Cyclooxygenase-2 (COX-2) Negatively Regulates Expression of Epidermal Growth Factor Receptor and Causes Resistance to Gefitinib in COX-2–Overexpressing Cancer Cells

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Abstract

Overexpression of cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFR) has been detected in many types of cancer. Although COX-2 and EGFR are closely related to each other, the exact mechanism of COX-2 in tumors has not been well understood. In this study, we investigated the relationship between COX-2 and EGFR in cancer cells. Using two cell lines stably overexpressing COX-2 (HCT-116-COX-2 and H460-COX-2) and a stable line of COX-2 knockdown MOR-P cells, we analyzed patterns of COX-2 and EGFR expression. To observe the effects of COX-2 on EGFR expression and activity, we did comparative analyses after treatment with various drugs (EGF, celecoxib, prostaglandin E_2, gefitinib, Ro-31-8425, PD98059, and SP600125) in HCT-116-Mock versus HCT-116-COX-2 cells and H460-Mock versus H460-COX-2 cells. Overexpression of COX-2 specifically down-regulated EGFR expression at the level of transcription. COX-2–overexpressing cells have a decreased sensitivity to gefitinib. COX-2 induced activation of extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK) but suppressed Akt activation. JNK inhibition by SP600125, a specific JNK inhibitor, resulted in restoration of EGFR levels in COX-2–overexpressing cells, whereas ERK inhibition by PD98059 did not. Overexpressed COX-2 negatively regulates EGFR expression via JNK activation, leading to gefitinib resistance. COX-2 may also regulate ERK activity independently of EGFR. Therefore, resistance of COX-2–overexpressing cells to gefitinib may be due to decreased expression of EGFR by JNK activation and EGFR-independent elevation of ERK activity by COX-2. The ability of COX-2 to inhibit EGFR expression and gefitinib effects may have significance in clinical cancer therapy. (Mol Cancer Res 2009;7(8):1367–77)

Received 1/5/09; revised 4/10/09; accepted 4/22/09; published OnlineFirst 8/11/09.
Grant support: National Cancer Center grant 0710380-1.
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doi:10.1158/1541-7786.MCR-09-0004

Introduction

Overexpression of cyclooxygenase-2 (COX-2) has been detected in malignant tumors of a variety of tissues, including colon, prostate, lung, breast, and uterine cervix (1-5). COX-2 is known to play an important role in carcinogenesis by stimulating growth, survival, invasion, metastasis, and angiogenesis of tumor cells (3, 4, 6-9), but its exact role in cancer development has not been well understood. COX-2 is a 72-kDa protein with 604 amino acids, localized primarily to the endoplasmic reticulum and nuclear membrane. COX-2 has an epidermal growth factor (EGF)–like domain at its NH_2 terminus that functions in Ca^{2+}-related signaling pathways such as the protein kinase C (PKC) pathway, whereas the COOH terminus has a peroxidase domain that acts in cellular oxidation and reduction. Thus, COX-2 is a bifunctional enzyme that has both cyclooxygenase and peroxidase activities (10, 11). Cyclooxygenase activity of COX-2 transforms arachidonic acid into intermediate prostaglandin G_2, which is subsequently converted to prostaglandin H_2 through peroxidase activity, and finally, prostaglandins [prostaglandin E_2 (PGE_2), prostaglandin D_2, prostaglandin $F_2\alpha$, thromboxane A, etc.] are produced by various synthases (6, 11, 12).

EGF receptor (EGFR) is a tyrosine kinase receptor that has critical functions in cellular gene expression, survival, differentiation, and proliferation (1, 13-15). Overexpression or mutation of EGFR activates cellular signaling pathways that induce characteristics of cancer cells, including angiogenesis, metastasis, and invasiveness (1, 13, 16, 17). In cancer patients, EGFR-induced signaling pathways have been implicated in disease progression, decreased survival, and resistance to cytotoxic agents or radiation (16-18). Therefore, EGFR is regarded as an important target molecule for cancer treatment.

Many studies have been done about the separate functions of either COX-2 or EGFR, and there is growing evidence that these two molecules are intimately related in cancer cells (1, 2, 16, 17, 19). However, a precise correlation between the two proteins is still not well understood. EGFR is known to induce COX-2 expression, and PGE_2, the major end product of COX-2, is also known to activate EGFR through various pathways (1). In addition, we, as well as other researchers, have reported that inhibitors of COX-2 and EGFR can exert a synergistic antineoplastic effect when administered together in some cancer cells (16, 17). Although it has been verified that the actions of COX-2 and EGFR are closely related in cells, as described above, expression patterns of COX-2 and EGFR are highly variable among cancer cell types and frequently seem to

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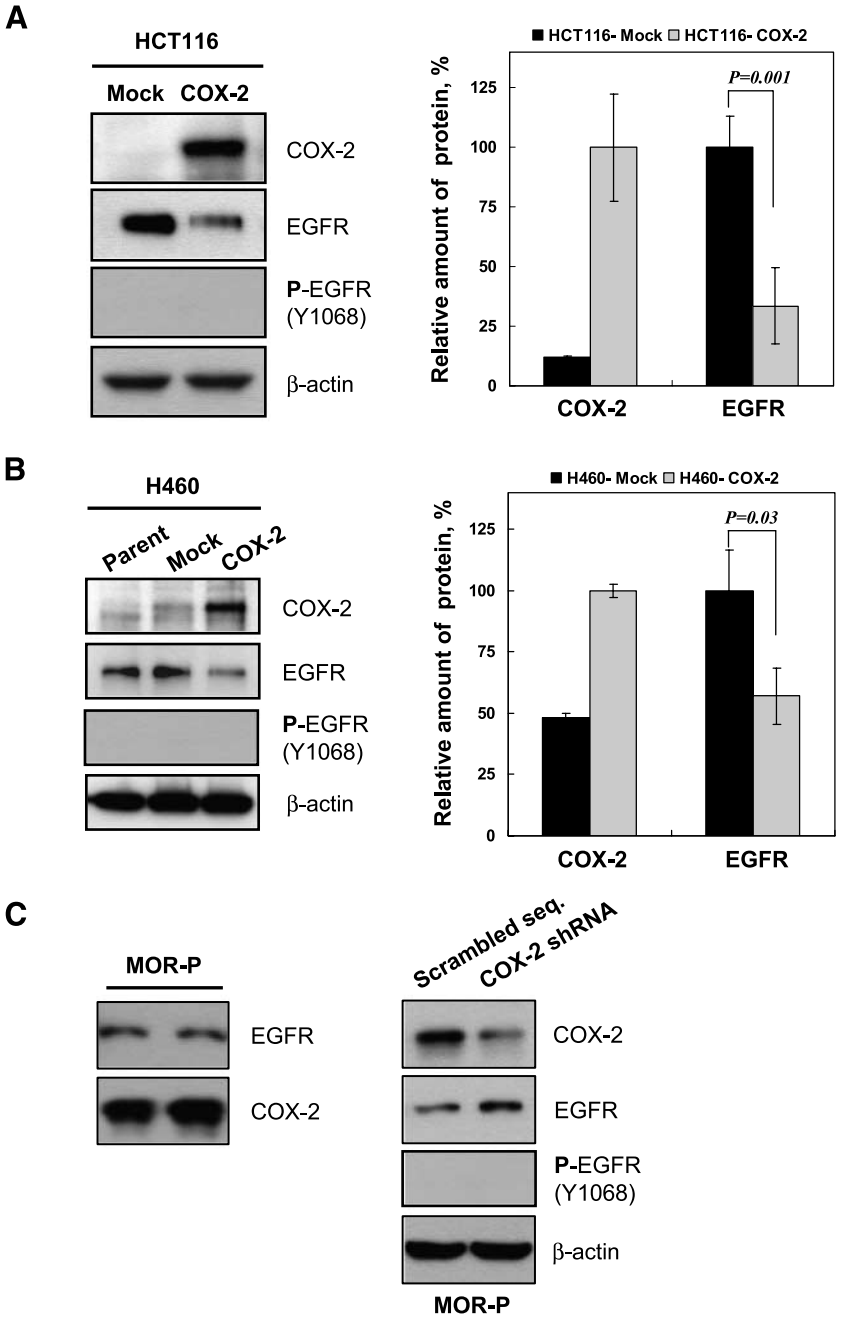

FIGURE 1. COX-2–overexpressing cells suppress expression of endogenous EGFR but COX-2 knockdown cells up-regulate it. Expression levels of COX-2 and EGFR were analyzed in selected COX-2 stable cell lines: HCT-116-Mock/HCT-116-COX-2 cells (**A**), H460-Mock/H460-COX-2 cells (**B**), and MOR-P/COX-2 knockdown MOR-P cells (**C**). An equal amount of protein was loaded in SDS-PAGE. COX-2, EGFR, and phospho-EGFR were detected with specific antibodies, and β-actin was used as loading control among samples. $P < 0.05$.

be independent of each other. These facts suggest that COX-2 and EGFR may independently or complementarily function under particular circumstances according to cell specificity (1, 20). Determining the precise type of relationship between the two proteins in a specific cancer type could provide useful clues for the development of new drugs or approaches (e.g., combined targeted strategies using COX-2–modulating and EGFR-modulating drugs for the treatment of cancer).

In the current study, we tried to further investigate the relationship between COX-2 and EGFR in several types of cancer cells. We propose that overexpressed COX-2 negatively regulates EGFR expression via c-Jun NH_2-terminal kinase (JNK) activation in tested cancer cells (HCT-116-COX-2 and H460-COX-2), causing resistance to gefitinib, an EGFR tyrosine kinase inhibitor.

Results

COX-2 Negatively Regulates EGFR Expression

To investigate the correlation between COX-2 and EGFR in tumor cells, we selected HCT-116 human colon adenocarcinoma cells that express no COX-2 protein but express a high level of EGFR protein. We developed a HCT-116 stable cell line constitutively overexpressing COX-2 (HCT-116-COX-2) and

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compared EGFR expression levels between the HCT-116-COX-2 and HCT-116-Mock cells. Interestingly, we found that EGFR expression levels were decreased in HCT-116-COX-2 cells compared with HCT-116-Mock cells (Fig. 1A). We further investigated whether EGFR down-regulation by COX-2 is specific to HCT-116-COX-2 cells or if it is a general phenomenon in other tumor cells. NCI-H460 human lung large cell carcinoma cells express a low level of COX-2 and express a high level of EGFR protein. Therefore, we also developed a NCI-H460 cell line constitutively overexpressing COX-2 (H460-COX-2; refs. 21, 22) and did the same comparison. EGFR expression in H460-COX-2 cells was also found to be down-regulated compared with mock cells much as it was in the HCT-116-COX-2 cells (Fig. 1B). Next, we evaluated whether endogenously overexpressed COX-2 functions similarly to ectopically overexpressed COX-2. We selected MOR-P lung cancer cells, which show high constitutive expression levels of endogenous COX-2 (Fig. 1C, *left*), and developed a stable COX-2 knockdown cell line using COX-2 short hairpin RNA (shRNA). When COX-2 was knocked down in MOR-P cells, EGFR expression was increased compared with control cells transfected with scrambled sequences (Fig. 1C, *right*). These results suggest that both endogenously and ectopically overexpressed COX-2 may down-regulate EGFR expression in cancer cells. Therefore, we suggest that down-regulation of EGFR expression by COX-2 may be a general function of COX-2 as a negative regulator in at least these tested tumor cell lines.

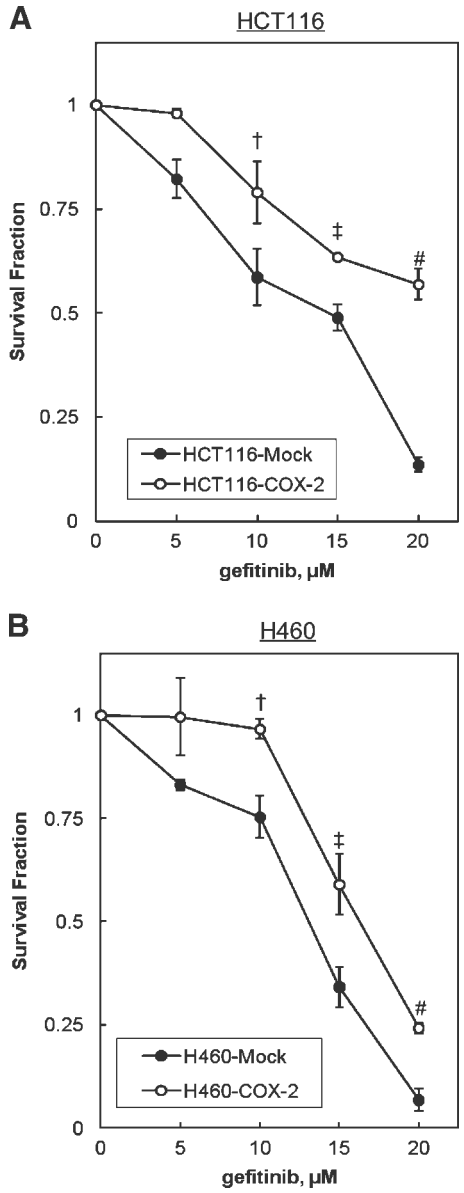

FIGURE 2. COX-2–overexpressing cells are resistant to gefitinib. The cells were exposed to a vehicle (0.1% DMSO) or to various gefitinib concentrations (0, 5, 10, 15, or 20 μmol/L) for 72 h, and then cell viability was analyzed by clonogenic assay. **A.** HCT-116-Mock versus HCT-116-COX-2. **B.** H460-Mock versus H460-COX-2. Points, mean of three independent experiments; bars, SE. **A.** †, $P = 0.03$; ‡, $P = 0.04$; #, $P = 0.02$. **B.** †, $P = 0.02$; ‡, $P = 0.01$; #, $P = 0.03$.

COX-2–Overexpressing Cells Are Resistant to Gefitinib, an EGFR-Specific Inhibitor

If COX-2 regulates EGFR expression, it may also alter the effect of EGFR-targeted inhibitors. Gefitinib is a specific EGFR tyrosine kinase inhibitor that is currently used clinically for the treatment of lung cancer. To compare the anticancer effect of gefitinib in cells expressing low or high levels of COX-2, cells were treated with gefitinib at various concentrations (0, 5, 10, 15, or 20 μmol/L) and cell viability was monitored by clonogenic assay. HCT-116-COX-2 cells showed relatively strong resistance to gefitinib compared with HCT-116-Mock cells (Fig. 2A). H460-COX-2 cells also showed a statistically significant resistance to gefitinib compared with H460-Mock cells (Fig. 2B). These results suggest that overexpression of COX-2 induces EGFR down-regulation, and as a result, cells become resistant to gefitinib.

COX-2 Down-Regulates Transcription of EGFR mRNA but not Translation

To understand the mechanism of COX-2–mediated EGFR down-regulation, we first investigated whether COX-2 activates degradation pathways of EGFR. Cells were incubated with proteasomal inhibitor MG132 (1 μmol/L) for the indicated times (0, 4, 8, 24, or 48 hours) at 37°C and then EGFR levels were compared with those of untreated cells. EGFR protein accumulated in cells treated with MG132 until 24 hours of treatment and thereafter decreased in both HCT-116-Mock and HCT-116-COX-2 cells (Fig. 3A). However, the difference in EGFR expression levels between the COX-2–overexpressing cells and mock cells was consistently maintained until 48 hours (Fig. 3A), implying that COX-2–induced EGFR down-regulation is not caused by EGFR degradation. Next, we analyzed EGFR mRNA levels by reverse transcription-PCR in tested cell lines. The levels of EGFR mRNA were reduced in both HCT-116-COX-2 and H460-COX-2 cells compared with their respective control cells (Fig. 3B; Supplementary Fig. S2). These results indicate that COX-2 down-regulates EGFR levels by inhibiting transcription of EGFR mRNA, but not translation.

COX-2–Overexpressing Cells Are Insensitive to EGF Stimulation

Next, we studied whether COX-2–induced EGFR down-regulation has an effect on EGFR activation. Cells were

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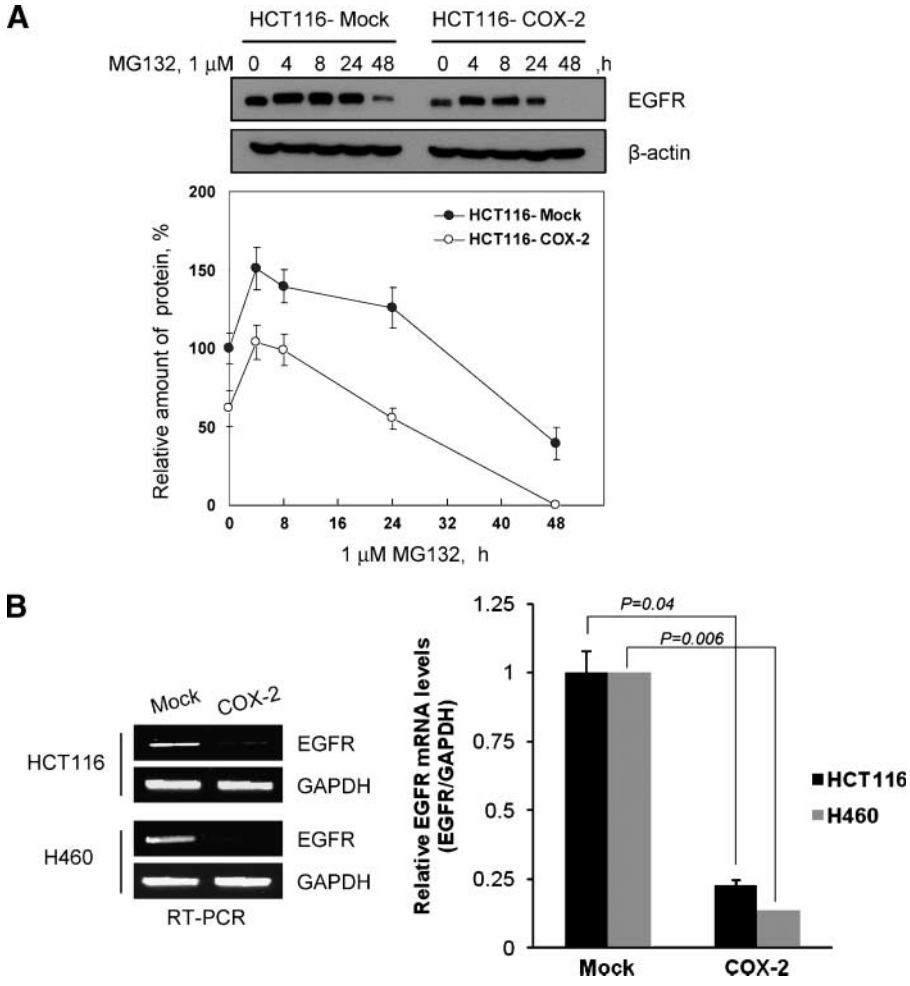

FIGURE 3. COX-2 down-regulates transcription of EGFR but not translation. **A.** HCT-116-Mock and HCT-116-COX-2 cells were treated with 1 μmol/L MG132 for indicated times (0, 4, 8, 24, or 48 h) at 37°C. An equal amount of protein was loaded in SDS-PAGE, and the accumulated EGFR was detected with specific antibody. **B.** Total RNA was isolated from HCT-116 (mock and COX-2) and H460 (mock and COX-2) cells, and then the EGFR mRNA levels were detected by reverse transcription-PCR (*RT-PCR*) as described in Materials and Methods. All measurements were done at least in triplicate.

stimulated with EGF (50 ng/mL) for the indicated times (0, 5, 10, 15, 30, 60, or 120 minutes) at 37°C and then phospho-EGFR levels were analyzed by Western blotting. Mock cells (HCT-116-Mock and H460-Mock) had a significantly higher initial level of EGFR activation than did COX-2–overexpressing cells (HCT-116-COX-2 and H460-COX-2), and EGFR levels were rapidly decreased within 5 minutes of EGF stimulation in all the tested cells as a result of receptor endocytosis following activation (Fig. 4A). We also compared EGFR activation between mock and COX-2 cells after cells were pretreated with gefitinib followed by EGF stimulation. Gefitinib efficiently blocked EGF-induced EGFR activation but not completely. The amount of EGF-stimulated EGFR activation after gefitinib pretreatment was also lower in COX-2–overexpressing cells compared with their mock cells (data not shown). These results may imply that EGFR activation is attenuated in COX-2–overexpressing cells because EGFR expression is inhibited in these cells.

COX-2 Overexpression Inhibits Akt Activation through the EGFR and Promotes Extracellular Signal-Regulated Kinase Activation Independently of the EGFR

EGFR is activated by various stimuli, thereby transmitting signals via downstream signaling cascades, including Akt, mitogen-activated protein kinases [MAPK; extracellular signal-regulated kinase (ERK), JNK, and p38], and signal transducer and activator of transcription 3. Therefore, we investigated the possibility that COX-2–mediated EGFR down-regulation regulates the activities of downstream signaling effectors of EGFR. We ascertained the basal activities of ERK and Akt, the most common EGFR downstream effectors, in HCT-116-Mock, HCT-116-COX-2, H460-Mock, and H460-COX-2 cells. Interestingly, we observed that Akt basal activity was inhibited in COX-2–overexpressing cells (HCT-116-COX-2 and H460-COX-2) compared with their mock cells, whereas ERK activity was increased (Fig. 4B). Furthermore, COX-2 knockdown MOR-P cells showed increased Akt phosphorylation and decreased ERK phosphorylation (Fig. 4C).

To further verify Akt or ERK regulation by COX-2, phosphorylation of Akt and ERK was analyzed after EGFR was inhibited by gefitinib or stimulated by EGF. EGF enhanced phosphorylation of Akt in both HCT-116-Mock and HCT-116-COX-2 cells, but phosphorylation was maintained for a much shorter time in HCT-116-COX-2 cells compared with mock cells. This difference may be due to EGFR down-regulation in HCT-116-COX-2 cells. In addition, Akt phosphorylation by EGF was almost completely inhibited in both cell lines by gefitinib pretreatment (Fig. 4D). These results suggest that Akt

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activity is governed by EGFR activity and COX-2–induced Akt inhibition may be a result of EGFR down-regulation.

EGF also enhanced ERK phosphorylation in HCT-116-Mock and HCT-116-COX-2 cells. However, EGFR inactivation by gefitinib did not affect the enhanced ERK phosphorylation in HCT-116-COX-2 cells (Fig. 4D). These results indicate that COX-2–induced ERK activation may be regulated in an EGFR-independent mode.

PGE_2 Does Not Mediate EGFR Down-Regulation by COX-2

To investigate how COX-2 induces EGFR down-regulation, we examined whether PGE_2, a major product of COX-2, mediates down-regulation of EGFR by COX-2. To inhibit production of PGE_2 by COX-2, cells were treated with celecoxib, a COX-2–specific inhibitor. Celecoxib effectively blocks production of PGE_2 from COX-2 (2, 5, 17). Although PGE_2 production was blocked by celecoxib, EGFR expression was still down-regulated in both HCT-116-COX-2 and H460-COX-2 cells (Fig. 5A). In addition, EGFR expression in HCT-116-Mock and H460-Mock cells was not decreased by addition of PGE_2 (Fig. 5A). These results indicate that PGE_2 itself does not mediate EGFR down-regulation.

According to several recent studies, PGE_2 can lead to PKC activation and activated PKC can inhibit EGFR signaling via phosphorylation of EGFR at Thr^{654} (23, 24). Therefore, we examined whether down-regulated EGFR expression seen in

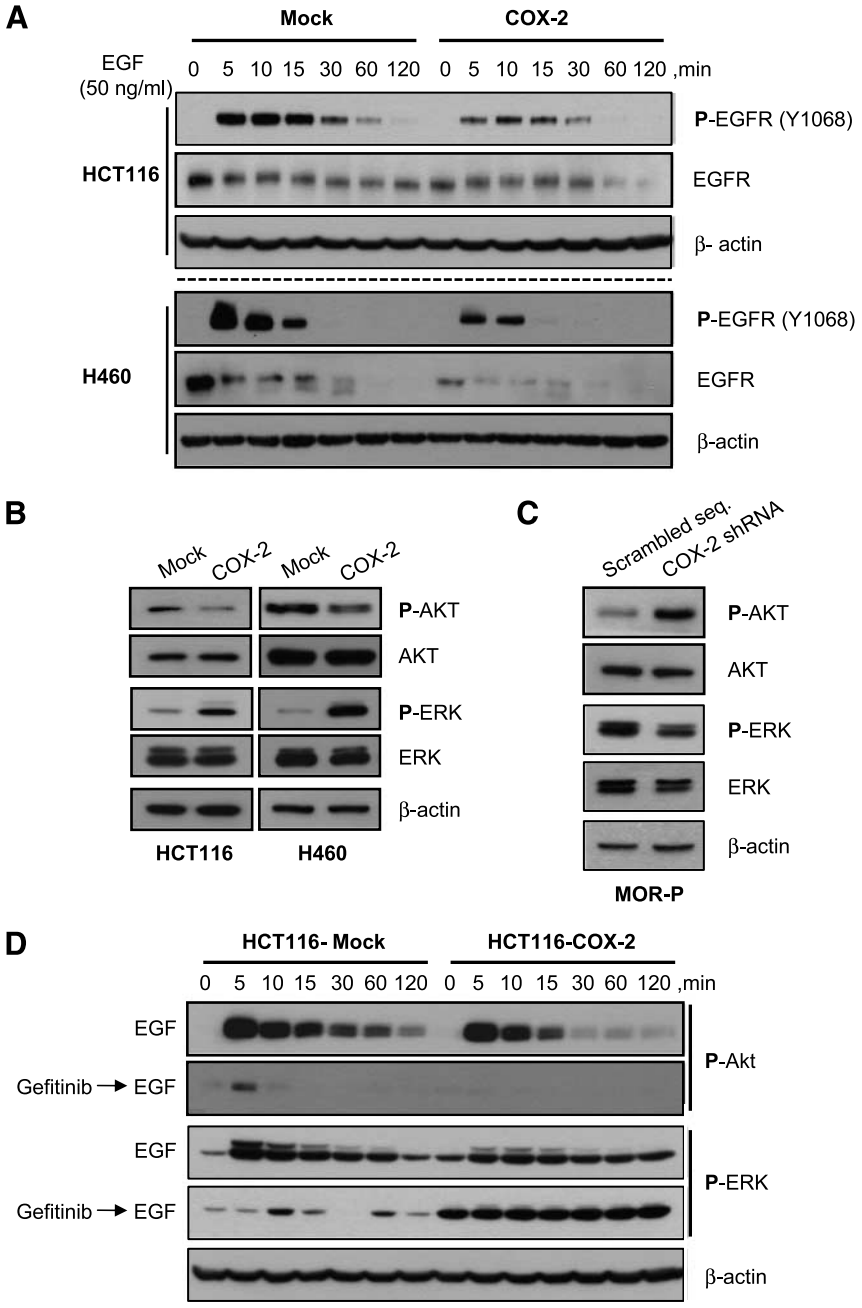

FIGURE 4. COX-2–overexpressing cells are insensitive to EGF stimulation by EGFR down-regulation. **A.** Serum-starved cells (HCT-116-Mock versus HCT-116-COX-2 and H460-Mock versus H460-COX-2) were pretreated with or without 10 μmol/L gefitinib for 4 h, then washed twice with serum-free medium, and then incubated with 50 ng/mL EGF for indicated times (0, 5, 10, 15, 30, 60, or 120 min). Treated cells were harvested after being washed twice with ice-cold PBS. Phospho-EGFR and EGFR were detected with specific antibodies. **B** and **C.** Level of phospho-Akt/Akt and phospho-ERK/ERK was analyzed in untreated HCT-116 cells (mock versus COX-2), H460 cells (mock versus COX-2), and MOR-P cells (scrambled sequence versus COX-2 shRNA). **D.** Cells pretreated with or without 10 μmol/L gefitinib were stimulated by 50 ng/mL EGF, and then levels of phospho-ERK/ERK and phospho-Akt/Akt were analyzed. β-Actin from combination samples of gefitinib and EGF was used as loading control. All measurements were done at least in triplicate. 0 h, 0.1% DMSO control.

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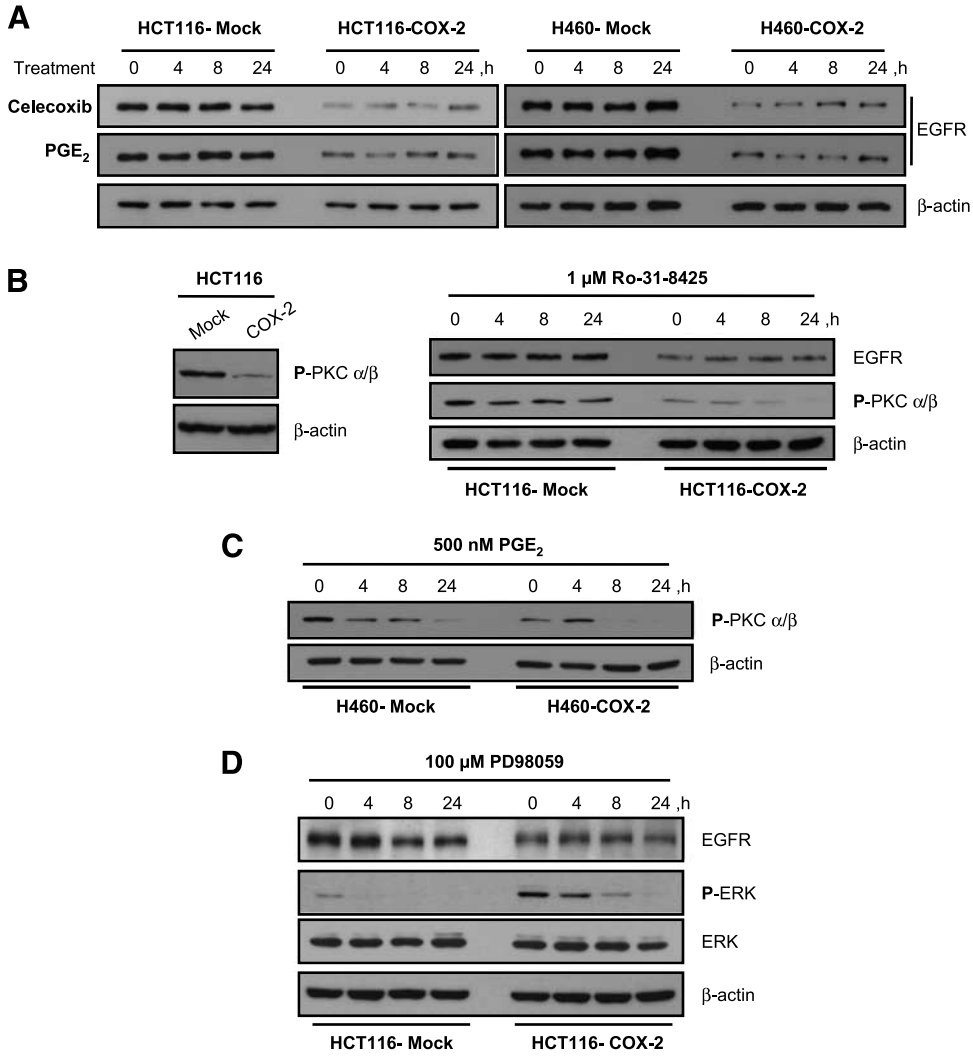

FIGURE 5. COX-2–induced PGE_2 and ERK activation are not involved in down-regulation of EGFR expression. **A.** Cells (HCT-116-Mock versus HCT-116-COX-2 and H460-Mock versus H460-COX-2) were incubated with 40 μmol/L celecoxib or 500 nmol/L PGE_2 for indicated times (0, 4, 8, or 24 h). Treated cells were harvested after being washed twice with ice-cold PBS, and an equal amount of protein was loaded in SDS-PAGE. Levels of EGFR in each sample group were detected with specific antibodies. **B.** Left, under basal condition, phospho-PKCα/β was analyzed in HCT-116-Mock versus HCT-116-COX-2 cells. Right, HCT-116-Mock and HCT-116-COX-2 cells were incubated with 1 μmol/L Ro-31-8425 for 24 h, and then EGFR and phospho-PKCα/β were detected with specific antibodies. **C.** H460-Mock and H460-COX-2 cells were incubated with 500 nmol/L PGE_2 for indicated times (0, 4, 8, or 24 h), and then phospho-PKCα/β was detected with specific antibodies. **D.** HCT-116-Mock and HCT-116-COX-2 cells were incubated with 100 μmol/L PD98059 for indicated times (0, 4, 8, or 24 h), and then phospho-ERK/ERK and EGFR were detected with specific antibodies. β-Actin was used as loading control. All measurements were done at least in triplicate. 0 h, 0.1% DMSO control.

COX-2–overexpressing cells was due to activation of PKCα/β by elevated PGE_2. However, we found that the level of phospho-PKCα/β in HCT-116-COX-2 cells was considerably lower than that of HCT-116-Mock cells (Fig. 5B, *left*), which is in contrast to the above hypothesis. In addition, when elevated phospho-PKCα/β in HCT-116-Mock cells was inhibited by Ro-31-8425, a specific PKC inhibitor, EGFR expression level was not changed (Fig. 5B, *right*). Furthermore, when exogenous PGE_2 was administered to H460-Mock cells, the phospho-PKCα/β levels were reduced (Fig. 5C). These results indicate that COX-2–produced PGE_2 is not associated with PKCα/β activation and therefore does not mediate EGFR down-regulation in the tested cancer cell lines.

COX-2–Induced ERK Activation Is Not Involved in Down-Regulation of EGFR Expression

We found COX-2–induced ERK activation to be independent of EGFR (Fig. 4D). PGE_2 has been shown to activate ERK (23) and EGFR is well known to regulate ERK activity, but ERK is also able to regulate EGFR activity by modulating production of its ligand (25, 26). Therefore, to investigate whether COX-2–induced ERK activation could negatively regulate EGFR expression, cells were treated with PD98059, an inhibitor of MAPK/ERK kinase 1/2, which is a kinase upstream of ERK. Although PD98059 completely blocked ERK activation in a time-dependent manner, expression of down-regulated EGFR in HCT-116-COX-2 cells was not recovered (Fig. 5D).

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This result indicates that COX-2–mediated ERK activation may not cause down-regulation of EGFR expression in HCT-116-COX-2 cells.

Taken together, the above results suggest that COX-2–induced EGFR down-regulation may be caused by pathways that do not involve PGE_2 and its related signaling molecules containing PKC and ERK.

COX-2–Activated JNK Negatively Regulates EGFR Expression

Several studies reported that c-Jun transcription factor, a substrate of JNK, positively regulates transcription of the *EGFR* gene (27-29). Therefore, we investigated whether JNK is involved in COX-2–induced EGFR down-regulation. The basal phosphorylation levels of JNK were analyzed in HCT-116-Mock versus HCT-116-COX-2 cells and H460-Mock versus H460-COX-2 cells. We found that COX-2–overexpressing cells showed increased phospho-JNK levels as well as JNK expression levels compared with their control cells (Fig. 6A, *top*). However, treatment with PGE_2 did not induce JNK activation in tested cancer cells (data not shown). We next examined whether COX-2–induced JNK activation affects EGFR expression. To inhibit JNK phosphorylation, cells were treated with 10 μmol/L SP600125, a specific JNK inhibitor, for 24 hours at 37°C and then EGFR expression levels were analyzed by Western blotting. SP600125 treatment decreased

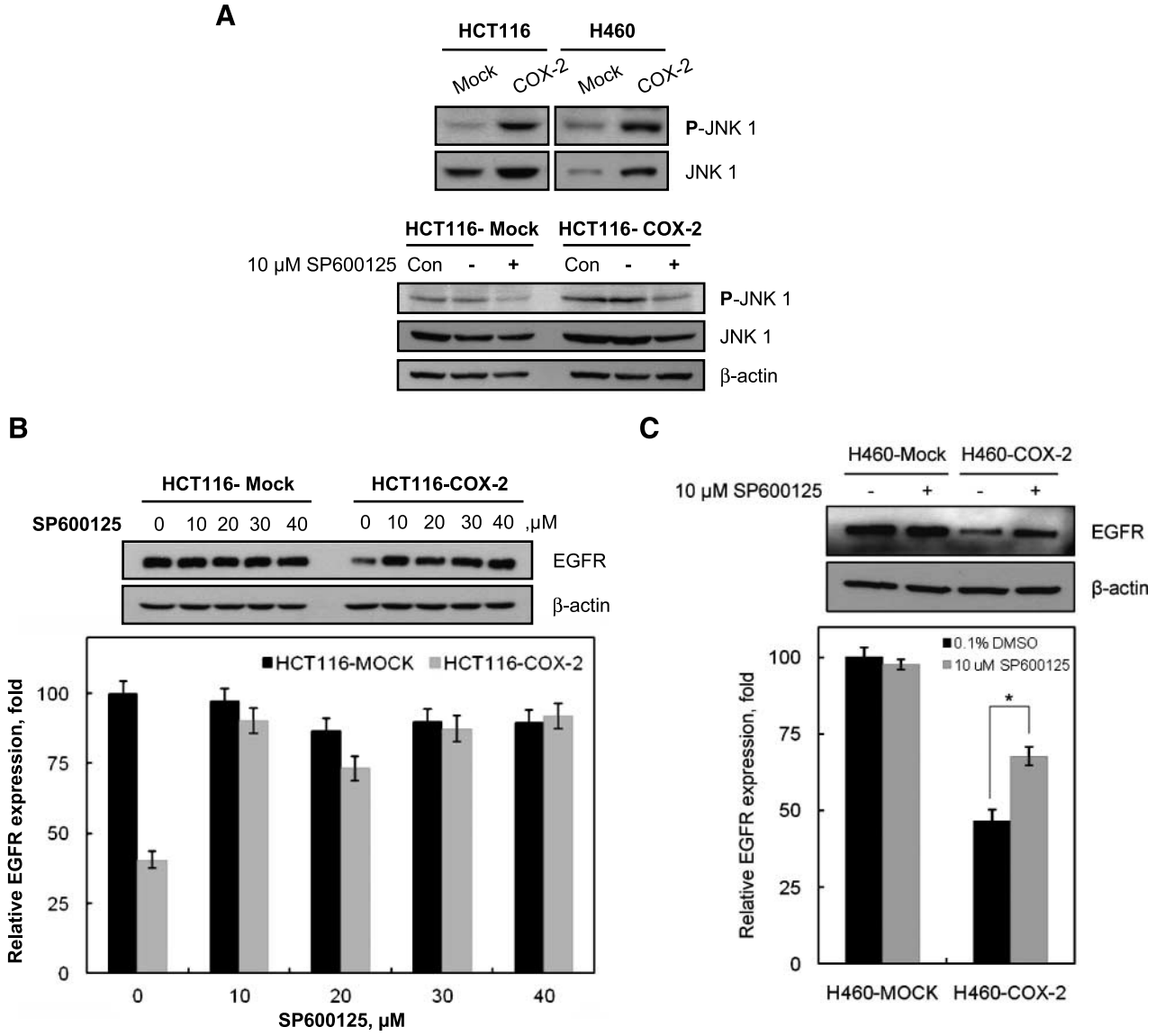

FIGURE 6. COX-2–activated JNK negatively regulates EGFR expression. **A.** Top, basal levels of phospho-JNK1 and JNK1 were analyzed in HCT-116-Mock versus HCT-116-COX-2 cells and H460-Mock versus H460-COX-2 cells. Bottom, HCT-116-Mock and HCT-116-COX-2 cells were incubated with 10 μmol/L SP600125 for 24 h, and phospho-JNK and JNK were detected with specific antibodies. Con, control without DMSO; –, 0.1% DMSO. **B.** HCT-116-Mock and HCT-116-COX-2 cells were incubated with SP600125 at various concentrations (0, 10, 20, 30, or 40 μmol/L) for 24 h. **C.** H460-Mock and H460-COX-2 cells were incubated with or without 10 μmol/L SP600125 for 24 h. Levels of EGFR were detected with specific antibodies, and β-actin was used as loading control. All measurements were done at least in triplicate. *, $P < 0.05$.

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JNK phosphorylation in HCT-116-COX-2 cells (Fig. 6A, *bottom*). Next, cells were incubated with SP600125 at various concentrations (0, 10, 20, 30, or 40 μmol/L) for 24 hours at 37°C, and EGFR expression levels were compared with those of control cells. When COX-2–induced JNK activation was inhibited by SP600125, the reduced EGFR expression in HCT-116-COX-2 cells was restored to the basal level of HCT-116-Mock cells, whereas EGFR expression in Mock cells was not found to be affected by SP600125 (Fig. 6B). Based on these results, H460-Mock and H460-COX-2 cells were treated with 10 μmol/L SP600125 for 24 hours at 37°C. EGFR expression in H460-COX-2 cells was also observed to recover following JNK inhibition (Fig. 6C). Our results indicate that activated JNK may mediate EGFR down-regulation in COX-2–overexpressing cancer cells.

Discussion

Up-regulation of COX-2 and EGFR has been detected in many types of cancer (4, 30). Although numerous reports suggest that COX-2 and EGFR are closely related to each other and play an important role in tumor development (1, 2, 16, 17, 19, 20), their exact mechanisms have not been well understood. PGE_2, an end product of COX-2, is known to up-regulate EGFR activity through the E-prostanoid receptor-cyclic AMP/protein kinase A-amphiregulin pathway or through an intracellular Src-mediated pathway independent of extracellular EGFR ligand release (1). Activated EGFR is also known to up-regulate COX-2 expression through an EGFR-Ras-MAPK-AP1-COX-2 cascade (2, 4, 16, 25). These data suggest that COX-2 and EGFR can regulate each other and imply that the influence of one molecule on the other in intracellular environments may be important to their sensitivity to each other's inhibitors. In this context, COX-2 may contribute to the sensitivity or resistance to EGFR inhibitors. However, there has been no report that compared EGFR expression, activity, or sensitivity with EGFR inhibitors between COX-2 low-expressing and high-expressing cells. Therefore, no confirmative data are available to indicate whether COX-2 has a consistent role in the intracellular environment to regulate EGFR or modify the sensitivity of a cell to EGFR inhibitors.

In the current study, we investigated the relationship of COX-2 and EGFR in cancer cells by analyzing the patterns of EGFR expression and phosphorylated EGFR in various COX-2 low-expressing or overexpressing cancer cell lines. Both HCT-116 colon adenocarcinoma cells and NCI-H460 lung large cell carcinoma cells express low levels of endogenous COX-2 (21). Interestingly, we found that ectopically overexpressed COX-2 down-regulated EGFR expression in both of these cancer cell lines, whereas knockdown of endogenous COX-2 in MOR-P cells resulted in increased EGFR expression (Fig. 1). We also found that MOR-P and LC2/ad cells highly expressing COX-2 were shown down-regulated EGFR expression, and NCI-H460 and VMRC-LCD cells almost not expressing COX-2 were shown up-regulated EGFR expression, indicating this relationship may be a general phenomenon in most cancer cells (Supplementary Fig. S1). However, many factors seem to regulate EGFR expression and activity, and then it may not be easy for any single factor (including COX-2) to determine absolute expression level of EGFR in cancer cells. Although current results clearly indicate that COX-2 and EGFR have intimate relationship, further studies are needed to verify whether COX-2 can be a major factor to regulate EGFR expression level of cancer cells.

PGE_2 is known to induce EGFR activation, which is followed by receptor endocytosis and degradation (25, 31). Accordingly, decreased EGFR levels by COX-2 may be a result of EGFR activation by COX-2–produced PGE_2 and resulting EGFR internalization and degradation. However, we did not observe elevated phosphorylation of EGFR in COX-2–overexpressing cells. Moreover, we found that EGFR down-regulation by COX-2 is not related to enhancement of EGFR degradation and that the phenomenon occurs at the level of transcription (Fig. 3). Therefore, we concluded that decreased EGFR expression does not occur by EGFR activation by COX-2–produced PGE_2 but instead may be the result of decreased EGFR gene transcription by COX-2. Modulation of EGFR expression by COX-2 has not been reported before, and this finding may have significance in the modulation of resistance or sensitivity of cancer cells to EGFR inhibitors. These results also suggest that interactions between COX-2 and EGFR can occur even in cells that originally do not express COX-2 and that this interaction may be a consistent mechanism to regulate cancer development and progression.

Gefitinib (Iressa, ZD1839) is a specific pharmacologic inhibitor that interrupts EGFR kinase activity (13, 32, 33) and is currently used in the treatment of advanced lung cancer as an important molecular-targeted therapeutic modality. However, this drug is responsive in only ~10% of lung cancer patients (26). Therefore, it has been a key issue to identify factors that govern resistance to gefitinib in cancer cells. EGFR expression levels and several mutations in EGFR domains are related to sensitivity of cancer cells to this drug (6, 13). However, there are many ongoing controversies on each of these subjects and it is thought that many more intracellular factors may be involved in the resistance or sensitivity to gefitinib. In the current study, we found that COX-2–overexpressing cells (HCT-116-COX-2 and H460-COX-2) showed increased resistance to gefitinib compared with their mock cells (Fig. 2). This interesting observation may be due to decreased expression of EGFR, a target molecule of gefitinib, or other factors regulated by COX-2. Krysan et al. (23) also suggested that COX-2 overexpression might be a contributor to cellular resistance to EGFR inhibitors. Our results suggest that regulation of COX-2 may be useful to increase the sensitivity of cancer cells to gefitinib, or they may imply that gefitinib should be used with patients who have COX-2 low-expressing tumors. However, further studies are needed to confirm this hypothesis and are currently under way.

Down-regulated EGFR expression in COX-2–overexpressing cells also inhibited activities of EGFR and its downstream molecules. As expected, activation of EGFR by EGF was weaker in COX-2–overexpressing cells (HCT-116-COX-2 and H460-COX-2) compared with their mock cells (Fig. 4A). Under basal conditions, both ectopic COX-2–overexpressing cell lines and one endogenous COX-2–overexpressing cell line showed elevated ERK phosphorylation and decreased Akt phosphorylation compared with their COX-2 low-expressing counterparts (Fig. 4B and C). ERK and Akt are well-known

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downstream effectors of EGFR (26, 34), and both showed enhanced phosphorylation following EGF stimulation of either HCT-116-Mock or HCT-116-COX-2 cells (Fig. 4D). However, ERK activation by COX-2 was shown to be independent of EGFR activity by showing that gefitinib did not inhibit the elevated phosphorylation of ERK in HCT-116-COX-2 cells (Fig. 4D). Krysan et al. (23) also reported that erlotinib, another EGFR inhibitor, did not inhibit PGE_2-induced ERK activation. ERK activation was also shown to be PGE_2 dependent in our study.[1] Taken together, ERK activation by COX-2 was induced by PGE_2 independent of EGFR. In contrast, Akt phosphorylation was found to be suppressed in HCT-116-COX-2 cells (Fig. 4B), and enhanced Akt phosphorylation stimulated by EGF was almost completely inhibited by gefitinib in these cells (Fig. 4D). Therefore, the inhibition of Akt phosphorylation by COX-2 seems to occur in an EGFR-dependent manner (Fig. 4D). These results suggest that EGFR down-regulation by COX-2 inhibits downstream signaling pathways containing Akt.

We searched for the underlying mechanism for EGFR down-regulation by COX-2. Several studies report that PGE_2 induces PKC phosphorylation and results in EGFR inactivation (23, 24). Therefore, we investigated whether COX-2 activates the PGE_2-PKC pathway, which suppresses EGFR signaling. However, EGFR down-regulation by COX-2 was not mediated by the PGE_2-PKC pathway in the tested cancer cells. This result suggests that COX-2 itself may down-regulate EGFR expression independently of its end product, PGE_2 (Fig. 5B and C). Although ERK is a signaling molecule downstream of EGFR, it can also act as upstream of EGFR (26). Therefore, we examined whether ERK activation by COX-2 can negatively regulate EGFR expression and found that COX-2–induced EGFR down-regulation seems to be unrelated to ERK activation (Fig. 5D). Taken together, the underlying mechanism of EGFR down-regulation by COX-2 in cancer cells does not seem to use the previously shown PGE_2-driven EGFR activation or inactivation pathways.

Several reports have shown that activated JNK positively regulates EGFR expression via c-Jun activation (27-29), and we investigated whether COX-2 regulates JNK activity, resulting in EGFR down-regulation in the tested cancer cells. Overexpressed COX-2 specifically activated JNK compared with control cells (Fig. 6A), and JNK activation was PGE_2 independent (data not shown). When COX-2–induced JNK activation was inhibited by a JNK inhibitor (SP600125), down-regulated EGFR expression was restored to the level of control cells. These results suggest that COX-2–induced JNK activation may negatively regulate EGFR expression in the tested cancer cells. Further studies are needed to understand how COX-2 activates JNK independently of PGE_2, how activated JNK down-regulates EGFR expression, and whether EGFR down-regulation by activated JNK is a phenomenon that is shown in only COX-2–overexpressing cells. Our novel finding that COX-2 induces JNK activation and leads to EGFR down-regulation may have important implications to our understanding of the complex functions of COX-2 and its relationship with EGFR in cancer cells.

In summary, we observed that overexpressed COX-2 inhibited EGFR expression and caused resistance to gefitinib in cancer cells. COX-2 induced activation of ERK through an EGFR-independent mechanism, whereas it inhibited Akt activation in an EGFR-dependent manner. EGFR down-regulation in COX-2–overexpressing cells seems to be mediated via JNK activation by COX-2. Our results suggest that COX-2 may influence the sensitivity of cells to EGFR inhibitors and may have important implications in the development of effective therapeutic strategies using COX-2–modulating or EGFR-modulating drugs or combinations thereof.

Materials and Methods

Materials

Celecoxib and gefitinib were provided by Pfizer, Inc. and AstraZeneca, respectively. PGE_2 was purchased from Cayman Chemical. MG132, Ro-31-8425, and SP600125 were purchased from Calbiochem. PD98059 and EGF were purchased from Sigma.

Cell Culture and Reagent Treatment

HCT-116 colon adenocarcinoma cells and NCI-H460 lung large cell carcinoma cell lines were purchased from the American Type Culture Collection. MOR-P lung cancer cells were kindly provided by Dr. Zhu (University of Sheffield, Sheffield, United Kingdom). COX-2–overexpressing HCT-116 and H460 stable cell lines (HCT-116-COX-2 and H460-COX-2) and their mock-transfected control cells (HCT-116-Mock and H460-Mock) were developed as described previously (22), and then the differences of mock versus COX-2 were compared within these stable cells. All tested cells were cultured in RPMI 1640 (Hyclone) containing 10% fetal bovine serum (Life Technologies), 50 units/mL penicillin (Life Technologies), 50 μg/mL streptomycin (Life Technologies), and 2 mmol/L L-glutamine (Life Technologies) at 37°C in an atmosphere of 5% CO_2 and 95% air. Cells were grown to 80% confluence in 75 cm^2 T-flasks up to passages 3 to 4. Cells were treated with drugs for indicated times (0, 4, 8, and 24 h) and then harvested after washing twice with ice-cold PBS. Final DMSO concentration in control medium without drug was maintained below 0.1%. A vehicle containing 0.1% DMSO was not toxic to the tested cells.

Development of COX-2 Knockdown MOR-P Stable Cell Line

MOR-P lung cancer cells were transfected with control pSM2 vector containing scrambled sequence or COX-2 shRNA-pSM2 vector (Open Biosystems) using Lipofectamine (Invitrogen) for 72 h at 37°C. After transfected cells were selected with puromycin (1 μg/mL; Calbiochem) for ~2 wk, according to manufacturer-provided protocol, the level of knocked down COX-2 was detected by Western blotting.

Reverse Transcription-PCR

Total cellular RNA was extracted with Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed for 1 h at 42°C in a reaction

[1] Unpublished data.

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mixture that contained 5 units RNase (GE Healthcare), 0.5 mmol/L deoxynucleotide triphosphate (Boehringer Mannheim), 2.5 μmol/L oligo(dT)$_{18}$ (Stratagene), 1× reverse transcriptase buffer, and 5 units reverse transcriptase (Qiagen). We conducted PCR using primers for EGFR (forward primer, 5′-TGGTCAGTTTTCTCTTGCAGTCGT-3′; reverse primer, 5′-CTCCAGAAGGTTGCACTTGTCCA-3′) in a PCR machine (GeneAmp PCR System 9700, Applied Biosystems). Cycling conditions consisted of 30 cycles, each cycle with a 30-s denaturation step at 95°C, followed by a 30-s annealing step at 62°C, and finally a 30-s extension step at 72°C. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control (forward primer, 5′-CAGGGCTGCTTTTAACTCTG-3′; reverse primer, 3′-GTCATGAGTCCTTCCACGATAC-5′).

Immunoblot Analysis

The cells were lysed on ice with lysis buffer [10 mmol/L Tris-Cl (pH 8.0), 100 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA] containing 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μg/mL pepstatin, 100 μg/mL phenylmethylsulfonyl fluoride, 10 mmol/L NaF, and 10 mmol/L Na_3VO_4 for 15 min. Protein concentration of cell lysate was measured by Bradford assay (Bio-Rad Laboratories). Proteins (30 μg) were separated by SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membrane, and probed with the following antibodies: a monoclonal antibody to COX-2 (BD Transduction), a polyclonal antibody to EGFR (Santa Cruz Biotechnology), a monoclonal antibody to phospho-EGFR (Y1068; BD Transduction), polyclonal antibody to ERK/phospho-ERK (Cell Signaling Technology), polyclonal antibody to Akt/phospho-Akt (Cell Signaling Technology), a polyclonal antibody to JNK (Cell Signaling Technology), and a monoclonal antibody to phospho-JNK (Santa Cruz Biotechnology) for overnight at a 1:1,000 dilution. The protein-antibody complexes were visualized with horseradish peroxidase–conjugated secondary antibody (Cell Signaling Technology) for 1 h at a 1:2,000 to 1:5,000 dilution. The blots were incubated for 3 min in an enhanced chemiluminescence Plus kit (GE Healthcare) and exposed to X-ray film (Hyperfilm, GE Healthcare). The membranes were also reprobed with β-actin antibody (Sigma) to normalize the differences between the samples. All experiments were done at least in triplicate.

In vitro *Clonogenic Assay*

The cells were serially diluted to the appropriate concentrations and plated out in 25 cm^2 cell culture T-flasks as described previously (16). The cells were incubated to attach for 24 h at 37°C. To measure cytotoxicity, the cells were exposed to a vehicle (0.1% DMSO) or to various gefitinib concentrations (0, 5, 10, 15, and 20 μmol/L) for 72 h at 37°C. The final DMSO concentration was adjusted to 0.1% in all flasks. The cells were allowed to grow in drug-free medium and maintained for an additional 6 d at 37°C to allow for the formation of colonies and then stained with 0.5% crystal violet (Sigma) in absolute methanol. The colonies were counted visually with a cutoff value of 50 viable cells. The surviving fraction was then calculated as described previously (16).

Statistical Analysis

Quantification of Western blot was done via TINA version 2.10e program or Multi Gauge v3.0 (Fuji Photo Film Co.) program. The data were analyzed by Student's *t* test to compare the two groups and then expressed as mean ± SD. Data from the clonogenic assay were also calculated as ±SE via the pooling of the results of three independent experiments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. K.A. Yoon (National Cancer Center) for real-time PCR.

References

1. Dannenberg AJ, Lippman SM, Mann JR, Subbaramaiah K, DuBois RN. Cyclooxygenase-2 and epidermal growth factor receptor: pharmacologic targets for chemoprevention. J Clin Oncol 2005;23:254–66.

2. Half E, Sun Y, Sinicrope FA. Anti-EGFR and ErbB-2 antibodies attenuate cyclooxygenase-2 expression and cooperatively inhibit survival of human colon cancer cells. Cancer Lett 2007;251:237–46.

3. Yang L, Amann JM, Kikuchi T, et al. Inhibition of epidermal growth factor receptor signaling elevates 15-hydroxyprostaglandin dehydrogenase in non-small-cell lung cancer. Cancer Res 2007;67:5587–93.

4. Singh B, Berry JA, Shoher A, Ayers GD, Wei C, Lucci A. COX-2 involvement in breast cancer metastasis to bone. Oncogene 2007;26:3789–96.

5. Subbaramaiah K, Dannenberg AJ. Cyclooxygenase-2 transcription is regulated by human papillomavirus 16 E6 and E7 oncoproteins: evidence of a corepressor/coactivator exchange. Cancer Res 2007;67:3976–85.

6. Grosch S, Maier TJ, Schiffmann S, Geisslinger G. Cyclooxygenase-2 (COX-2)-independent anticarcinogenic effects of selective COX-2 inhibitors. J Natl Cancer Inst 2006;98:736–47.

7. Chang YW, Marlin JW, Chance TW, Jakobi R. RhoA mediates cyclooxygenase-2 signaling to disrupt the formation of adherens junctions and increase cell motility. Cancer Res 2006;66:11700–8.

8. Tsai WC, Tsai ST, Jin YT, Wu LW. Cyclooxygenase-2 is involved in S100A2-mediated tumor suppression in squamous cell carcinoma. Mol Cancer Res 2006;4:539–47.

9. Watwe V, Javle M, Lawrence D, et al. Cyclooxygenase-2 (COX-2) levels before and after chemotherapy: a study in rectal cancer. Am J Clin Oncol 2005;28:560–4.

10. Kiefer JR, Pawlitz JL, Moreland KT, et al. Structural insights into the stereochemistry of the cyclooxygenase reaction. Nature 2000;405:97–101.

11. Im JY, Kim D, Paik SG, Han PL. Cyclooxygenase-2-dependent neuronal death proceeds via superoxide anion generation. Free Rad Biol Med 2006;41:960–72.

12. Cipollone F. COX-2 and prostaglandins in atherosclerosis. Lupus 2005;14:756–9.

13. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.

14. Xu H, Yu Y, Marciniak D, et al. Epidermal growth factor receptor (EGFR)-related protein inhibits multiple members of the EGFR family in colon and breast cancer cells. Mol Cancer Ther 2005;4:435–42.

15. Chen Z, Zhang X, Li M, et al. Simultaneously targeting epidermal growth factor receptor tyrosine kinase and cyclooxygenase-2, an efficient approach to inhibition of squamous cell carcinoma of the head and neck. Clin Cancer Res 2004;10:5930–9.

16. Park JS, Jun HJ, Cho MJ, et al. Radiosensitivity enhancement by combined treatment of celecoxib and gefitinib on human lung cancer cells. Clin Cancer Res 2006;12:4989–99.

17. Zhang X, Chen ZG, Choe MS, et al. Tumor growth inhibition by simultaneously blocking epidermal growth factor receptor and cyclooxygenase-2 in a xenograft model. Clin Cancer Res 2005;11:6261–9.

18. Sminia P, Stoter TR, van der Valk P, et al. Expression of cyclooxygenase-2 and epidermal growth factor receptor in primary and recurrent glioblastoma multiforme. J Cancer Res Clin Oncol 2005;131:653–61.

19. Lanza-Jacoby S, Burd R, Rosato FE, Jr., et al. Effect of simultaneous

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inhibition of epidermal growth factor receptor and cyclooxygenase-2 in HER-2/neu-positive breast cancer. Clin Cancer Res 2006;12:6161–9.

20. Choe MS, Zhang X, Shin HJ, Shin DM, Chen ZG. Interaction between epidermal growth factor receptor- and cyclooxygenase 2-mediated pathways and its implications for the chemoprevention of head and neck cancer. Mol Cancer Ther 2005;4:1448–55.

21. Shin YK, Park JS, Kim HS, et al. Radiosensitivity enhancement by celecoxib, a cyclooxygenase (COX)-2 selective inhibitor, via COX-2-dependent cell cycle regulation on human cancer cells expressing differential COX-2 levels. Cancer Res 2005;65:9501–9.

22. Kang HK, Lee E, Pyo H, Lim SJ. Cyclooxygenase-independent down-regulation of multidrug resistance-associated protein-1 expression by celecoxib in human lung cancer cells. Mol Cancer Ther 2005;4:1358–63.

23. Krysan K, Reckamp KL, Dalwadi H, et al. 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. Cancer Res 2005;65:6275–81.

24. Wang XQ, Yan Q, Sun P, et al. Suppression of epidermal growth factor receptor signaling by protein kinase C-α activation requires CD82, caveolin-1, and ganglioside. Cancer Res 2007;67:9986–95.

25. Zhang L, Jiang L, Sun Q, et al. Prostaglandin E(2) enhances mitogen-activated protein kinase/Erk pathway in human cholangiocarcinoma cells: involvement of EP1 receptor, calcium and EGF receptors signaling. Mol Cell Biochem 2007.

26. Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 2007;26:3291–310.

27. Mialon A, Sankinen M, Soderstrom H, et al. DNA topoisomerase I is a co-factor for c-Jun in the regulation of epidermal growth factor receptor expression and cancer cell proliferation. Mol Cell Biol 2005;25:5040–51.

28. Crotty T, Cai J, Sakane F, Taketomi A, Prescott SM, Topham MK. Diacylglycerol kinase δ regulates protein kinase C and epidermal growth factor receptor signaling. Proc Natl Acad Sci U S A 2006;103:15485–90.

29. Yujiri T, Ware M, Widmann C, et al. MEK kinase 1 gene disruption alters cell migration and c-Jun NH_2-terminal kinase regulation but does not cause a measurable defect in NF-κB activation. Proc Natl Acad Sci U S A 2000;97:7272–7.

30. Xu Z, Choudhary S, Voznesensky O, et al. Overexpression of COX-2 in human osteosarcoma cells decreases proliferation and increases apoptosis. Cancer Res 2006;66:6657–64.

31. Nishimura Y, Yoshioka K, Bereczky B, Itoh K. Evidence for efficient phosphorylation of EGFR and rapid endocytosis of phosphorylated EGFR via the early/late endocytic pathway in a gefitinib-sensitive non-small cell lung cancer cell line. Mol Cancer 2008;7:42.

32. Cappuzzo F, Hirsch FR, Rossi E, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 2005;97:643–55.

33. Vokes EE, Choy H. Targeted therapies for stage III non-small cell lung cancer: integration in the combined modality setting. Lung Cancer 2003;41 Suppl 1:S115–21.

34. Javle MM, Yu J, Khoury T, et al. Akt expression may predict favorable prognosis in cholangiocarcinoma. J Gastroenterol Hepatol 2006;21:1744–51.

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