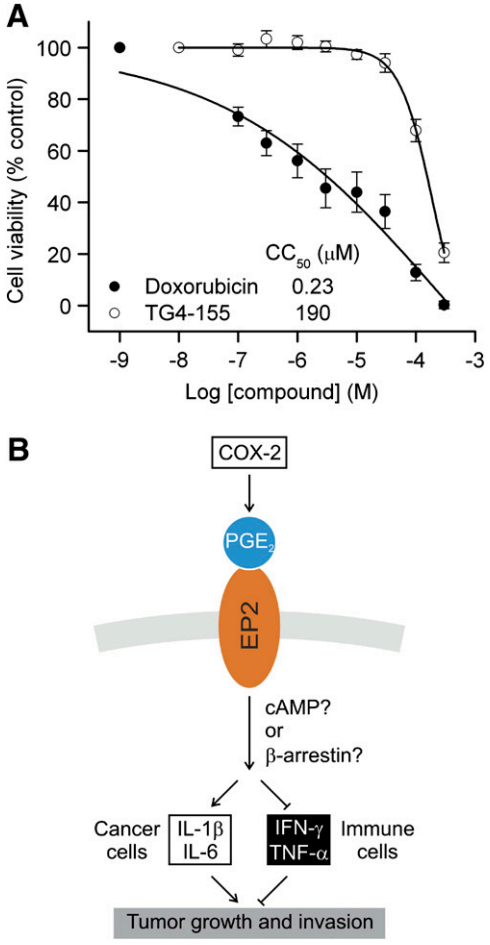

Fig. 4. EP2 receptor mediates cancer cell activities possibly via an inflammatory mechanism. (A) Low cytotoxicity of EP2 antagonist TG4-155. Cytotoxicity of TG4-155 was tested in C6G cells with the CellTiter-Glo luminescent cell viability assay. TG4-155 did not show significant cytotoxicity with CC_{50} = 190 μM; doxorubicin as positive control with CC_{50} = 0.23 μM. Data are shown as mean ± S.E.M. ($n \geq 6$ independent experiments). (B) Model proposed for the inflammatory action of EP2 signaling in cancer cell activities. COX-2 increases PGE_2 level in tumor tissues. PGE_2 signaling through the EP2 receptor upregulates tumor-promoting cytokines, including IL-1β and IL-6 in cancer cells, while downregulating antitumor cytokines such as IFN-γ and TNF-α in immune cells (Yamane et al., 2000; Walker and Rotondo, 2004; Li et al., 2006; Oxford et al., 2010); therefore, tumor growth and invasion are facilitated. A selective EP2 antagonist might reduce inflammation in tumor tissues, thus repress tumor growth.

Discussion

Recent studies using EP2 receptor-deficient mice implicated PGE_2-EP2 signaling in tumor progression and tumor-associated angiogenesis (Sonoshita et al., 2001; Yang et al., 2003; Chang et al., 2005a; Sung et al., 2005; Kamiyama et al., 2006; Brouxhon et al., 2007). Genetic ablation of prostaglandin receptors has been useful but complicated by the possibility of developmental and other homeostatic adjustments (Narumiya and FitzGerald, 2001; Hirata and Narumiya, 2011). Therefore, selective small molecule modulators for prostaglandin receptors would be a valuable complement to genetic strategies. In this report, we revealed a PGE_2-EP2 signaling pathway in human prostate cancer cells (Fig. 1). Using a novel potent EP2 receptor antagonist TG4-155, we further demonstrated the involvement of EP2 receptor in cancer cell proliferation and invasion (Fig. 2) and an inflammatory cascade among these cancer cells (Fig. 3). TG4-155 is 14-fold more potent on the EP2 than the DP1 receptor, and the expression level of DP1 mRNA in PC3 cells is 11-fold lower than that of EP2 (Fig. 1, B and D). Thus most of the effects of TG4-155 on prostanoid receptors can be attributed to inhibition of EP2, although DP1 can potentially contribute. Our results and data mining suggest that pharmacological inhibition of prostaglandin receptors might represent a novel strategy to repress tumorigenesis likely via an anti-inflammatory mechanism (Fig. 4B), although the effect of EP2 antagonists on other tumor activities such as angiogenesis and metastasis in tumor models awaits study.

The mechanism underlying the regulation of tumor development by EP2 is not clear, but could involve downstream β-arrestin signaling (Chun et al., 2009; Chun et al., 2010; Yun et al., 2011). The chronic inflammation mediated by EP2 signaling might also play a critical role in tumorigenesis given that inflammation has been widely recognized as a prominent risk factor for cancer (Dannenberg and Subbaramaiah, 2003; Aggarwal et al., 2006). Inflammatory events create a local microenvironment that fosters genomic alterations and promotes the neoplastic processes involving proliferation, survival, and migration. Tumor cells often release many cytokines and chemokines to attract monocytes and macrophages. The infiltrating macrophages in turn secrete growth factors that promote tumor progression and recruit secondary leukocytes to enhance and maintain this mutual promotion between inflammation and tumor. As the major inflammatory mediator derived from COX-2, PGE_2 via EP2 receptor can induce a host of proinflammatory mediators including cytokines, chemokines, iNOS, and COX-2 itself, which in turn facilitates cell proliferation, cell survival, angiogenesis, invasion, and metastasis, thereby promoting tumorigenesis (Aggarwal et al., 2006).

EP2 activation can significantly induce proinflammatory cytokines, such as IL-1β and IL-6, in cancer cells (Fig. 3A). IL-1β has been recognized for its capability of promoting tumor growth, invasiveness, and angiogenesis (Saijo et al., 2002; Voronov et al., 2003; Grivennikov et al., 2010). Cancer patients usually have elevated levels of IL-6, which is related to progression of many types of cancer, including prostate, colorectal, breast, and ovarian cancers (Plante et al., 1994;

Smith et al., 2001; Chung and Chang, 2003; Knupfer and Preiss, 2007; Grivennikov et al., 2010). On the other hand, EP2 receptor activation failed to induce the two antitumor cytokines in cancer cells, IFN-γ and TNF-α (Fig. 3A). Interestingly, EP2 receptor activation by butaprost suppresses IFN-γ synthesis in natural killer T cells (Walker and Rotondo, 2004; Oxford et al., 2010); similarly, EP2 activation downregulates TNF-α expression in immune cells such as neutrophils and macrophages (Yamane et al., 2000; Li et al., 2006; Oxford et al., 2010). Both INF-γ and TNF-α are well-recognized for their roles in inducing apoptotic cell death and inhibiting tumorigenesis. EP2 receptor activation by butaprost did not reduce these two antitumor cytokines in PC3 cells, probably because of their low basal levels in the in vitro culture setting (Fig. 3A). Nonetheless, blockade of EP2 signaling by small molecule antagonists can mitigate chronic inflammation in damaged tissues and might provide a novel therapeutic strategy for cancer treatment with interest waning in the use of COX-2 inhibitors in recognition of their detrimental cardiovascular and cerebrovascular side effects (Fig. 4B) (Abraham et al., 2007).

Authorship Contributions

Participated in research design: Jiang, Dingledine.

Conducted experiments: Jiang.

Performed data analysis: Jiang.

Wrote or contributed to the writing of the manuscript: Jiang, Dingledine.

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Address correspondence to: Dr. Jianxiong Jiang, Department of Pharmacology, Emory University School of Medicine, 1510 Clifton Road, Room 5002, Atlanta, GA 30322. E-mail: jjiang3@emory.edu