Prostaglandin E_2 Activates Mitogen-Activated Protein Kinase/Erk Pathway Signaling and Cell Proliferation in Non–Small Cell Lung Cancer Cells in an Epidermal Growth Factor Receptor–Independent Manner

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Abstract

Cyclooxygenase 2 (COX-2) overexpression is found in a wide variety of human cancers and is linked to all stages of tumorigenesis. Elevated tumor COX-2 expression is associated with increased angiogenesis, tumor invasion, suppression of host immunity and promotes tumor cell resistance to apoptosis. Previous reports have linked the COX-2 product prostaglandin E_2 (PGE_2) to the abnormal activation of the mitogen-activated protein kinase/Erk kinase pathway. Here we show that PGE_2 is able to rapidly stimulate Erk phosphorylation in a subset of non–small cell lung cancer (NSCLC) cell lines. This effect is not evident in bronchial epithelial cells. In contrast to previous reports in colon cancer, we found that Erk activation as well as cellular proliferation induced by PGE_2 was not inhibited by pretreatment of the cells with epidermal growth factor receptor (EGFR) inhibitors. Activation of the Erk pathway by PGE_2 was also resistant to src kinase inhibitors but sensitive to the protein kinase C inhibition. PGE_2 effects are mediated through four G protein–coupled receptors. Selective inhibition of EP receptors revealed the possible involvement of Ca^{2+}-dependent signaling in PGE_2-mediated activation of Erk. Our data indicate the presence of an EGFR-independent activation of the mitogen-activated protein kinase/Erk pathway by PGE_2 in NSCLC cells. These findings provide evidence for the possible link between tumor COX-2 overexpression and elevated Erk-mediated cancer cell proliferation and migration. Importantly, these findings suggest that COX-2 overexpression may contribute to EGFR inhibitor resistance in NSCLC. (Cancer Res 2005; 65(14): 6275-81)

Introduction

Cyclooxygenases (COX) are the rate-limiting enzymes in prostanoid synthesis, which convert arachidonic acid into prostaglandin H_2, a substrate for specific prostaglandin synthases (1). Two isoforms of COX have been isolated and characterized—the ubiquitously expressed COX-1 and inducible COX-2 (2, 3).

Studies of human cancers have revealed frequent overexpression of COX-2 in a variety of different malignancies. High-level constitutive COX-2 expression has been detected in colorectal (4, 5), prostate (6), lung (7–9), breast (8, 10), and other cancers. Overexpression of COX-2 can stimulate epithelial cell growth and invasion (11, 12) and promote cellular survival (13, 14). Nonsteroidal antiinflammatory drugs as well as specific COX-2 inhibitors promote cancer cell apoptosis and are therefore being evaluated for cancer chemoprevention and therapy (15).

Prostaglandins serve as autocrine and paracrine mediators and are involved in a variety of biological processes. A COX-2 metabolite abundantly present in the lung cancer microenvironment, prostaglandin E_2 (PGE_2) is an important mediator of immunoregulation and epithelial survival. PGE_2 exerts its multiple effects through four G protein–coupled receptors (GPCR; ref. 3). It has been shown previously that GPCRs are able to trans-activate the epidermal growth factor receptor (EGFR) pathway leading to the promotion of cancer cell growth and motility (16, 17).

In the present study, we investigated the mechanisms of PGE_2-mediated mitogen-activated protein kinase (MAPK)/Erk activation in non–small cell lung cancer (NSCLC) cells. We report that PGE_2 treatment rapidly induces Erk phosphorylation in a subset of NSCLC cell lines and that this induction is resistant to EGFR inhibitors. In this subset of NSCLC cell lines, we found that PGE_2 treatment stimulated cell proliferation that was resistant to EGFR inhibitors. Our analysis of the possible cross-talk mechanisms between the GPCR and MAPK/Erk signal transduction pathways suggests the involvement of protein kinase C (PKC)-dependent signaling.

This is the first documentation of PGE_2-mediated EGFR-independent activation of the MAPK/Erk pathway. These findings suggest that COX-2 overexpression may be an important contributor to EGFR inhibitor resistance in NSCLC. Our results provide a strong rationale for the pharmacologic inhibition of PGE_2 synthesis in combination with EGFR inhibitors in NSCLC therapy.

Materials and Methods

Reagents. 16,16-Dimethyl-PGE_2 was purchased from Cayman Chemicals (Ann Arbor, MI). EGFR inhibitor Erlotinib (Tarceva; working concentration 2 μmol/L) was generously provided by OSI Pharmaceuticals (Farmingdale, NY). EGF, EGFR inhibitor PD153035 also known as AG 1517 (working concentration 1 μmol/L), PKC inhibitor Ro-31-8425 (1 μmol/L), protein kinase A (PKA) inhibitor KT5720 (10 μmol/L), MAP/ERK kinase inhibitor U0126 (10 μmol/L), src inhibitors PP2 (1 μmol/L) and SU6656 (2 μmol/L), EP1/2 receptor antagonist AH6809 (50 μmol/L), cyclic AMP (cAMP)-specific phosphodiesterase inhibitors Rolipram (10 μmol/L) and IBMX (0.5 mmol/L), adenylate cyclase activators Forskolin (20 μmol/L)

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and cholera toxin (2.5 μg/mL) and G_i protein inhibitor pertussis toxin (50 ng/mL) were purchased from Calbiochem (San Diego, CA). Other reagents were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise specified.

Cell culture. Human squamous cell carcinoma NSCLC cell lines H157 (National Cancer Institute, Bethesda, MD), RH2 (previously established in our laboratory; ref. 18), and adenocarcinoma cell line H460 (American Type Culture Collection, Rockville, MD), were grown in RPMI supplemented with 10% fetal bovine serum (Gemini Biological Products, Calabasas, CA). NCI-H292, NCI-H1975, NCI-H226, NCI-H2122, NCI-H1703, H125, H358, SKLU-1, NCI-H1437, NCI-H441, NCI-H520, A427, NCI-H647, NCI-H522, NCI-H1650, NCI-H1299, SW 900, NCI-H661, NCI-H1975, and Calu-3 NSCLC cell lines were purchased from the American Type Culture Collection. H3255 cells were a generous gift from Bruce E. Johnson (Dana-Farber Cancer Institute, Boston, MA). For the experiments, cells were plated at 3.5×10^5 cells per well in six-well plates, incubated overnight in RPMI + 10% fetal bovine serum, serum-starved for 2 hours, and treated as indicated in a fresh serum-free medium. To study the effect of PGE_2 on Erk activation, cells were treated with PGE_2 (10 μg/mL) for 10 minutes with or without inhibitors and for the time course of PGE_2 effects—for 5, 15, 30, 60 and 120 minutes. As a control of EGF pathway activation, cells were treated with EGF (50 ng/mL) alone or with EGFR tyrosine kinase inhibitors PD153035 (1 μmol/L) or Erlotinib (2 μmol/L). For inhibitor studies, cells were pretreated with the respective inhibitors at the working concentrations indicated above for 1 hour in serum-free medium prior to PGE_2 addition.

Western blotting. Cells were washed with PBS and lysed with modified radioimmunoprecipitation assay buffer. Protein concentrations were measured using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Western blotting was done as previously described (14). Briefly, proteins were separated in SDS-PAGE and transferred to the nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked in 5% Blotto (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS + 0.1% Tween 20 and incubated with anti-phospho-Erk 1, 2 (p-Erk) mouse monoclonal antibodies (Cell Signaling, Beverly, MA) or anti-actin rabbit polyclonal antibodies (Sigma) diluted 1:1,000 or 1:5,000, respectively, in blocking solution. The membranes were then treated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, 1:10,000) and developed using the SuperSignal West Pico kit (Pierce).

RT-PCR for EP receptors. RNA was isolated from NSCLC cell lines using the RNAEasy kit (Qiagen, Valencia, CA) and reverse-transcribed using the SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was done using the Taq polymerase from New England Biolabs (Beverly, MA). The following primer pairs (F—forward, R—reverse primers 5′-3′, amplicon sizes are in parentheses) were used for EP receptor and β-actin amplification: EP1F GGTATCATGGTGGTGTCGTG, EP1R GGCCTC-TGGTTGTGCTTAGA (324 bp); EP2F GCCACGATGCTCATGCTCTTCGCC, EP2R CTTGTGTTCTTAATGAAATCCGAC (655 bp); EP3F CGTGTCGCG-CAGCTACCGGCG, EP3R CGGGCCACTGGACGGTGTACT (398 bp); EP4F GGGCTGGCTGTCACCGACCTG, EP4R GGTGCGGCGCATGAACTGGCG (485 bp); β-actinF CCATCGAGCACGGCATCGTC, β-actinR TCCAGACG-CAGGATGGCATG (363 bp). PCR conditions were 3 minutes at 95°C followed by 35 cycles (25 for β-actin): 1 minute at 95°C, 1 minute at 58°C, and 1 minute at 72°C.

Cell proliferation assay. Proliferation of NSCLC cells was studied using ^{3}H-thymidine incorporation assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Promega Corp., Madison, WI) and colorimetric bromodeoxyuridine (BrdUrd) incorporation ELISA (Roche, Indianapolis, IN). Cells were plated in 96-well plates at 2×10^3 (H157 and H460) or 1.5×10^3 (RH2) cells per well in 96-well plates, incubated overnight in RPMI + 10% fetal bovine serum and treated with PGE_2 (10 μg/mL) in fresh RPMI + 1% fetal bovine serum for 24, 48, or 72 hours. Where applicable, one of the EGFR inhibitors (1 μmol/L PD153035 or 2 μmol/L Erlotinib), specific COX-2 inhibitor sc58236 (5 μmol/L) or a combination of both were added to the cells. For PGE_2 and EGFR inhibitors, combined treatment cells were pretreated with PD153035 or Erlotinib for 1 hour prior to PGE_2 addition. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and BrdUrd incorporation ELISA were done as recommended by the manufacturers. For ^{3}H-thymidine incorporation assay, ^{3}H-thymidine (Amersham Biosciences) was added at 1 μCi/mL for 4 hours on the last day of PGE_2 treatment. After the medium was removed, cells were washed twice with cold PBS, treated with 5% trichloroacetic acid for 30 minutes at 5°C, solubilized with 0.5 N NaOH and assayed on β scintillation counter for incorporation of ^{3}H.

Results

Prostaglandin E_2 cross-activates the mitogen-activated protein kinase/Erk signal transduction pathway. To determine the effect of PGE_2 on Erk activation, we screened a panel of 24 NSCLC cell lines. To exclude the input of EGFR signaling on Erk phosphorylation, cells were serum-deprived for 2 hours. After 2 hours of serum starvation, the phosphorylation of Erk in most NSCLC cell lines was virtually undetectable. Stimulation with PGE_2 (10 μg/mL) strongly induced Erk phosphorylation in a time-dependent manner in a subset of NSCLC cells. In dose titration experiments, we observed similar phosphorylation of Erk in response to PGE_2 concentration as low as 0.1 μg/mL (data not shown). We selected three cell lines with a strong Erk phosphorylation response (H157, H460, and RH2) and used them for further studies. Hereafter, these cell lines are referred to as "PGE_2-responsive." Mutational analysis of EGFR tyrosine kinase domains (exons 18-21) of these cell lines revealed no mutations. In six other NSCLC cell lines, Erk was phosphorylated to a lesser extent in response to PGE_2 treatment (data not shown). The panel of cell lines that were analyzed for PGE_2 response, included three cell lines that have mutated EGFR tyrosine kinase domains-H3255 and H1975 (both − 2573T → G substitution in exon 21) and H1650 (15 bp in-frame deletion in exon 19). We observed no Erk phosphorylation following PGE_2 treatment in H3255 and H1650, and moderate phosphorylation in H1975 (data not shown).

Notable increase of phospho-Erk in PGE_2-responsive cells was detected 5 minutes following PGE_2 treatment, reached its maximum at ~15 minutes and returned to the basal level at ~30 minutes after the treatment (Fig. 1). Whereas this effect was observed in the PGE_2-responsive subset of NSCLC cells (8 out of 23), it was not evident in BEAS-2B bronchial epithelial cells (Fig. 2). Both EGF- and PGE_2-driven Erk phosphorylation was completely abolished by the MAP/ERK kinase inhibitor U0126 (data not shown). The time-dependent pattern of Erk phosphorylation by PGE_2 was similar to that seen following EGF exposure (50 ng/mL; data not shown), suggesting that PGE_2 cross-activates the MAPK/Erk signal transduction cascade rather than modulating the activity of the Erk negative regulator, MAPK phosphatase or stimulating the proteolytic release of EGFR ligands in the medium by proteinases (19). These results indicate that PGE_2-dependent signaling can cross-talk with the MAPK/Erk pathway and induce the activation of the downstream effector kinase Erk that is responsible for modulation of various cellular processes.

Prostaglandin E_2-mediated Erk phosphorylation is resistant to epidermal growth factor receptor inhibitors. We investigated the effect of highly specific EGFR inhibitors PD153035 (IC_{50} = 25 pmol/L) and Erlotinib (IC_{50} = 2 nmol/L) that rapidly suppress EGFR autophosphorylation. Stimulation of bronchial epithelial or NSCLC cells with EGF (50 ng/mL) induced rapid Erk phosphorylation that was completely blocked by pretreatment of the cells with the EGFR inhibitors (Fig. 2). However, in PGE_2-responsive

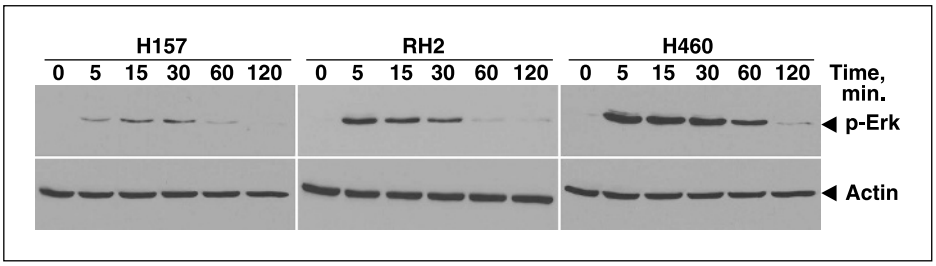

Figure 1. Time course of PGE_2-dependent Erk 1, 2 phosphorylation in NSCLC. H157, RH2, and H460 NSCLC cells were serum-starved and treated with PGE_2 (10 μg/mL) in serum-free medium for the indicated periods of time (in minutes). Phosphorylation of Erk-1, 2 (p-Erk) was assessed by Western blotting (*top*). The blot was washed and reprobed with anti-actin antibodies to ensure equal loading (*bottom*).

NSCLC cell lines, PGE_2-induced Erk phosphorylation was resistant to EGFR inhibition. Combined stimulation of these NSCLC cells with EGF and PGE_2 did not produce a significant difference in Erk phosphorylation (Fig. 2).

Prostaglandin E_2-mediated Erk phosphorylation is cyclic AMP-independent and resistant to src inhibition. In order to elucidate the mechanisms of PGE_2-mediated MAPK/Erk pathway activation, we studied the effect of elevated cAMP production and PKA activation on Erk phosphorylation. Pretreatment of the NSCLC cells with the potent specific (K_i = 56 nmol/L) PKA inhibitor KT5720 did not prevent PGE_2-dependent Erk phosphorylation (Fig. 3*A*). Similarly, treatment of the cells with cAMP level–modulating agents Rolipram (inhibitor of cAMP-specific phosphodiesterase) and Forskolin (activator of adenylate cyclase) alone or in combination with PGE_2 did not affect Erk phosphorylation (Fig. 3*B*). There was also no change in Erk phosphorylation observed with other cAMP-elevating agents, such as cholera toxin (activates G_s proteins), IBMX (phosphodiesterase inhibitor) and pertussis toxin (EP3 antagonist, G_i protein suppressor; data not shown). To clarify the role of src tyrosine kinase on PGE_2-mediated Erk phosphorylation, the NSCLC cells were pretreated with potent inhibitors of src family kinases SU6656 and PP2 prior to PGE_2 stimulation. Neither of the src inhibitors blocked the PGE_2-induced Erk phosphorylation (Fig. 4).

Prostaglandin E_2-mediated Erk phosphorylation is inhibited by protein kinase C inhibition. We next analyzed the role of PKC in PGE_2-dependent Erk phosphorylation. NSCLC cells were pretreated with the selective and potent (IC_{50} = 15 nmol/L) PKC inhibitor Ro-31-8425 prior to PGE_2 stimulation. We found that inhibition of PKC significantly blocks PGE_2-dependent Erk phosphorylation in NSCLC cells (Fig. 5*A*). In agreement with this finding, desensitization of PKC by treatment with 200 nmol/L phorbol-12,13-dibutyrate for 24 hours, completely inhibited PGE_2-dependent Erk phosphorylation (data not shown). To corroborate the involvement of PKC in MAPK/Erk pathway cross-activation by PGE_2, we used the EP1/EP2 receptor antagonist AH6809. The NSCLC cells used in this study express the EP1, EP3, and EP4 receptors but do not express the EP2 receptor (Fig. 5*C*). Therefore, in our model, AH6809 blocked only EP1 receptors, which are responsible for cellular calcium influx (3) and activation of Ca^{2+}-dependent PKC isoforms. Consistent with our findings using PKC inhibition by Ro-31-8425, blocking the EP1 receptors by AH6809 notably suppressed the PGE_2-dependent Erk phosphorylation (Fig. 5*B*).

Prostaglandin E_2 induces epidermal growth factor receptor inhibitor-resistant cell proliferation in non–small cell lung cancer cells. To further investigate the physiologic effects of PGE_2-dependent Erk phosphorylation, we studied the proliferation dynamics of NSCLC cells treated with PGE_2, EGFR inhibitors and a COX-2 inhibitor. To determine the capacity of EGFR inhibition to block PGE_2-dependent cell proliferation, cells were pretreated with Erlotinib or PD153035 prior to PGE_2 stimulation. We observed a significant increase of NSCLC cell proliferation in response to PGE_2 treatment that was resistant to EGFR inhibition (Fig. 6). Treatment with EGFR inhibitors or sc58236 alone had only a modest effect on cell proliferation. However, treatment with the combination of both drugs significantly decreased proliferation of NSCLC cells compared with either drug alone or nontreated control cells.

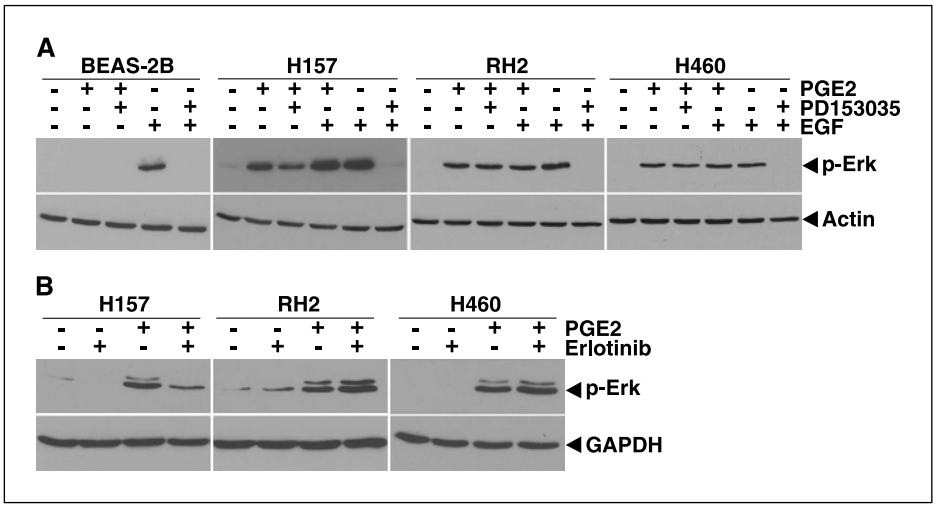

Figure 2. PGE_2-induced Erk phosphorylation is not inhibited by EGFR inhibitors Erlotinib and PD153035 in NSCLC cells. PGE_2-induced Erk phosphorylation is not evident in bronchial epithelial cells. BEAS-2B human bronchial epithelial cells and H157, RH2, and H460 NSCLC cells were serum-starved and treated with PGE_2 (10 μg/mL) or EGF (50 ng/mL) in serum-free medium for 10 minutes. Where applicable, cells were pretreated with PD153035 (1 μmol/L; *A*) or Erlotinib (2 μmol/L; *B*) for 1 hour prior to PGE_2 or EGF stimulation. Blots were probed for p-Erk-1, 2 (*top*) and actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*bottom*).

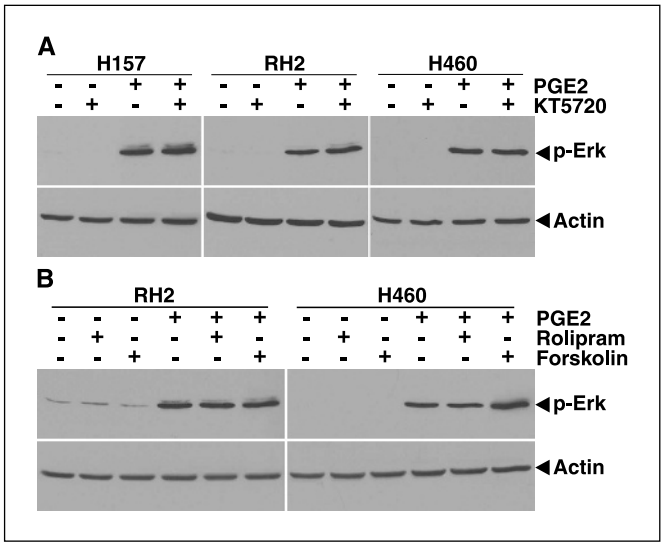

Figure 3. PGE_2-induced Erk phosphorylation in NSCLC cells is cAMP-independent. H157, RH2, and H460 NSCLC cells were serum-starved and treated with PGE_2 (10 μg/mL) in serum-free medium for 10 minutes. Where applicable, cells were pretreated with PKA inhibitor KT5720 (10 μmol/L; *A*), cAMP-specific phosphodiesterase inhibitor Rolipram (10 μmol/L) or adenylate cyclase activator Forskolin (20 μmol/L; *B*) for 1 hour prior to PGE_2 exposure. Neither of the PKA-modulating agents, either alone or in combination with PGE_2, affected Erk phosphorylation as assessed by Western blot (*top*, *A* and *B*). Blots were reprobed with anti-actin antibody (*bottom*).

Discussion

EGFR is frequently overexpressed in a variety of malignancies and plays an important role in tumor cell growth, proliferation, and metastasis (20–23). Because of its important role in tumor progression, EGFR is also a promising target for cancer therapy (24–26). However, only a limited population of cancer patients responds to EGFR inhibitor therapy. Recent studies indicate that sensitivity to EGFR tyrosine kinase inhibitors is due to activating somatic mutations in the tyrosine kinase domain (27–29). Whereas the identification of these activating EGFR mutations is important, they are apparently present in a minority of lung cancer patients. Thus, the definition of resistance pathways in tumors expressing wild-type EGFR is of paramount importance. Investigation of the cross-talk between EGFR and other signal transduction pathways that can overcome pharmacologic inhibition of EGFR by cross-activation of its downstream effectors could identify additional targets for combination therapies. GPCRs belong to the largest and most ubiquitous superfamily of membrane receptors that signal by activating heterotrimeric G proteins, thus controlling a broad range of physiologic functions (30). Here we studied the PGE_2, GPCR-dependent signaling that can overcome EGFR TK inhibition in NSCLC.

It is well-established that human malignancies frequently overexpress COX-2 and produce high levels of the COX-2 metabolite PGE_2 (4–10). PGE_2 exerts its multiple actions through four GPCRs designated EP1, EP2, EP3, and EP4 (3) that can stimulate epithelial cell growth and invasion (11) and promote cellular survival (13). Studies of the receptor subtypes have shown that the EP2 and EP4 receptor signaling is mediated by G_s G proteins and leads to activation of adenylate cyclase and elevated cAMP synthesis. In contrast, EP3 signaling through G_i, inhibits adenylate cyclase and cAMP synthesis. The EP1 receptor acts via G_q protein and upon activation, increases cellular Ca^{2+} level that in turn leads to PKC activation (31). GPCRs can cross-activate the MAPK/Erk pathway through different cross-talk points (32). Recent reports suggest that GPCR-dependent MAPK/Erk pathway cross-activation may be mediated by either intracellular cross-talk (16, 17, 33, 34) or proteolytic release of EGFR agonists (19).

Here, we report that in NSCLC cells, PGE_2 induced EGFR-independent Erk phosphorylation within minutes of treatment and stimulated increased cellular proliferation in a longer term assays. The EGFR inhibitors Erlotinib and PD153035 completely abolished the EGF-induced MAPK/Erk pathway activation but were unable to suppress PGE_2-mediated Erk phosphorylation (Fig. 2) in a subset of NSCLC cells. The rapid phosphorylation of Erk suggests that it is executed via intracellular activation of kinase cascades rather than proteolytic release of EGFR ligands as has been previously described (35). The time-dependent pattern of Erk phosphorylation by PGE_2 (Fig. 1) was similar to the pattern of Erk phosphorylation after EGF treatment. This suggests the presence of *de novo* phosphorylation of Erk rather than modulation of another locus in the pathway such as Erk phosphatase activity (36). To investigate the molecular mechanisms of this MAPK/Erk pathway activation by PGE_2 in NSCLC, we analyzed several possible cross-talk points. Previous reports suggested that activation of EP2 and EP4 receptors and the subsequent increase of cAMP synthesis and PKA activity was operative in cancer progression, modulating either Raf or src activities (11, 37, 38). However, in our experiments, inhibition of PKA by the specific inhibitor KT5720 did not prevent Erk activation by PGE_2 in PGE_2-responsive NSCLC cells (Fig. 3*A*). Similarly, modulation of cAMP levels in NSCLC cells by an inhibitor of cAMP-dependent phosphodiesterase or an activator of adenylate cyclase, did not have any effect on PGE_2-dependent Erk phosphorylation (Fig. 3*B*) arguing against possible PKA-independent effects of cAMP or involvement of EP2 or EP4 receptor activation. Other reagents that continuously elevate cAMP levels, such as G_s proteins stimulating cholera toxin, phosphodiesterase

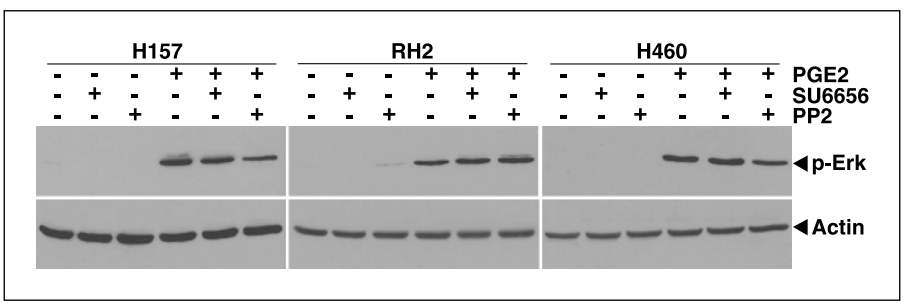

Figure 4. PGE_2-induced Erk phosphorylation is resistant to src kinase inhibitors. H157, RH2, and H460 NSCLC cells were serum-starved and treated with PGE_2 (10 μg/mL) in serum-free medium for 10 minutes. Where applicable, cells were pretreated with src inhibitors SU6656 (2 μmol/L) or PP2 (1 μmol/L) for 1 hour prior to PGE_2 induction. Blots were probed for p-Erk (*top*) or actin (*bottom*).

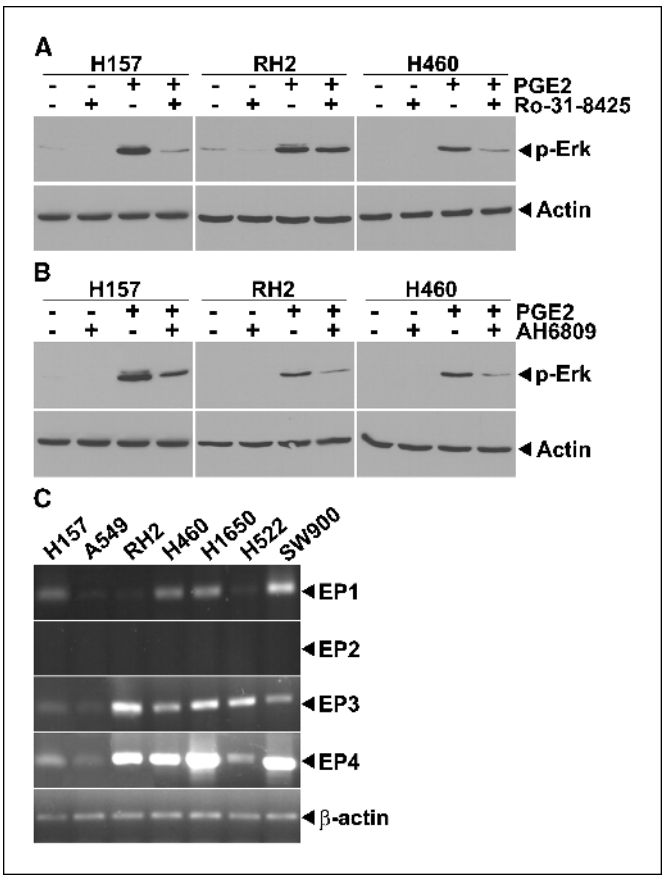

Figure 5. PGE_2-induced Erk phosphorylation is PKC-dependent and mediated by EP1 receptors. H157, RH2, and H460 NSCLC cells were serum-starved and treated with PGE_2 (10 μg/mL) in serum-free medium for 10 minutes. Where applicable, cells were pretreated with PKC inhibitor Ro-31-8425 (1 μmol/L; *A*) or EP1/2 receptor antagonist AH6809 (50 μmol/L; *B*) for 1 hour prior to PGE_2 induction. Blots were probed for p-Erk (*top*, *A* and *B*) or actin (*bottom*). *C*, EP receptor expression in different NSCLC cell lines by RT-PCR.

inhibitor IBMX and EP3 antagonist, G_i protein suppressor pertussis toxin, did not alter phosphorylation of Erk by PGE_2 (data not shown). These data indicate that PGE_2-dependent activation of the MAPK/Erk pathway is not predominantly mediated by cAMP in PGE_2-responsive NSCLC cells.

Several reports have suggested involvement of src tyrosine kinase, induced by G_q- or G_i-coupled GPCRs, in MAPK/Erk pathway activation (16, 17, 33). We therefore analyzed the contribution of src in PGE_2-dependent Erk phosphorylation using two potent inhibitors of src kinase, PP2 and SU6656. We found that in contrast to these previous reports, activation of the MAPK/Erk pathway by PGE_2 in NSCLC cells was resistant to src inhibitors (Fig. 4). These observations indicate that the analyzed pathway cross-activation in NSCLC cells is not src-dependent.

Given that activation of EP1 receptors initiates Ca^{2+} influx and increase in Ca^{2+}-dependent PKC activity, we determined the effect of PKC inhibition on PGE_2-mediated Erk phosphorylation. Previous studies have shown that activated PKC can phosphorylate Raf-1 either directly or by activation of small GTPase Ras, which in turn activates the MAPK/Erk cascade and supports cell growth (39–41). In our experiments, pretreatment with the PKC inhibitor Ro-31-8425 abrogates the PGE_2-dependent Erk phosphorylation (Fig. 5*A*). In accord with this data, the use of EP1/EP2 receptor inhibitor, AH6809, significantly suppressed PGE_2-dependent Erk phosphorylation in NSCLC cell lines (Fig. 5*B*). RT-PCR analysis revealed that the NSCLC cell lines used in our study did not express the EP2 receptor (Fig. 5*C*). Thus, this narrows the effect of AH6809 to EP1 receptor inhibition, which is therefore responsible for PKC activation by PGE_2. These results are consistent with the data of Watanabe et al. who have shown the importance of EP1 receptor in colon carcinogenesis (42). Incomplete inhibition of PGE_2-dependent Erk phosphorylation by AH6809 may indicate the low potency of the inhibitor in suppression of EP1 receptor activation. Alternatively, this could imply activation of other EP receptors. However, our findings seem to implicate EP1 receptor signaling in these events. For example, agents that mimic EP4 stimulation did not induce Erk activation and EP2 receptor expression was not evident in tested cell lines. Some reports indicate that PKC, upon activation by 12-*O*-tetradecanoylphorbol-13-acetate, is able to phosphorylate Thr^{654} in the juxtamembrane domain of EGFR exon 17, thus modulating the receptor signaling (43). As EGFR mutations have recently been found critical for modulation of receptor activation (27–29), we searched for mutations in exon 17 in cell lines responsive and nonresponsive to PGE_2 stimulation and found that all of them had a wild-type exon 17 (data not shown). Taken together, our results suggest that activation of the MAPK/

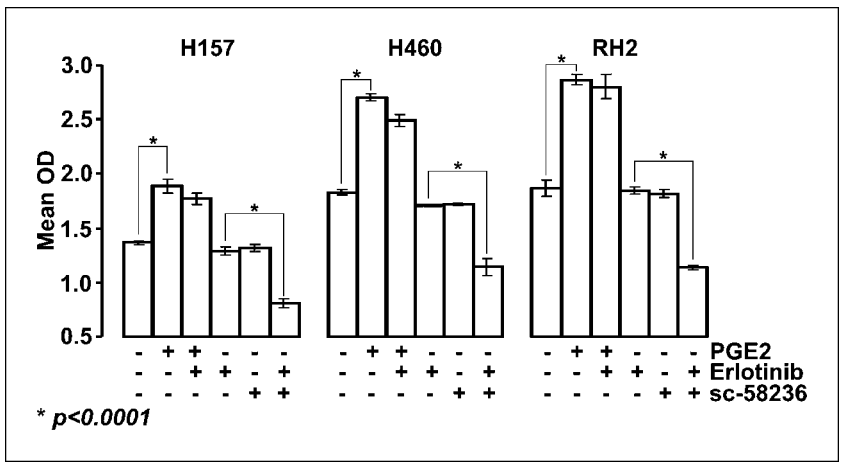

Figure 6. Combination treatment of PGE_2-responsive NSCLC cells with COX-2 and EGFR inhibitors significantly reduces cell proliferation compared with either drug alone. H157, RH2, and H460 NSCLC cells were treated with PGE_2 (10 μg/mL), EGFR inhibitor Erlotinib (2 μmol/L), COX-2 inhibitor sc58236 (5 μmol/L), PGE_2 + Erlotinib, or Erlotinib + sc58236. For PGE_2 + Erlotinib treatment, cells were pretreated with Erlotinib for 1 hour prior to addition of PGE_2. Cell proliferation was assessed after 72 hours using the BrdUrd incorporation ELISA kit according to the manufacturer's protocol (Roche). Columns, mean; bars, ±SD.

Erk pathway by PGE_2 in NSCLC cells depends on EP1 receptor activation and is mediated by PKC at the post-receptor level. However, the exact mechanism of the initiation of MAPK/Erk pathway activation at the EP receptor level including the possible role of GPCR Gβγ subunits (30, 32) in PGE_2 signal transmission will require further investigation.

The MAPK/Erk signal transduction pathway controls a number of vital cellular processes, such as proliferation, migration, survival and angiogenesis (44, 45). To investigate the physiologic effects of PGE_2-driven Erk phosphorylation, we studied the effect of PGE_2 treatment on proliferation of NSCLC cells. Consistent with the previous reports (11, 16) that showed the proliferative effect of PGE_2, we were able to stimulate cell proliferation by PGE_2 treatment in PGE_2-responsive NSCLC cell lines (Fig. 6). This PGE_2-dependent stimulation was resistant to EGFR inhibitor treatment. Treatment of the cells with the EGFR or COX-2 inhibitors alone had only minimal effect on cell proliferation. Strikingly, the combination of these drugs reduced cell proliferation significantly, suggesting that COX-2 overexpression may be an important contributor to EGFR inhibitor resistance in NSCLC. These results provide evidence of functional significance of PGE_2-dependent activation of MAPK/Erk pathway in NSCLC cells.

Finally, our data highlight the complex network of cross-signaling between the GPCR and MAPK/Erk pathways (46–48). This PGE_2-mediated cross-talk can shift the balance between the stimulatory and inhibitory responses in NSCLC toward pro-survival and proliferative phenotypes that ultimately may determine the cancer cell resistance to pharmacologic EGFR inhibition. This is the first indication of an EGFR inhibitor–resistant MAPK/Erk pathway activation by PGE_2 in NSCLC. The functional manifestation of this activation was an enhanced cellular proliferative response to PGE_2 that was resistant to EGFR inhibition. Here we also describe a novel EP1/PKC-mediated mechanism of PGE_2-dependent Erk cascade activation in NSCLC. Collectively, these findings suggest that overproduction of PGE_2 in the tumor environment may lead to EGFR inhibitor resistance in NSCLC. Thus, COX-2 inhibitors may play a role in augmenting the efficacy of EGFR tyrosine kinase inhibitor therapy in patients with NSCLC. Based on our current findings, clinical evaluation of this combination therapy is in progress (49).

Acknowledgments

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