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Cyclooxygenase-2 Is Up-Regulated by Interleukin-1β in Human Colorectal Cancer Cells via Multiple Signaling Pathways[1]

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ABSTRACT

Overexpression of cyclooxygenase-2 (COX-2) has been observed in human colorectal cancer. COX-2 expression in human tumors can be induced by growth factors, cytokines, oncogenes, and other factors. The mechanisms regulating COX-2 expression in human colon cancer have not been completely elucidated. We hypothesized that the proinflammatory cytokine interleukin-1β (IL-1β) mediates COX-2 expression in HT-29 human colon cancer cells. Treatment of HT-29 cells with IL-1β induced expression of COX-2 mRNA and protein in a time- and dose-dependent manner. Inhibitors of the extracellular signal-regulated kinase 1/2, c-Jun NH_2-terminal kinase, P38 mitogen-activated protein kinase, and nuclear factor-κB (NF-κB) signaling pathways blocked the ability of IL-1β to induce COX-2 mRNA. In contrast, Wortmannin, a phosphoinositide 3-kinase inhibitor upstream of protein kinase B/Akt, led to a slight increase in COX-2 mRNA expression after IL-1β treatment. Electrophoretic mobility shift assay on nuclear extracts demonstrated that IL-1β induced NF-κB DNA binding activity in HT-29 cells, and the activated NF-κB complex was eliminated after treatment with an inhibitor of NF-κB. Supershift assay indicated that the two NF-κB subunits, p65 and p50, were involved in activation of NF-κB complex by IL-1β stimulation. The stability of COX-2 mRNA was not altered by IL-1β treatment. These data demonstrate that IL-1β induces COX-2 expression in HT-29 cells through multiple signaling pathways and NF-κB.

INTRODUCTION

The conversion of arachidonic acid to prostaglandins and other eicosanoids is controlled by the two COX[3] isoforms, COX-1 and COX-2 (1). COX-1 is constitutively expressed in many human tissues and is thought to be involved in physiological functions via synthesis of prostaglandins. In contrast, COX-2 is undetectable or present at very low levels under basal conditions but is rapidly induced in response to inflammation in a wide variety of cell types, including cancer cells.

Various human tumor tissues have been reported to contain high concentrations of prostaglandins (2). Recent experimental evidence suggests that COX-2 may play an important role in colorectal carcinogenesis (3). COX-2 mRNA is overexpressed in 80–90% of human colorectal carcinomas and in 40–50% of premalignant adenomas (3, 4). COX-2 may contribute to the growth and metastatic potential of colorectal cancer by increasing expression of the antiapoptosis factor BCL-2 and up-regulating specific angiogenic factors (5, 6). A decrease in COX-2 activity results in decreased tumor growth in colorectal tumor models (COX-2 knockout mice and use of COX-2-selective inhibitors; Ref. 7). However, regulation of COX-2 in human colon cancer cells has not been fully elucidated.

The proinflammatory cytokine IL-1β is synthesized in tumor-infiltrating immune cells as well as endothelial cells. Studies from our laboratory have demonstrated that IL-1β induces expression of the potent angiogenic factors vascular endothelial growth factor and IL-8 in human colon cancer cells and pericytes (Refs. 8, 9 and unpublished data). In this study, we investigated the effect of IL-1β on COX-2 mRNA and protein expression in the human colorectal cell line HT-29. Furthermore, we investigated the mechanisms involved in COX-2 induction by IL-1β.

MATERIALS AND METHODS

Cell Line and Culture Condition. HT-29 human colon cancer cells were obtained from American Type Culture Collection (Manassas, VA). HT-29 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, penicillin-streptomycin, vitamins, sodium pyruvate, L-glutamine, and nonessential amino acids (Life Technologies, Inc., Grand Island, NY) at 37°C in 5% CO_2 and 95% air.

Materials and Antibodies. IL-1β, epidermal growth factor, insulin-like growth factor-I, and TNF-α were purchased from R&D Systems, Inc. (Minneapolis, MN). Phosphorylated Erk 1/2 (Thr^{202}/Tyr^{204}), JNK (Thr^{183}/Tyr^{185}), P38 (Thr^{180}/Tyr^{182}) MAPK, Akt (Ser^{473}) and total Erk 1/2, JNK, Akt, and P38 MAPK antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). SB203580 (P38 MAPK inhibitor) and SP600125 (JNK inhibitor) were purchased from Calbiochem Co. (La Jolla, CA). U0126 and PD98059 (MEK inhibitors) were purchased from New England Biolabs, Inc. (Beverly, MA). Actin antibody, Act D, Wortmannin, and TLCK were purchased from Sigma (St. Louis, MO). COX-2, NF-κB, p65, p50, p52, c-Rel antibodies, and nonspecific rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Western Blot Hybridization. Cells were lysed with protein lysis buffer [20 mM sodium phosphate (pH 7.4), 150 mM sodium chloride, 1% Triton X-100, 5 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 1 μg/ml leupeptin, and 500 μM Na_3VO_4].

The protein was subjected to electrophoresis on polyacrylamide gel and transferred to a nylon membrane (Millipore Corp., Bedford, MA) as described previously (9). After blocking with 5% milk in 0.1% Tween 20 in PBS, the membranes were probed with primary antibodies. The membranes were then washed and treated with the secondary antibody labeled with horseradish peroxidase. Protein bands were visualized using a commercially available chemiluminescence kit (Amersham Corp.).

Isolation of mRNA and Northern Blot Analysis. Total RNA was extracted from 80% confluent tumor cells growing in culture using TRIzol reagent according to the manufacturer's protocol (Life Technologies, Inc.). Northern blot analysis was performed as described previously (9). Each cDNA probe was radiolabeled with [α-^{32}P]deoxyribonucleotide triphosphate by random primer technique using a commercially available kit (Amersham Corp.). After prehybridization of blots for 3–4 h at 65°C in rapid hybridization buffer (Amersham Corp.), the membranes were hybridized overnight at 65°C with the cDNA probe for COX-2 (Cayman Chemical Co.) or glyceraldehyde-3-phosphate dehydrogenase (internal control; American Type Culture Collection). The probed nylon membranes were washed at 65°C with 30 mM NaCl, 3 mM

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[3] The abbreviations used are: COX, cyclooxygenase; IL, interleukin; TNF-α, tumor necrosis factor α; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; Act D, actinomycin D; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; TLCK, *N*-tosyl-Lys-chloromethyl ketone; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift analysis; PI3k, phosphatidylinositol 3′-kinase.

sodium citrate (pH 7.2), and 0.1% SDS. Autoradiography was then performed. Densitometric analysis was performed using NIH Image 1.62 software to quantify the results of Northern/Western blot analyses in the linear range of the film. Glyceraldehyde-3-phosphate dehydrogenase and actin were used as internal controls for loading.

Transcriptional Activity and mRNA Half-Life Studies. To determine whether the increase in COX-2 mRNA in colon cancer cells was attributable to an increase in transcription, HT-29 cells were incubated in the presence or absence of Act D (5 μg/ml) for 2 h before exposure to IL-1β, and total RNA was extracted from the cells after treatment with IL-1β for 2 h. Control cells were treated with Act D without IL-1β. To investigate the effect of IL-1β on the half-life of COX-2 mRNA, HT-29 cells were incubated in the presence or absence of IL-1β (10 ng/ml) for 2 h. Additional transcription was blocked by adding Act D (5 μg/ml). Total RNA was extracted from the cells at 0, 0.5, 1.5, 3, 6, and 24 h after the addition of Act D, and Northern blot analysis was done. The half-life of COX-2 mRNA was determined by plotting relative COX-2 mRNA expression levels on a semilogarithmic axis *versus* time (Cricket Software, Malvern, PA).

Extraction of Nuclear Protein and Electrophoretic Mobility Shift Assay. HT-29 cells (80% confluent) were incubated in 5% fetal bovine serum-containing medium overnight and then treated with IL-1β (10 ng/ml) for indicated time points. Nuclear protein extraction and EMSA were performed as described previously (9). EMSA was performed with the Gel Shift Assay System (Promega, Madison, WI). Briefly, oligonucleotide containing the consensus sequence for NF-κB (5′-AGTTGAGGGGACTTTCCCAGG-3′) was end-labeled with [γ-^{32}P]AMP (3000 Ci/mmol; Amersham Corp.) using T4 polynucleotide kinase and was then purified in Microspin G-25 columns (Sigma) and used as probes for EMSA. The gel was dried and subjected to autoradiography. In competition studies, a 100-fold excess of unlabeled oligonucleotide was included in the reaction mixture along with the radiolabeled probe. For supershift experiments, affinity-purified rabbit antibodies (2 μg/reaction) to p50, p65, p52, and c-Rel and nonspecific IgG were incubated in the standard reaction mixture at room temperature for 45 min before the labeled oligonucleotide was added.

RESULTS

Effect of IL-1β Treatment on COX-2 mRNA and Protein Expression in HT-29 Cells. Initially, we sought to determine cytokines or growth factors that increase COX-2 mRNA expression in HT-29 human colorectal cancer cells. Cells were incubated with epidermal growth factor, insulin-like growth factor-I, IL-1β, or TNF-α for various times. Expression of COX-2 mRNA increased after incubation of HT-29 cells with either IL-1β or TNF-α. Expression was highest at 4 h and remained elevated by 24 h (Fig. 1). To confirm that this finding was not restricted to a single cell line, we examined COX-2 expression in a second colon cancer cell line, KM12L4. Similar to results with HT-29 cells, IL-1β and TNF-α increased COX-2 mRNA expression in KM12L4 cells (data not shown).

To additionally examine the effects of IL-1β on COX-2 expression in HT-29 cells, cells were incubated in the presence of IL-1β (10 ng/ml) for 0, 1, 2, 4, 6, 12, or 24 h, and COX-2 mRNA and protein expression were determined. IL-1β increased COX-2 mRNA expression ~6-fold at 1 h (Fig. 2*A*), and it remained increased by at least 4-fold for up to 24 h. Similarly, COX-2 protein expression was increased ~2–3-fold by 1–2 h with IL-1β treatment and increased to 5-fold at 24 h (Fig. 2*B*). To examine the effect of the dose of IL-1β on COX-2 expression, HT-29 cells were incubated in various concentrations of IL-1β for 2 h (COX-2 mRNA) or 4 h (COX-2 protein). COX-2 mRNA expression was increased after incubation of cells with IL-1β at 1 ng/ml, with no additional increase observed at higher concentrations (Fig. 3*A*). COX-2 protein expression was increased after incubation with 1 ng/ml and remained elevated at higher doses of IL-1β (Fig. 3*B*).

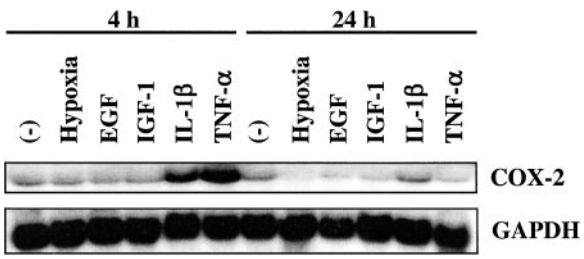

Fig. 1. Expression of COX-2 mRNA by Northern blot analysis in HT-29 cells treated with cytokines, growth factors, or hypoxia exposure. COX-2 mRNA increased after incubation of cells with IL-1β or TNF-α for 4 h.

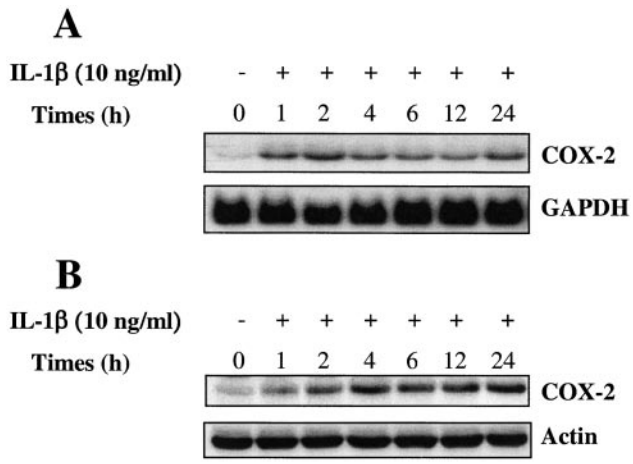

Fig. 2. Time course for induction of COX-2 mRNA and protein by IL-1β in HT-29 cells. HT-29 cells were incubated in the presence of IL-1β (10 ng/ml) for various periods. *A*, COX-2 mRNA (Northern blot) increased after incubation of cells with IL-1β at 1 h, and it remained elevated for at least 24 h. *B*, COX-2 protein (Western blot) increased after incubation of cells with IL-1β by 1–2 h and remained elevated for at least 24 h.

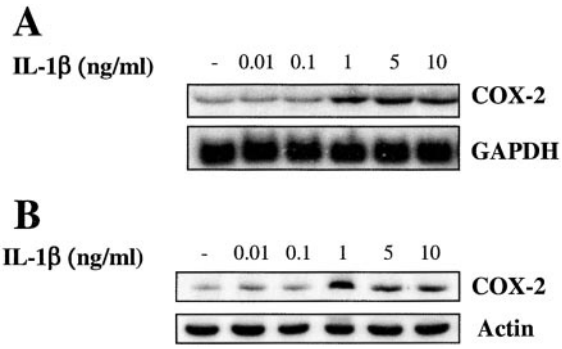

Fig. 3. Effect of IL-1β concentration on induction of COX-2 mRNA and protein in HT-29 cells. HT-29 cells were incubated in various concentrations of IL-1β for 2 h (mRNA) or 4 h (protein), and samples were harvested. *A*, the level of COX-2 mRNA (Northern blot) increased after incubation with IL-1β at 1 ng/ml, with no additional increase at higher concentrations. *B*, COX-2 protein was induced by IL-1β at 1 ng/ml but did not increase additionally at higher concentrations.

Effect of Inhibition of MAPKs and Phosphoinositide 3-Kinase on COX-2 mRNA Expression. To determine the signaling intermediates activated by IL-1β, HT-29 cells were incubated in 10 ng/ml IL-1β for 0, 5, 15, 30, or 60 min, and phosphorylated and total Erk 1/2, JNK, Akt, and P38 MAPK were examined by Western blot analysis. The phosphorylation of Erk 1/2 was induced within 15 min, was highest at 30 min, and returned to the basal level by 60 min (Fig. 4). The phosphorylation of JNK was induced within 30 min and had returned to the basal level by 60 min. Phosphorylation of Akt was essentially unchanged by IL-1β treatment. The phosphorylation of P38 MAPK was not induced by IL-β (Fig. 4).

To identify which of the induced signaling pathways were required

for COX-2 induction by IL-1β, HT-29 cells were pretreated with kinase inhibitors 1 h before treatment with IL-1β (10 ng/ml) for 2 h. We found that PD98059, an inhibitor of MEK1, U0126, an inhibitor of MEK1/2, SP600125, an inhibitor of JNK, and SB203580, an inhibitor of P38 MAPK, blocked IL-1β induction of COX-2 mRNA. In contrast, Wortmannin, a PI3k inhibitor, slightly increased COX-2 expression (Fig. 5*A*).

Recently, it was shown that high concentrations of SB203580 not only inhibited P38 MAPK activity but also inhibited the activity of JNK isoforms (10). SB203580 reduced P38 MAPK activity at doses that did not effect JNK MAPK activity (data not shown; 0.1–1 μM; Refs. 10, 11). To confirm that SB203580 at these doses inhibited P38 MAPK, we treated HT-29 cells with various doses of SB203580 ranging from 0.01 to 25 μM for 1 h before the addition of IL-1β. SB203580 blocked induction of COX-2 by IL-1β at a concentration as low as 0.1 μM (Fig. 5*B*). SB203580 at similar doses inhibited constitutive P38 activation by 40–60% (data not shown).

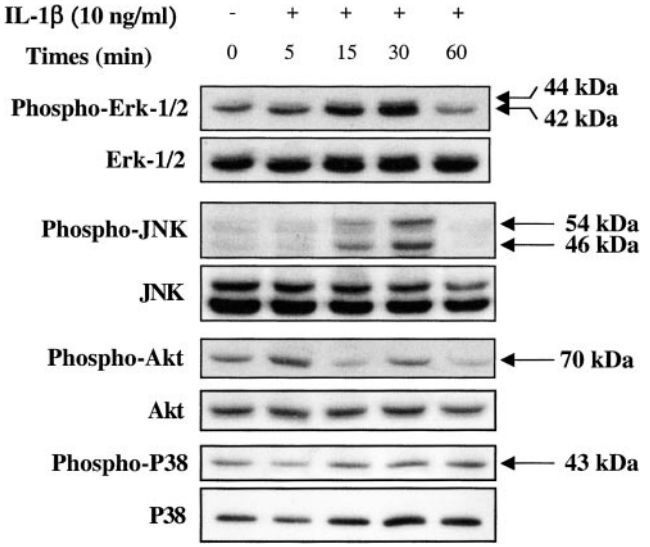

Fig. 4. The phosphorylation of Erk 1/2, JNK, and P38 MAPKs and Akt by IL-1β in HT-29 cells. Cells were exposed to IL-1β (10 ng/ml) for various times and lysed for Western blot analyses of signaling intermediates.

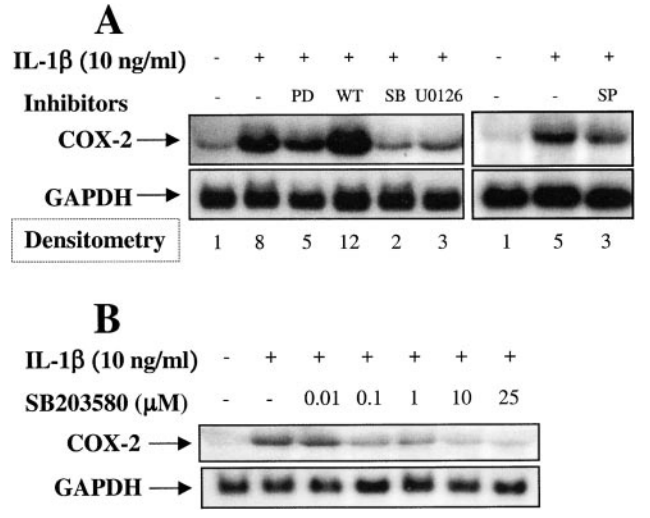

Fig. 5. Effect of inhibition of signaling pathways on COX-2 mRNA expression induced by IL-1β in HT-29 cells. *A*, HT-29 was pretreated with different kinase inhibitors [U0126, PD98059 (PD), SB203580 (SB), SP600125 (SP), and Wortmannin (WT)] 1 h before treatment with IL-1β (10 ng/ml for 2 h). Inhibition of MEK1/2, JNK, and P38 MAPKs blocked induction of COX-2 by IL-1β, and blocking PI3k slightly increased induction of COX-2. *B*, HT-29 cells were treated with various doses of SB (0.01–25 μM) for 1 h before the addition of IL-1β. COX-2 induction by IL-1β was blocked by SB at a concentration as low as 0.1 μM.

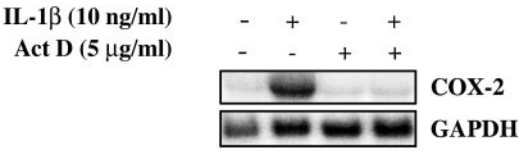

Fig. 6. Effects of IL-1β on transcriptional activity. HT-29 cells were incubated in the presence or absence of Act D (5 μg/ml) 2 h before exposure to IL-1β. Induction of COX-2 mRNA expression by IL-1β (Northern blot) was blocked by Act D.

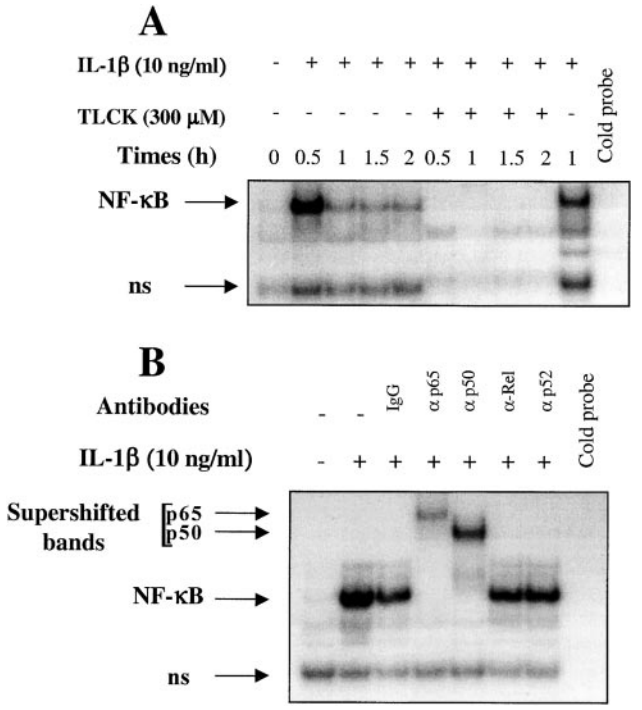

Fig. 7. Effect of IL-1β on induction of NF-κB DNA binding. *A*, activation of NF-κB binding by IL-1β in HT-29 cells was inhibited with NF-κB-specific inhibitor (EMSA). Formation of NF-κB complexes was induced with IL-1β treatment. The NF-κB binding complex was inhibited by the NF-κB inhibitor TLCK. Unlabeled NF-κB consensus oligonucleotide 100-fold in excess was added as a cold competitor. *B*, supershift of NF-κB binding complexes was demonstrated by anti-p65 and anti-p50 antibodies. Nuclear proteins extracted from HT-29 cells stimulated with IL-1β were treated with anti-p65, anti-p50, anti-Rel, or anti-p52 antibodies or nonspecific rabbit IgG and analyzed for NF-κB binding complexes. ns, nonspecific.

Effects of IL-1β on Transcriptional Activity and mRNA Half-Life. To determine the mechanism by which IL-1β induced COX-2 mRNA expression, transcription was blocked with Act D in HT-29 cells before the addition of IL-1β. This blockade of transcription completely abolished induction of COX-2 mRNA by IL-1β (Fig. 6). To determine the effect of IL-1β on the half-life of COX-2 mRNA, HT-29 cells were incubated in the presence or absence of IL-1β for various times, and additional transcription was blocked with Act D. The half-life of COX-2 mRNA was similar to that in cells treated with IL-1β as in controls (data not shown).

Effect of IL-1β on NF-κB Activation and Effect of Inhibition of NF-κB on COX-2 mRNA Expression. To determine whether IL-1β led to activation of NF-κB, EMSA was performed on nuclear extracts from HT-29 cells incubated with IL-1β (10 ng/ml) for 0, 30, 60, 90, or 120 min, and the consensus oligonucleotides for the NF-κB binding sites were used as labeled probes. The transcription factor NF-κB was translocated by IL-1β treatment at 30 min. Activation of NF-κB was inhibited by the NF-κB-specific inhibitor TLCK (Fig. 7*A*). Supershift

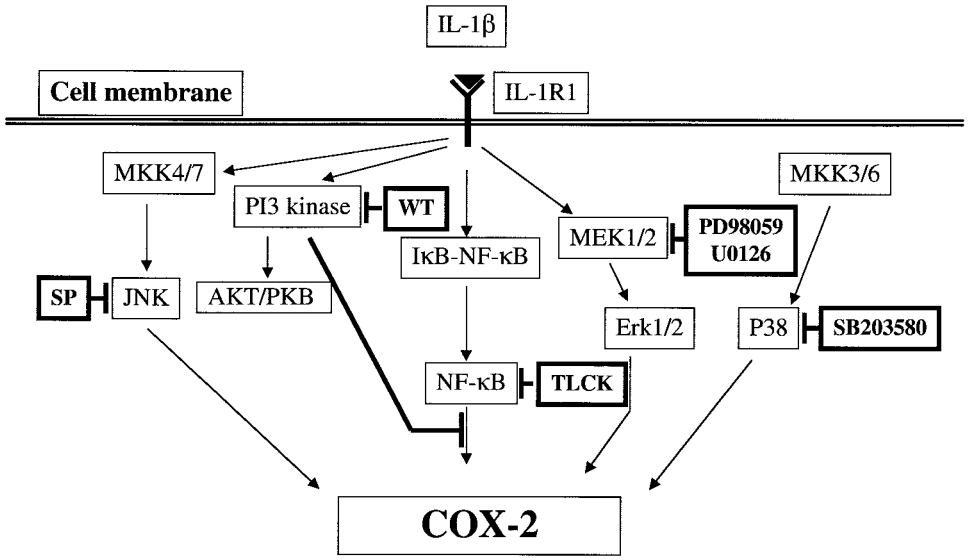

Fig. 8. Possible intracellular signaling pathways for IL-1β induced COX-2 expression in HT-29 cells. Note that IL-1β does not lead to activation of P38, but P38 constitutive activity is important for COX-2 basal and inducible expression.

assay indicated that both NF-κB subunits, p65 and p50, were induced by IL-1β (Fig. 7*B*).

To determine the role of the NF-κB pathway in COX-2 mRNA induction, HT-29 cells were pretreated with three NF-κB inhibitors (TLCK, *N*-α-tosylphenylalanyl-chloromethyl ketone, and pyrrolidine dithiocarbamate) 1 h before treatment with IL-1β. All three NF-κB inhibitors blocked COX-2 induction in a dose-dependent fashion (data not shown).

DISCUSSION

COX-2 is an inducible isoenzyme that can be up-regulated in numerous pathologic conditions, including inflammation and cancer. COX-2 is thought to play an important role in colorectal cancer progression (12, 13). Kargman *et al.* (14) showed that COX-2 was detected in human colon tumors but was not detectable in noncancerous colon tissues from the same patient. In contrast, COX-1 is a constitutively expressed protein involved in eicosanoid metabolism and homeostasis in almost all tissues (15, 16). COX-2 overexpression has been associated with resistance to apoptosis that leads to phenotypic changes and changes in that increase the metastatic potential of colorectal cancer (5, 17). Although COX-2 is not a direct angiogenic factor, it does induce production of specific prostaglandins that lead to induction of vascular endothelial growth factor and possibly other angiogenic factors (6). In this study, we used HT-29 human colon cancer cells to analyze COX-2 induction by IL-1β and to determine the mechanisms by which this occurs.

IL-1β is a potent immunoregulatory and proinflammatory cytokine. Recently, IL-1β levels were associated with macrophage infiltration and angiogenin levels in human colon cancer (18). Two basic IL-1 receptors have been identified. The IL-1 type I receptor initiates signal transduction upon binding its ligand (19), whereas the type II receptor, which lacks an intracellular signaling domain, does not transduce a signal. We have previously shown by using reverse transcriptase-PCR that both HT-29 and KM12L4 colon cancer cells express the mRNA for the type I receptor (data not shown).

IL-1β signaling involves numerous intracellular mediators, including the Erk 1/2, JNK, P38 MAPK, and PKB/Akt pathways (20, 21). Several signaling pathways have been implicated in the regulation of COX-2 expression by IL-1β. In human gastric cancer cells, both P38 MAPK and Erk 1/2 are upstream signaling intermediates that regulate induction of COX-2 expression by IL-1β (22). Regulation of COX-2 induction is also dependent on the activation of protein kinase C in human endothelial cells (23). Guan *et al.* (24) have demonstrated that JNK/stress-activated protein kinase and P38 MAPK signaling cascades are required for IL-1β-induced COX-2 expression and prostaglandin E_2 synthesis in rat renal mesangial cells. To determine the signaling pathways that mediate induction of COX-2 by IL-1β in HT-29 human colon cancer cells, we determined the signaling intermediates that were activated upon IL-1β induction. We then used pharmacological inhibitors of various signaling pathways to determine whether blockade of the activity of specific pathways would block induction of COX-2 by IL-1β. We found that inhibition of Erk 1/2, JNK, and P38 MAPK signaling pathways blocked IL-1β COX-2 expression. It has previously been demonstrated that activation of NF-κB leads to induction of COX-2 in macrophages, colon, and lung epithelial cells (25–27). Consistent with this finding, our data indicate that the use of NF-κB inhibitors can block COX-2 induction by IL-1β.

In the HT–29 colon cancer cell line that we used in the current study, others have shown that inhibition of PI3k can activate NF-κB binding ability (28). Our studies demonstrated that the PI3k inhibitor Wortmannin led to induction of COX-2 mRNA (Fig. 5*A*), and IL-1β activated both the PI3k/Akt and NF-κB pathways in HT-29 colon cancer cells. On the basis of the above, the PI3k pathway might negatively regulate COX-2 expression. The blockade of the PI3k pathway may increase NF-κB binding, which in turn may up-regulate COX-2 expression.

In conclusion, our study demonstrates that multiple signaling pathways are involved in COX-2 induction by IL-1β, including the Erk 1/2, JNK, and P38 MAPK. Although IL-1β did not lead to P38 activation, COX-2 induction by IL-1β was dependent upon constitutive P38 MAPK activity as an inhibitor of P38 activity blocked COX-2 induction by IL-1β (Fig. 8). Our study also demonstrated that the transcription factor NF-κB is involved in COX-2 induction by IL-1β. Thus, targeting the pathways that mediate COX-2 may be useful as a component of therapeutic strategies in patients with colon cancer.

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