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Differential Regulation of Cyclooxygenase-2 in Nontransformed and Ras-Transformed Intestinal Epithelial Cells[1]

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Abstract

To determine signaling pathways responsible for modulation of COX-2 expression in nontransformed and transformed epithelial cells, we studied a rat intestinal epithelial (RIE) cell line expressing constitutively active Ras and RhoA. Expression of COX-2 protein was higher in RIE-RhoA(63L) (four-fold) and RIE-Ras(12V) (seven-fold) cells than in parental cells. Prior work suggests that Ras hyperactivity induces the expression of transforming growth factor (TGF) β and increases epidermal growth factor (EGF)–related peptide signaling—possible mechanisms for increased COX-2 expression. Expression of COX-2 was stimulated by TGFβ and TGFα in RIE and RIE-Rho(63L) cells, but not further stimulated in RIE-Ras(12V) cells. PD153035, an inhibitor of EGF receptor tyrosine kinase, and PD98059, an inhibitor of Erk, attenuated COX-2 expression in RIE and RIE-RhoA(63L). However, the high levels of COX-2 expression in RIE-Ras(12V) cells were not inhibited by either compound. Titration with a pan-neutralizing anti-TGFβ antibody did not decrease COX-2 in RIE-Ras(12V) cells, even with concurrent EGFR inhibition. Thus, stimulation of the EGF receptor is important in the modulation of COX-2 expression in nontransformed RIE and RIE-RhoA(63L) cells. In Ras-transformed cells, signaling by additional Ras effector pathways, perhaps the RhoA pathway, must be invoked. Identification of these *pathways* is critical for therapeutic manipulation of COX-2 expression.

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Keywords: Ras, RhoA, transforming growth factor β, COX-2, epidermal growth factor.

Introduction

Cyclooxygenases (COXs), or prostaglandin–endoperoxide synthetases, catalyze the rate-limiting step in the synthesis of prostaglandins and other eicosanoids from arachidonic acid [1]. COX-1 and COX-2 are recognized as constitutive and inducible COX isoenzymes, respectively. Increased expression of COX-2 and prostaglandins has been widely observed in human [2] and rodent intestinal tumors [3,4] when compared with normal adjacent mucosa, whereas COX-1 expression is not differentially expressed. Among the consequences of elevated COX-2 expression and increased prostaglandin production are stimulation of angiogenesis, inhibition of apoptosis, and stimulation of cellular proliferation, all of which favor tumorigenesis. Inhibitors of COX-2 activity such as aspirin, nonsteroidal anti-inflammatory agents (NSAIDs), and specific COX-2 antagonists significantly reduce the occurrence of colorectal carcinoma in both animal and human studies (reviewed in Ref. [5]). In addition, experimental animal and human data show that individuals with colon adenomas occurring in the context of a defect in the adenomatous polyposis coli (APC) gene product show a significant reduction in the number of adenomas following treatment with NSAIDs or more specific COX-2 inhibitors [6,7]. Collectively, these observations indicate that understanding the regulation of COX-2 activity is linked to unraveling the pathogenesis of colorectal cancer and to identifying strategies for its treatment and prevention.

In cell culture systems, a wide variety of growth factors and cytokines induce COX-2 expression, including epidermal growth factor (EGF)–related peptides [8], keratinocyte growth factor [9], basic fibroblast growth factors [10], transforming growth factor β (TGFβ) [11], hepatocyte growth factor [12], platelet-derived growth factor [13], interferon γ [14], and gastrin [15], among many others. Inasmuch as many of these growth factors are overexpressed in colorectal polyps and cancers, it is perhaps not unexpected that these same tumors frequently demonstrate markedly elevated levels of COX-2 and prostaglandins. Many growth factors stimulate cell surface membrane–associated tyrosine kinase receptors that activate cellular Ras and its associated effector pathways. Activating mutations in the K-ras protooncogene are found in approximately 50% of colorectal cancers, and multiple cell culture

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studies have reported the overexpression of COX-2 in the context of the mutant activation of Ras [16–18]. In some cell culture models, this induction appears to occur through increased COX-2 transcription [16] and an increase in the half-life of the COX-2 mRNA by activation of protein kinase B1 [19]. In addition to the activation of Ras, activation of other small GTPases such as RhoA may also increase COX-2 transcription by a Ras-independent pathway [17,20].

Clarification of the signaling pathways by which oncogenic Ras upregulates COX-2 may be important in understanding the pathogenesis of aberrant growth-regulatory pathways in the intestine. In its activated, GTP-bound state, Ras stimulates a complex network of downstream pathways potentially involved in COX-2 upregulation [21], including stimulation of EGF-related peptide synthesis, activation of EGFRs [22], and activation of TGFβ transcription [23]. Activation of EGF signaling by Ras is especially interesting in light of recent studies highlighting synergy between the inhibition of COX-2 activity and the blockade of the EGFR signaling in models of colorectal neoplasia [24].

Herein, we examine the differential regulation of COX-2 expression in transformed and nontransformed intestinal epithelial cell model systems. Emphasis is given to the importance of epidermal growth factor receptor (EGFR) intrinsic tyrosine kinase activity and TGFβ signaling in the regulation of COX-2 expression by Ras and Rho GTPases pathways in RIE-1 intestinal epithelial cells.

Materials and Methods

Cell Lines and Reagents

RIE-1 rat intestinal epithelial cells were obtained from Ken Brown (Cambridge, UK) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum [25]. Dr. Robert Coffey (Vanderbilt University) kindly supplied RIE-Ras(12V) and RIE-Rho cells, which have been described in more detail elsewhere [26]. Briefly, RIE-Ras(12V) cells were stably transfected with pSV2-H-*ras*(12V) containing human sequences encoding the transforming H-Ras(12V) protein. RIE-RhoA(63L) cells were established by stable transfection with mutant active RhoA(63L) (pZIP-RhoA-63L). Unless otherwise noted, the cells used in our studies were propagated from pools of multiple G418-resistant clones (> 50). Ras hyperactivity in the RIE-Ras(12V) cells was confirmed based on binding of GTP-Ras to immobilized Raf-1, as previously reported by our laboratory [27]. In selected experiments, double stable transfectants were used. For these studies, RIE-Ras(12V) cells were transfected with dominant-negative mutant RhoA(N19) (kindly supplied by Dr. Gary Bokoch). RhoA(N19) was subcloned into pcDNA3.1/Zeo and transfected into RIE-Ras(12V) cells using the Lipofectamine 2000 protocol supplied by Invitrogen Life Technologies (Carlsbad, CA). Stable clones were double-selected in Zeocin and G418. Expression of RhoA(N19) was verified by Western blot analysis using both anti-HA and anti-RhoA antibodies. Multiple clones were selected for testing.

EGF was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). TGFβ1 was obtained from R&D Systems (Minneapolis, MN). The following signaling inhibitors were obtained from Calbiochem-Novabiochem Corp.: PD153035 (EGFR tyrosine kinase inhibitor), PD98059 (MEK inhibitor), and Y-27632 (p160ROCK inhibitor). U0126 was obtained from Promega Corporation (Madison, WI). EKI-785 was a kind gift from Dr. Robert Coffey. A rabbit polyclonal anti–human COX-1 antibody and a goat polyclonal anti–human COX-2 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal anti–cyclin D1 antibody was obtained from Santa Cruz Biotechnology.

Cell Proliferation Assays

^{3}H thymidine incorporation assays were carried out in 24-well tissue culture plates as described previously [28]. Cells were seeded at a density of 20,000 cells/well, allowed to attach for at least 24 hours, then treated as described in the figure legend. A ^{3}H thymidine (Amersham Life Sciences, Arlington Heights, IL) pulse (1 μCi/well) was provided between the 18th and 21st hours of treatment. Radioactivity incorporated into trichloroacetic acid–insoluble material was determined by scintillation counting and results are presented as the mean ± SEM for triplicate or quadruplicate measurements. Experiments were repeated at least three times.

Immunoprecipitation and Western Blot Analysis

Cells in 12-well plates or 100-mm culture dishes were solubilized in lysis buffer (20 mM Tris–HCl, pH 7.4, 120 mM NaCl, 100 mM NaF, 200 μM Na_3VO_4, 4 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5% NP-40, and 2 mM benzamidine) for 30 minutes at 4°C. The lysate was clarified by centrifugation and the supernatant was incubated with primary antibody overnight at 4°C. Immunoprecipitates were incubated with protein A-agarose for 1.5 hours and washed repeatedly in phosphate-buffered saline containing 0.05% NP-40. The immune complexes were eluted in SDS sample buffer and transferred onto PVDF membranes in 25 mM Tris, 192 mM glycine, and 20% methanol buffer at 30 V overnight. Membranes were then blocked, probed with the diluted antibody of interest, and then incubated with horseradish peroxidase–conjugated anti-IgG. The ECL Plus detection system (Amersham Life Sciences) was used to detect antigen–antibody complexes.

Prostaglandin E_2 (PGE_2) Assay

Cells were plated in 24-well plates and grown in serum-containing medium for 24 to 28 hours. The medium was replaced with serum-free DMEM for 24 hours, which was then collected for measurements of basal levels of secreted PGE_2 by enzyme-linked immunoabsorbent assay (ELISA; Cayman Chemical, Ann Arbor, MI).

Northern Analysis

Total cellular RNA was extracted using Trizol. RNA samples (20 μg/lane) were loaded into 1% agarose/formaldehyde gels and separated by electrophoresis and blotted onto nitrocellulose membranes. COX-1 and COX-2 cDNA probes

were labeled by random primer extension using Redivue with α-^{32}P dCTP and the Rediprime DNA labeling system from Amersham Life Sciences. After hybrizidation and washing, the membranes were subjected to autoradiography. The integrity of the RNA was assessed by 18 rRNA signals.

Results

Expression and Activity of COXs in RIE, RIE-Ras(12V), and RIE-Rho(63L) Cells

COX-2 expression is increased in cells transformed by oncogenic activation of Ras [16,17,29,30]. Induction of COX-2 by RhoA activation has recently been reported, but is less well studied [17]. As previously shown, COX-2 expression in RIE-1 cells stably transfected and transformed with activated Ras showed a marked increase by immunoblot analysis (Figure 1). The level of COX-2 expression was also examined in cells that overexpress mutant-activated RhoA. These cells, which are nontransformed, exhibit four-fold greater COX-2 expression than parental RIE-1 cells, but less than that observed for transformed RIE-Ras(12V) cells. Expression of COX-1, a constitutive COX, is equivalent in these three cell lines. To supplement these observations, the level of PGE_2, a product of COX activity, was measured to determine if the level of COX-2 protein observed in the RIE-Ras(12V) and RIE-RhoA cells lines is associated with an increase in prostaglandin synthesis. The level of PGE_2 in RIE-Ras(12V) cells was 70-fold greater than the parental RIE-1 cell line, whereas levels in RIE-RhoA(63L) cells were two-fold greater. Although PGE_2 has previously shown to be the predominant prostaglandin synthesized in the context of Ras transformation, similar detailed studies have not been performed in cells overexpressing RhoA and it is plausible that other prostaglandins are synthesized in greater abundance. Notwithstanding, these results confirm increased expression of COX-2 protein and activity in both RIE-Ras(12V) and RIE-RhoA(63L) cells, when compared with the parental RIE-1 cell line.

In nontransformed cells, proliferation is regulated, in part, by cell density and confluence, whereas in transformed cells, contact inhibition is lost. Expression of EGF-related peptides, EGFRs, and EGF-signaling proteins is also regulated by epithelial cell density [31,32]. Therefore, we examined the expression of COX-2 protein as a function of RIE-1, RIE-Ras(12V), and RIE-RhoA(63L) cell confluence (Figure 2). In the nontransformed RIE-1 and RIE-RhoA(63L) cells, expression of COX-2 was decreased by approximately 60% when measured 48 hours after cells reached confluence. By contrast, the transformed RIE-Ras(12V) cells showed little difference in their high level of COX-2 expression, even at 72 hours—a time when the cells have become densely confluent. Thus, the high levels of COX-2 expressed in RIE-Ras(12V) cells occur independent of cellular confluence, whereas contact inhibition in nontransformed cells is associated with decreased COX-2 expression.

Regulation of COX-2 Expression by EGF-Related Peptides and TGFβ

It is well established that COX-2 levels are increased by EGF-related peptides and TGFβ in a variety of cell lines,

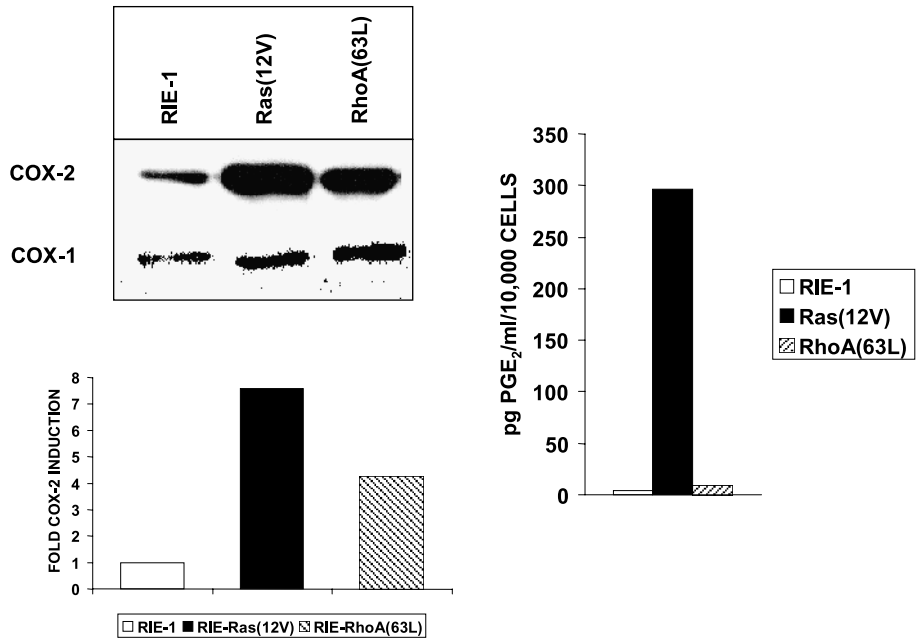

Figure 1. *Expression of COX-2 protein and determination of PGE_2 levels in intestinal epithelial cell lines. Total cellular lysates from RIE-1, RIE-Ras(12V), and RIE-RhoA(63L) cells growing in subconfluent cultures were isolated, and the basal expression of COX-2 and COX-1 was examined by Western blot analysis. The bar graph shows the relative expression of COX-2 in each cell line determined by densitometry. The graph on the right shows PGE_2 levels in serum-free cell culture medium collected from each cell line. PGE_2 levels were measured by ELISA as described in the Materials and Methods section.*

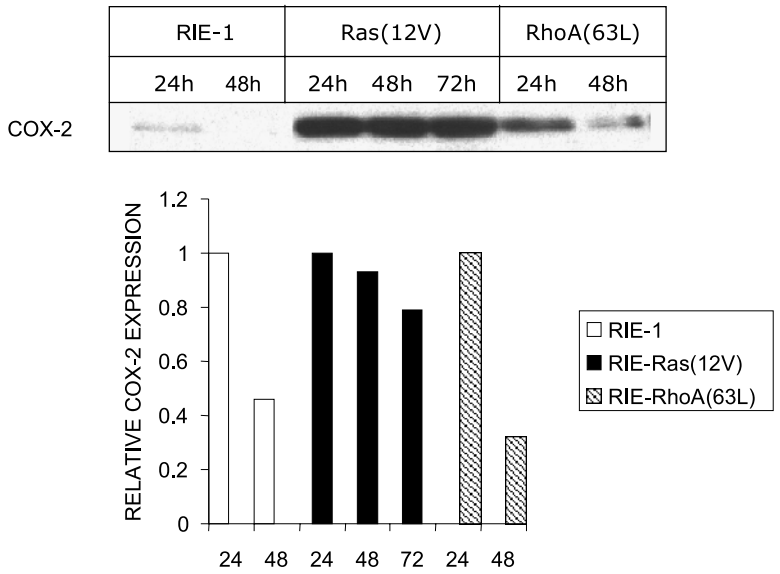

Figure 2. *Regulation of COX-2 expression as a function of cell density. Cultures of RIE-1, RIE-Ras(12V), and RIE-RhoA(63L) cells were grown to the point at which they just reached confluence. This time point was considered time 0. Continued postconfluent growth of cells was allowed to occur and, at 24-hour intervals thereafter, cell lysates were isolated, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a single gel, and subjected to Western blot analysis for COX-2 expression. Because RIE-Ras(12V) cells did not show downregulation of COX-2 expression 48 hours after reaching confluence, an additional 24 hours of postconfluence growth was permitted. Quantified results, obtained by densitometry, are shown in the bar graph.*

including the RIE-1 line used herein. To investigate the relative induction of COX-2 in RIE-1 cells overexpressing activated Ras(12V) and RhoA(63L), we compared the induction of COX-2 in response to treatment with TGFα and TGFβ. Consistent with prior reports [8], COX-2 was rapidly and markedly induced in RIE cells following treatment with TGFα, approximately 13-fold after 8 hours of treatment (Figure 3). Similarly, COX-2 was induced in the nontransformed RIE-RhoA(63L) cell line, albeit less than the parental cell line. The RIE-Ras(12V) cell line, which already has a markedly increased level of COX-2, showed no further induction in response to treatment with TGFα.

Although the level of COX-2 induction by TGFβ was less prominent than for TGFα, qualitatively, the observations are similar [i.e., COX-2 is rapidly induced in the nontransformed cell lines, but not further induced in the transformed RIE-Ras(12V) cell line] (Figure 3). These results indicate that increased levels of COX-2 expression characteristic of RIE-Ras(12V) cells cannot be further induced in response to autocrine growth-regulatory factors that are commonly overexpressed in transformed intestinal epithelial cells. The data suggest that overexpression of EGF-related peptides, such as TGFα or TGFβ, is possibly a major driving force for COX-2 overexpression in RIE-Ras(12V) cells, and that

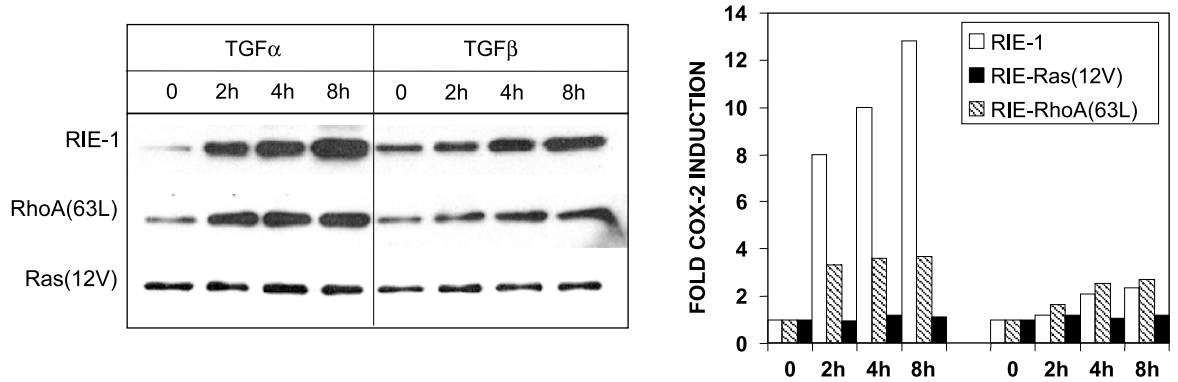

Figure 3. *Induction of COX-2 protein expression by TGFα and TGFβ. Rapidly growing cultures of RIE-1, RIE-Ras(12V), and RIE-RhoA(63L) cells were treated with 2 ng/ml TGFα or 2 ng/ml TGFβ1 for the indicated time period. Total cellular lysates were collected, and COX-2 protein expression was examined by Western analysis using a COX-2–specific antibody. These experiments were performed three separate times and representative results are shown. As shown in Figures 1 and 2, the relative level of COX-2 expression is different for each cell line. In this figure, autoradiographic exposures for each cell line were varied to optimize exposure levels and results were quantified by densitometry.*

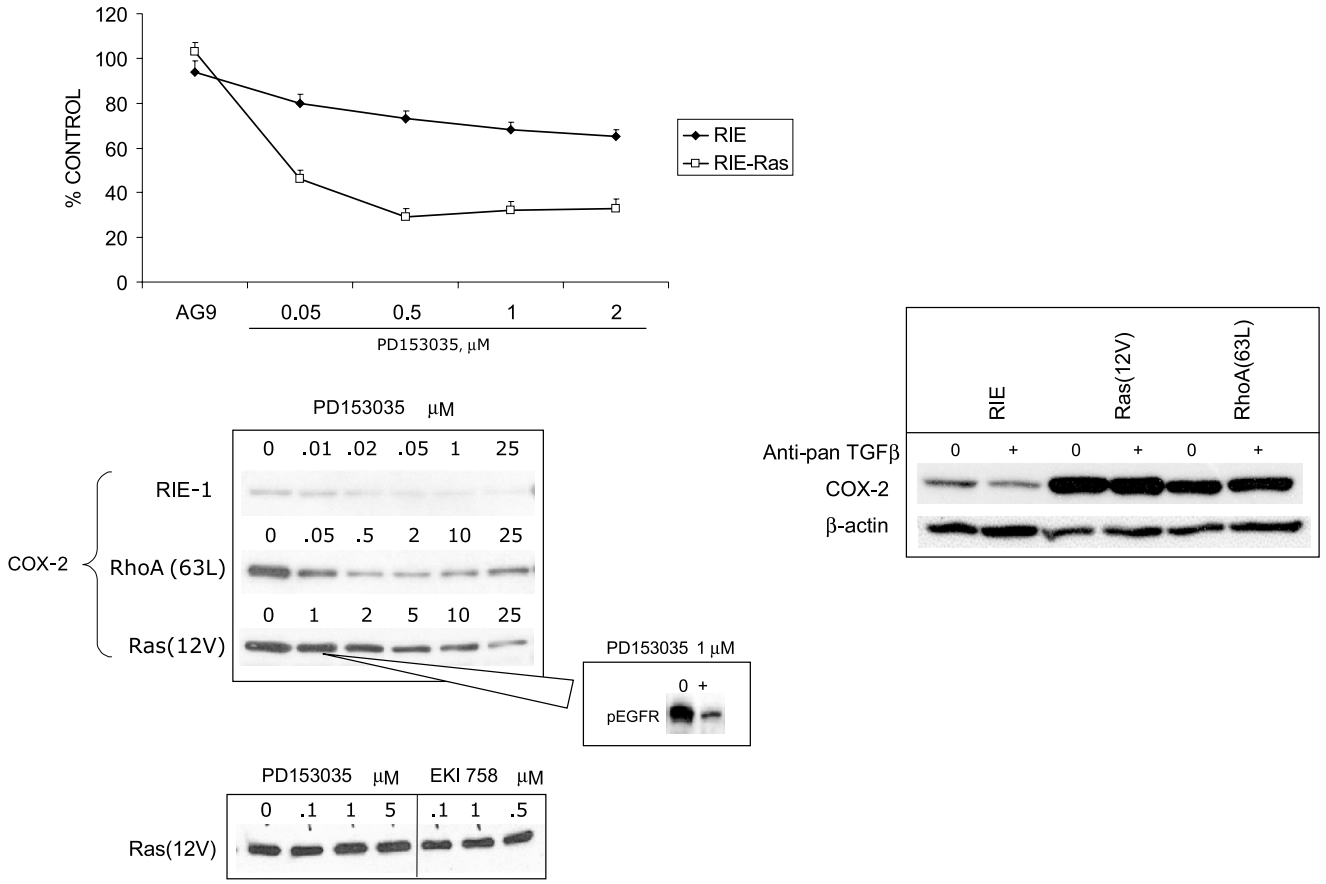

Figure 4. *EGFR tyrosine kinase inhibitors decrease ^{3}H thymidine incorporation but not COX-2 levels in Ras-transformed cells. RIE-Ras(12V) cells were rapidly treated with the indicated concentration of the tyrosine kinase inhibitor, PD153035, in serum-free medium. Eighteen hours later, a 3-hour pulse of ^{3}H thymidine (1 μCi/well) was provided, followed by measurements of thymidine incorporation into TCA-insoluble materials, as described in the Materials and Methods section. AG9, an inactive structural analogue of AG1478, was used as a control in these experiments, and identical experiments with AG1478 were performed (not shown). Results, shown in the top panel, are expressed as percent of ^{3}H thymidine incorporation in cells treated with dimethyl sulfoxide alone. Rapidly growing cultures of RIE-1, RIE-Ras(12V), and RIE-RhoA(63L) cells were treated for 24 hours with the highly specific tyrosine kinase inhibitor, PD153035, at concentrations between 0.01 and 25 mg/ml. Note that the inhibitor concentrations varied for each cell line. Total cellular lysates were collected and COX-2 protein expression was examined by Western blot analysis using a COX-2–specific antibody. These experiments were performed three separate times and representative results are shown. As shown in Figures 1 and 2, the relative level of COX-2 expression is different for each cell line. In this figure, autoradiographic exposures for each cell line were varied to optimize exposure levels. Because of the apparent insensitivity of RIE-Ras(12V) cells to PD153035, its activity as a tyrosine kinase inhibitor was checked in separate experiments, under identical conditions, using an alternate EGFR tyrosine kinase inhibitor, EKI-758. Again, reduced levels of COX-2 are not observed (bottom right). The inset shows that at a concentration of 1 μg/ml, PD153035 markedly inhibited EGFR phosphorylation, but not COX-2 expression, as shown by the arrow. In the experiment shown in the right panel, cells were grown to near confluence and treated with a pan-neutralizing anti-TGFβ antibody for 24 hours. COX-2 protein expression in each cell line was measured by Western blot analysis.*

addition of these peptides does not further increase the expression of COX-2.

Characterization of EGFR Tyrosine Kinase Inhibition in the RIE-Ras(12V) Cell Line

In our studies, the RIE-1 intestinal epithelial cell line is utilized as a model for normal intestinal epithelial cells, whereas RIE-Ras(12V) cells are used as a model for transformed cells. Prior studies have shown that parental RIE-1 cells are morphologically nontransformed and do not form colonies in soft agar, nor do they form tumors in nude mice [26]. In contrast, RIE cells that overexpress mutant-activated Ras(12V) assume a transformed morphology, form colonies in soft agar, and rapidly form tumors in nude mice [26]. These characteristics have been attributed, in part, to production of EGF-related peptides. To determine the effect of EGFR kinase inhibition on the growth and morphology of RIE-Ras(12V) cells, PD153035 (AG1517) was used in ^{3}H thymidine incorporation assays. As shown in Figure 4, DNA synthesis was decreased by concentrations of the tyrosine kinase inhibitor between 0.05 and 2 μM, whereas 2 μM AG9, an inactive tyrphostin analogue, had no significant effect. Similar results were observed with the tyrosine kinase inhibitor, tyrphostin (AG1478). No effect was observed on the morphology of cells. Based on the near equivalence of tyrphostin and PD153035, further experiments described in this manuscript were performed using the latter compound. The inset in Figure 4 shows a marked decreased in the tyrosine phosphorylation of EGFR in RIE-Ras(12V) cells at a PD153035 concentration of 1 μM. Collectively, these concentrations are consistent with effective tyrosine kinase inhibitor concentrations reported

previously [33–35], and substantiate the design of subsequent experiments on COX-2 expression reported herein. The results also show the critical dependence of increased EGFR tyrosine kinase activity on cellular proliferation in RIE-Ras(12V) cells.

Effect of EGFR Tyrosine Kinase Inhibition and TGFβ Inhibition on COX-2 Expression

To test the hypothesis that COX-2 overexpression in RIE-Ras(12V) cells is due to the high level of expression of EGF-related peptides characteristic of these cells, RIE-1, RIE-Ras(12V), and RIE-RhoA(63L) cells were treated for 24 hours with PD153035 at concentrations known to decrease the phosphorylation of EGFR (Refs. [33–35]; Figure 4, *inset*) and inhibit DNA synthesis RIE-Ras(12V) cell lines (Figure 4, *top panel*). The results show that COX-2 expression in parental RIE-1 cells is highly sensitive to the EGFR tyrosine kinase inhibitor, with half-maximal inhibition occurring between 0.02 and 0.05 μM PD153035 (Figure 4). RIE-Rho cells are somewhat less sensitive, with a half-maximal inhibition between 0.05 and 0.5 μM. By contrast, COX-2 levels are resistant to EGFR tyrosine kinase inhibition up to 5 μM in transformed cells overexpressing mutant-activated Ras. This relative (100-fold) resistance in RIE-Ras(12V) cells was confirmed by treatment with EKI-758, a structurally distinct EGFR tyrosine kinase inhibitor (Figure 4, *bottom panel*). Collectively, these results indicate that the basal levels of COX-2 in RIE-1– and RIE-RhoA(63L)–expressing cells are maintained, in part, by autocrine stimulation of the EGFR tyrosine kinase activity. Despite the high levels of production of EGF-related peptides in RIE cells that stably overexpress activated Ras, stimulation of the EGFR by these autocrine growth factors is not independently responsible for the maintenance of high levels of COX-2 expression characteristic of this cell line.

COX-2 expression is also stimulated by TGFβ in many cell types, potentially explaining the increased COX-2 expression observed in Ras-transformed cells, as Ras activates the transcription of the TGFβ gene [23]. To confirm this in RIE cells, TGFβ levels in 72-hour conditioned medium were determined by ELISA and were approximately increased 10-fold in RIE-Ras(12V) cells compared with the parental RIE cell line (RIE = 21.1 pg/ml per 10^4 cells *versus* Ras(12V) = 191.9 pg/ml per 10^4 cells; RIE-RhoA(63L) cells = not performed). A pan-neutralizing anti-TGFβ antibody that recognizes all three isoforms of mammalian TGFβ was added to the cell culture medium in each of the three cell lines under study in an attempt to interrupt autocrine TGFβ-stimulated COX-2 expression. Addition of the pan-neutralizing antibody (1 μg/ml) for 24 hours did not diminish COX-2 expression in any of the cell lines (Figure 4), although this was sufficient to reduce basal levels of Smad2 phosphorylation (not shown). Simultaneous treatment of RIE-Ras(12V) cells with pan-neutralizing anti-TGFβ antibody and PD153035 also did not reduce COX-2 expression. This result indicates that autocrine production of TGFβ does not contribute to the basal levels of COX-2 expression in nontransformed or transformed cell lines.

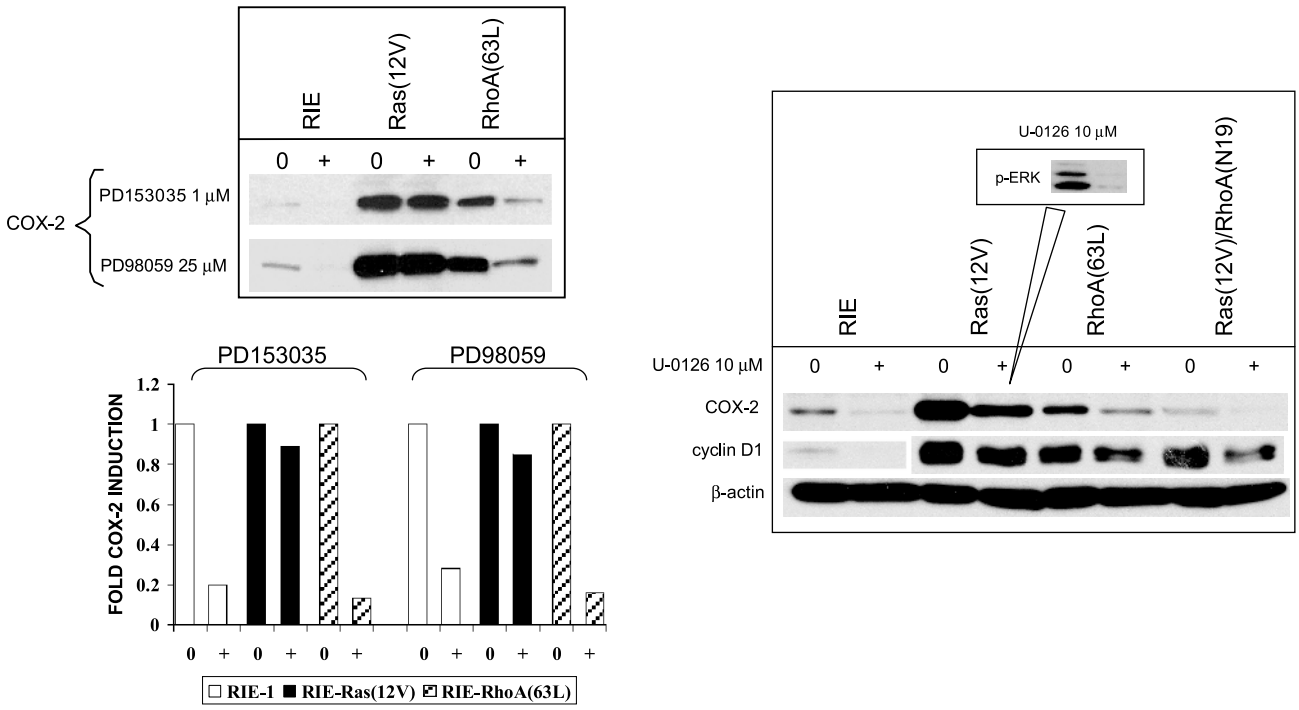

Figure 5. *Effect of Erk pathway inhibition on COX-2 expression. Rapidly growing, subconfluent RIE-1, RIE-Ras(12V), RIE-RhoA(63L), and RIE-Ras(12V)/RhoA(N19) cells were treated for 24 hours with the indicated concentration of the EGFR tyrosine kinase inhibitor, PD153035, and the MEK inhibitor, PD98059 (left panel), or with U0126, a more potent inhibitor of MEK (right panel). Total cellular lysates were isolated from each cell line and loaded onto SDS-PAGE gels. Western blot analysis was performed for COX-2 and cyclin D1 expression. This experiment was repeated three times with similar results.*

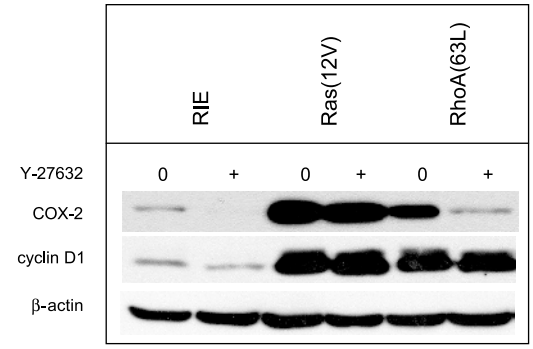

Figure 6. *Effect of Rho inhibition on COX-2 expression. Rapidly growing, subconfluent RIE-1, RIE-Ras(12V), and RIE-RhoA(63L) cells were treated for 24 hours with the indicated concentration of Y-27632, an inhibitor of the p160ROCK limb of the RhoA pathway. Total cellular lysates were isolated from each cell line and loaded onto SDS-PAGE gels. Western blot analysis was performed for COX-2 and cyclin D1 expression. This experiment was repeated three times with similar results.*

Effect of MEK and p160ROCK Inhibition on COX-2 Expression

Activation of Ras stimulates a variety of diverse intracellular signaling pathways, the most extensively studied of which is the canonic Raf/MEK/Erk pathway. Inhibitors of MEK were used to determine the involvement of this pathway in the stimulation of COX-2 expression in intestinal epithelial cell lines. Preliminary experiments (not shown) confirmed that 25 μM PD98059 significantly inhibited MEK activity in the cells used in this study, consistent with published reports. Similarly, PD98059 inhibited basal COX-2 expression in parental RIE-1 cells, as well as inhibited the intermediate level of COX-2 expression in cells expressing activated RhoA (Figure 5). However, inhibition of COX-2 by PD98059 did not occur in cells overexpressing activated Ras. This observation was further explored by using U0126, an alternate, more potent, and selective inhibitor of MEK activity [36]. Using U0126 at a concentration of 10 μM, inhibition of MEK was virtually complete as shown in the inset in Figure 5, but inhibition of COX-2 expression was modest and did not decrease to the lower level of expression observed in parental RIE or RIE-RhoA(63L) cells. Thus, despite the expression of oncogenic Ras at a level sufficient to cause transformation, activation of Erk was not fully and independently responsible for the maintenance of high levels of COX-2 expression characteristic of this cell line.

As shown in Figure 5, transfection of RIE-Ras(12V) cells with a dominant-negative RhoA(N19) construct markedly decreased COX-2 levels, as we have reported previously [37]. The selectively of dominant-negative Rho(A) on expression was apparent by the lack of reduced cyclin D1 in dually transfected cells. When the dual transfectant was treated with U0126, decreased MEK activity further reduced COX-2 expression, suggesting that Rho and MEK jointly regulate COX-2 expression.

We previously found that activated RhoA and activated Raf function cooperatively to transform intestinal epithelial cells and promote the acquisition of growth characteristics highly reminiscent of Ras-transformed cells [37]. Additionally, we found that dominant-negative expression of RhoA in RIE-Ras(12V) cells markedly inhibits elevated COX-2 expression and increased PGE_2 levels characteristic of cells expressing hyperactive Ras, but did not affect levels of other proteins relevant to cellular proliferation, such as cyclin D1 [37]. To determine if the p160ROCK Rho effector pathway is involved in the stimulation of COX-2 expression, we treated RIE-1, RIE-Ras(12V), and RIE-RhoA(63L) cells with an inhibitor of p160ROCK, a downstream effector Rho-kinase. Although 10 μM Y-27632 reduced basal COX-2 expression in RIE cells by approximately 70%, significant inhibition in RIE-RhoA(63L) and RIE-Ras(12V) cells was not observed, and no alterations in the expression of cyclin D1 were observed, as shown in Figure 6. These results suggest that other RhoA effector pathways, independent of p160ROCK, are involved in the regulation of COX-2 expression in Ras-transformed intestinal epithelial cells.

Discussion

COX-2 is believed to play an important role in the progression of colorectal cancer. Its expression is markedly induced in human colorectal tumors and is not detectable in adjacent normal mucosa [2,38]. Although increased COX-2 expression is not sufficient for neoplastic transformation of cultured intestinal epithelial cells [39], reduction of COX-2 activity in cell culture models, experimental animals, and human research subjects decreases transformed cell behavior and tumor growth. For example, the growth of subcutaneously implanted lung carcinoma tumor cells in COX-$2^{-/-}$ mice is attenuated [40] when compared with COX-2–expressing littermates. The growth of small intestinal tumors is significantly diminished in COX-$2^{-/-}$ mice that are also deficient in the APC tumor suppressor gene product [41]. Similarly, the use of the COX-2 inhibitor, celocoxib, in persons with FAP diminishes the number of *existing* adenomas by about 25% [6], although standard doses of sulindac do not prevent the *development* of adenomas [42]. Thus, there is a connection between COX-2 activity and transformed cell behavior, which is explained, in part at least, by the effect of COX-2–derived prostaglandins on cellular proliferation, inhibition of apoptosis, increased invasiveness, and stimulation of angiogenesis. Thus, understanding how COX-2 levels are regulated and how its activity can be modified to therapeutic advantage has become a major area of investigation in the past decade.

In quiescent G_0 cells, COX-2 expression is low or not detectable; its levels are induced by growth factors, such as EGF-related peptides and TGFβ, and are super-induced by the protein synthesis inhibitor, cyclohexamide [8]. Thus, COX-2 is regulated as an immediate early gene in nontransformed intestinal epithelial cells and other nontransformed cells [8]. Diverse intracellular signal transduction pathways tightly regulate COX-2 expression in normal epithelial cells by the regulation of gene transcription, mRNA stability, and protein translation [43]. In the setting of cellular

transformation, COX-2 is constitutively overexpressed due to the activation of diverse intracellular signaling pathways. This is particularly true in the case of cells overexpressing mutant-activated Ras. For example, Ras upregulates COX-2 expression by activation of the transcription and stabilization of COX-2 mRNA by ERK, JNK, p38, Rho, and Akt signaling (reviewed in Ref. [43]). Activation of COX-2 expression by other small GTPases, such as RhoGTPases, also occurs [17] in some cells by a p160ROCK– and NF-κB–dependent mechanism, although there is a great deal of cell type specificity in this response [20]. Notwithstanding this growing body of information on intracellular signaling pathways involved in the regulation of COX-2 expression, little significant attention has been given to growth factor pathways that specifically activate COX-2 expression in Ras-transformed cells.

Because overexpression of EGF-related peptides and TGFβ contributes to the Ras-transformed phenotype [22, 44,45], in the present work, we examined the relative importance of these growth factors in the regulation of COX-2 expression in the nontransformed rat intestinal epithelial cell line RIE-1, and in RIE-1 cells transformed by stable transfection of oncogenic Ras. In addition, regulation of COX-2 in RIE cells overexpressing mutant-activated RhoA was also examined because of emerging reports suggesting that RhoA transcriptionally activates COX-2 expression and our own work showing that simultaneous expression of activated RhoA and activated Raf in RIE cells mimics many of the characteristics of Ras-transformed cells [37].

EGFR activation by autocrine EGF-related peptides is a potentially attractive mechanism for the upregulation of COX-2 expression in Ras-transformed intestinal epithelial cells because upregulation of these peptides commonly occurs in a variety of Ras-transformed epithelial cell types, including intestinal epithelial cells [22] and keratinocytes [45]. In fact, a number of characteristics of Ras-transformed cells can be specifically attributed to signaling through by the EGFR. For example, Ras-mediated induction of vascular endothelial growth factor and tumor angiogenesis [46], growth in soft agar and morphologic transformation [22,26], and sensitivity to Ras inhibition by farnesyltransferase inhibitors [44] can be linked to the production of EGF-related peptides such as transforming growth factor α, heparin-binding EGF-like growth factor, and amphiregulin in RIE-1 cells. In the present work, we investigated the potential role of EGFR activation in contributing to the high levels of COX-2 in RIE-Ras(12V) cells. Treatment of nontransformed RIE-1 and RIE-RhoA cells with EGFR tyrosine kinase inhibitors reduced COX-2 expression in a dose-dependent fashion, indicating the stimulation of EGFR tyrosine kinase, presumably by autocrine production of TGFα, amphiregulin, betacellulin, and HB-EGF, which we have previously found in RIE-1 cells [47]. However, unlike the observations cited above, we found that COX-2 induction by stable Ras overexpression in RIE-Ras(12V) cells cannot be solely attributed to EGFR activation, despite a demonstrated inhibitory effect of EGFR tyrosine kinase inhibitors on Ras-transformed cell growth and EGFR phosphorylation. Clearly, an additional pathway for the regulation of COx-2 expression must be invoked.

TGFβ increases COX-2 expression in RIE-1 cells. In our studies, TGFβ did not further increase the already elevated levels of COX-2 expression in Ras-transformed cells. We also found that inhibition of TGFβ signaling by anti–TGFβ-neutralizing antibodies did not reduce COX-2 levels in Ras-transformed cells. Our findings contrast with the observations of Sheng et al. [29], who rapidly induced Ras expression in RIE cells using an isopropyl-1-thio-β-D-galactopyranoside (IPTG)–inducible construct. In this system, TGFβ further induced COX-2 expression and anti–TGFβ-neutralizing antibodies reduced, but did not eliminate, IPTG-induced COX-2 expression. The divergence between our study and the Sheng et al. study is likely due to technical differences in the systems under study. Inducible systems are excellent for the analysis of the immediate signaling effects of oncogenic Ras overexpression, are but less attractive for understanding cellular consequences of the sustained overexpression of oncogenic Ras that typifies neoplasia. Conversely, because induction of genomic instability by oncogenic Ras is a well-known phenomenon and, indeed, all *in vitro* cell culture systems are susceptible to genomic instability [48], it is feasible that other genetic alterations contribute to variations in the regulation of COX-2 expression reported in various published studies, especially when rodent cell lines are used.

Emerging data suggest the regulation of COX-2 expression by the small GTPase RhoA [17,20]. Recently, we found that dominant-negative expression of RhoA in RIE-Ras(12V) cells markedly and specifically inhibits elevated COX-2 expression and increases PGE_2 levels characteristic of cells expressing hyperactive Ras [37]. This suggests that RhoA activation is necessary for increased COX-2 expression in the presence of mutant-activated Ras. In support of this, herein, we report that levels of COX-2 protein expression in RIE cells that stably overexpress mutant-activated RhoA are increased relative to parental RIE-1 cells, are but less than RIE-Ras cells. In addition, stable expression of a dominant-negative RhoA construct in RIE-Ras(12V) reduces COX-2 expression to relatively low levels seen in parental cells. Further, this residual COX-2 expression is reduced by a pharmacologic inhibitor of Erk activation, suggesting that the canonic Ras/Raf/MEK,Erk pathway and Rho jointly regulate COX-2 activation. Identification of the specific RhoA effector pathway(s) involved in the regulation of COX-2 expression will be problematic as more than 30 potential effectors of Rho-GTPases have been identified and, in most instances, downstream targets of these various Rho effectors are not yet known [49]. Also not investigated in our system is the potential role of other Rho proteins such as RhoB, RhoC, and Rac1, which share overlapping effector systems with RhoA [50]. Candidate effectors include mDIA, rhotekin, rhophilin, and PRK, among others. In the current study, because of the facile availability of a highly specific pharmacologic inhibitor (Y-27632), we studied p160ROCK, which has recently been identified as a RhoA effector pathway involved in a variety of functions such as actin stress fiber assembly [51] and tight junction formation [52]. Our findings indicate that ROCK does not mediate the induction of COX-2

in Ras-transformed cells. However, further support for the involvement of the RhoA pathway in the regulation of COX-2 expression is suggested by our work, indicating that additional investigation of this complex pathway is needed to fully understand the role of RhoA in the regulation of COX-2 expression in normal and transformed cells.

In summary, stimulation of the EGFR is an important inducer of basal COX-2 expression in nontransformed culture intestinal epithelial cells and in cells overexpressing activated RIE-RhoA(63L). By contrast, in cells transformed by overexpression of mutant-activated Ras, increased EGFR signaling and overproduction of TGFβ do not account for increased COX-2 expression. Emerging data suggest that RhoA activation in Ras-transformed cells is a critical key regulator of COX-2 expression, although the precise mechanism by which this occurs requires further investigation. Identification of these complex pathways will be important in the design of therapeutic strategies to manipulate COX-2 expression in the gastrointestinal tract.

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