Expression of Cyclooxygenase-2 Is Regulated by Glycogen Synthase Kinase-3β in Gastric Cancer Cells*

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Cyclooxygenase-2 (COX-2) expression is a marker of poor prognosis in gastric cancer patients, and its inhibition suppresses gastric tumorigenesis in experimental animal models. The mechanism that leads to COX-2 overexpression in this tumor type is unknown. We have now shown that inhibition of phosphatidylinositol 3-kinase by LY294002 suppresses both basal and phorbol myristate acetate-induced COX-2 expression in TMK-1 and MKN-28 gastric cancer cells. Furthermore, inhibition of glycolgen synthase kinase-3β (GSK-3β) by SB415286 induced expression of COX-2 mRNA and protein as well as the enzyme activity in the gastric cancer cells. The effect of SB415286 was confirmed by the use of two additional GSK-3β inhibitors, lithium chloride and SB216763. SB415286 had a modest 1.6-fold stimulatory effect on a 2-kb COX-2 promoter reporter construct, but more importantly, it was shown to block the decay of COX-2 mRNA. In contrast to modulation of phosphatidylinositol 3-kinase/Akt/GSK-3β pathway, inhibitors of mitogen-activated protein kinases (MEK 1/2, p38, JNK) or the mammalian target of rapamycin did not alter COX-2 expression in gastric cancer cells. Our data show that inhibition of GSK-3β stimulates COX-2 expression in gastric cancer cells, which seems to be primarily facilitated via an increase in mRNA stability and to a lesser extent through enhanced transcription.

Gastric cancer is the second most lethal cancer worldwide (1), and risk factors for this disease include diet, Helicobacter pylori infection, and genetic background (2, 3). There exist two major histological entities of gastric adenocarcinoma, i.e. intestinal and diffuse types, which exhibit distinct epidemiological, etiological, and genetic properties. Use of aspirin and other nonsteroid anti-inflammatory drugs reduces the risk for gastrointestinal malignancies, among them gastric cancer (4–8). Cyclooxygenase (COX) enzyme, the primary target of nonsteroid anti-inflammatory drugs, catalyzes the conversion of arachidonic acid (AA) to prostanooids that are further metabolized to various biologically active prostanoids by the various synthase/isomerase enzymes. Two distinct genes encode COX enzymes, referred to as COX-1 and COX-2. Expression of COX-1 is constitutive in most tissues, whereas expression of COX-2 is considered to be inducible by polypeptide growth factors, mediators of inflammation, and carcinogens. The major prostanooid produced by the epithelium and by malignancies derived from this cell type is prostaglandin E2 (PGE2) (9, 10). There exist three distinct types of PGE2 synthases (PGES), i.e. microsomal PGES (mPGES) 1 and 2 and cytosolic PGE2 (11), that have been identified in gastric cancer cells (12, 13).

Altered COX-2 gene expression has been observed in a variety of human cancers (5, 8), including gastric cancer (7, 14). Furthermore, COX-2 expression is an independent prognostic factor in gastric cancer patients (15, 16). In mice, transgenic overexpression of COX-2 and mPGES-1 leads to formation of hyperplastic gastric tumors (17). Moreover, a selective COX-2 inhibitor, celecoxib, suppresses gastric tumor formation in trefoil factor 1-deficient mice (18) and in a chemically induced model in rats (19).

Pathways linked to Ras, protein kinase C, mitogen-activated protein kinases, and PI3K/Akt have been shown to modulate COX-2 expression in colorectal, breast, and other cancer cells (20, 21). It is, however, unknown whether these pathways regulate expression of COX-2 in gastric cancer. Growing evidence indicates that COX-2 overexpression is a result of both increased transcription and enhanced mRNA stability (22–25). In the present study, we investigated the signaling pathways that lead to increased COX-2 expression in gastric cancer cells and examined the molecular mechanisms underlying COX-2 overexpression.

EXPERIMENTAL PROCEDURES

Cell Culture—Gastric adenocarcinoma cell lines originated either from intestinal-type (MKN-28) or from diffuse-type (TMK-1) tumors (a kind gift from Hiroshi Yokozaki, Kobe University Graduate School of Medicine, Kobe, Japan) (26). The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (PromoCell GmbH, Heidelberg, Germany), 2 mM L-glutamine, and antibiotics (Bio Whittaker) and maintained at 37 °C at 5% CO2 in air. Once these cells had reached ~80% confluence, experiments were performed on fresh culture medium or medium containing 0.5% fetal calf serum.

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and interleukin-1β (10 ng/ml) was from R&D Systems Inc. (Minneapolis, MN). The cells were treated with or without the signal transduction inhibitors with or without addition of PMA or interleukin-1β.

**RNA Isolation and Northern Blot Analysis**—Total RNA was extracted with the TRIzol reagent (Invitrogen) and analyzed by Northern blotting as previously described (14, 22). Loading was controlled by staining of ribosomal 28S RNA with ethidium bromide.

**Real-time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**—RNA was extracted with the TRIzol reagent (Invitrogen), and 1 μg of total RNA was converted to cDNA with Moloney-murine leukemia virus reverse transcriptase (Promega, Madison, WI) by random primers (Invitrogen) in a volume of 50 μl for 1 h at 37 °C. The reaction was heat inactivated (95 °C, 3 min) and filled to a final volume of 200 μl. Real-time PCR reactions were performed with the Gene Amp® 5700 Sequence detection system by use of Assays-on-Demand™ gene expression products according to the vendor’s protocol (Applied Biosystems, Foster City, CA). In brief, for each reaction 0.75–1.5 μl of cDNA template were mixed with 1.25 μl of COX-2 primers and probes labeled with a reporter dye (PTGS2, Assays-on-Demand®). TaqMan® Universal PCR Mastermix (12.5 μl) and RNase-free water were added to a final volume of 25 μl. Human TATA box-binding protein or 18 S rRNA served as an endogenous control to account for differences in reverse transcription of total RNA. PCR cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C denaturation for 15 s and 60 °C annealing/extension for 1 min. Each sample was measured in triplicate, and data were analyzed by the delta-delta method for comparing relative expression results (ratio, 2−ΔΔCT sample−ΔΔCT control) (27).

**Protein Extraction and Western Blot Analysis**—Proteins were extracted and subjected to Western blot analysis as described earlier (12). In brief, 30–70 μg of total protein extracts were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked (4 °C, overnight) with Tris-buffered saline-Nonidet P-40 containing 5% nonfat milk powder and then incubated (1 h, room temperature) with mouse monoclonal anti-human-COX-2 (1:1000, for TMK-1 cells), mouse monoclonal anti-COX-1 (1:500), rabbit polyclonal anti-mPGES-1, anti-mPGES-2, anti-cytosolic PGES (1:1000) (Cayman Chemical Co., Ann Arbor, MI), goat polyclonal anti-β-actin (1:500) or goat polyclonal anti-human-COX-2 (1:1000, for MKN-28 cells) (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse monoclonal anti-phospho- and total COX-2 antibodies (1:1000; Amersham Biosciences), or donkey anti-goat (1:2000; Santa Cruz) antibodies conjugated to horseradish peroxidase for 1 h at room temperature. The proteins were visualized by enhanced chemiluminescence (ECL Western blotting analysis system; Amersham Biosciences) or with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and the Multimage™ FC Light Cabinet/FluorChem™ 8800 digital imaging system (Alpha Innotech Corp., San Leandro, CA).

**Akt Kinase Activity Assay**—TMK-1 cells were cultured in 25-cm² cell culture flasks until 80% confluence. The cells were incubated in plain culture medium or with LY294002 (20 μM) for 24 h. Cell extracts were made according to the manufacturer’s protocol (Akt activity assay kit; Calbiochem). Briefly, TMK-1 cells were trypsinized, pelleted, washed once with ice-cold phosphate-buffered saline, and lysed in ice-cold kinase extraction buffer. The pellet was incubated on ice for 5 min and then centrifuged at 13,000 × g at 4 °C for 10 min. The protein concentration was measured with the BCA protein assay kit (Pierce). Akt antibody (2 μl) was first added to the cell lysates containing a total of 300 μg of protein extract and incubated in continuous rotation for 45 min at room temperature. Akt-antibody complexes were collected by rotating them with protein-A agarose for 1 h at room temperature. The immunocomplexes were pelleted and washed twice with the kinase extraction buffer and once with kinase assay buffer provided by the manufacturer (Calbiochem). The immunoprecipitate was then incubated with a recombinant GSK-3α protein in the presence of ATP at 30 °C for 3 h, after which 30 μl of the supernatant was collected and resuspended in 4 × SDS sample buffer. Immunodetection was carried out by the same protocol as described for phospho-Akt but by use of an antibody specific for phospho-Ser-21 GSK-3α at a dilution of 1:1000 (Santa Cruz Biotechnology). Equal loading was assayed by use of rabbit polyclonal anti-GSK3α (Santa Cruz Biotechnology) at a dilution of 1:500. For both these primary antibodies, horseradish peroxidase-conjugated secondary antibody raised against rabbit was used at a dilution of 1:2000 (Amersham Biosciences).

**PGE₂ Measurement**—The cells were plated on 6- or 12-well cell culture dishes. These cultures were first preincubated with or without a selective COX-2 inhibitor NS-398 (5 μM; Cayman Chemical), PI3K inhibitor LY294002 (20 μM), or GSK-3β inhibitor SB415286 (20 μM) for 2 h in 1–2 ml of the culture medium, after which incubation was continued with or without PMA (10 ng/ml) for 6–24 h. The medium was then removed, and the cells were washed once with RPMI 1640 and incubated further with 0.5–1 ml of AA (10 μM; Sigma-Aldrich) in RPMI 1640 for 15–20 min. PGE₂ was analyzed by using an enzyme immunoassay according to the protocol supplied by the manufacturer (Cayman Chemical).

**Transfection of Reporter Constructs**—We used the COX-2 promoter (1840 bp upstream to 123 bp downstream of the transcriptional start site) in a pGL3-Basic vector (Promega) (28) and a pRL-TK Renilla luciferase reporter construct as a transfection control (Promega). For transient transfection, cells were plated on 24-well tissue culture clusters at a split ratio of 1/5 to 1/10 and grown overnight. COX-2 promoter construct (0.2 μg) was co-transfected with the Renilla construct (0.04 μg) with FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer’s protocol. Briefly, FuGENE reagent was added to serum-free RPMI 1640 and incubated with plasmid DNA for 30 min at room temperature. A transfection reaction mixture was gently added to the cells containing 0.5 ml of complete growth medium. The transfection medium was removed after 4 h, and the cells were grown for 18 h in serum-reduced medium (0.5% fetal calf serum) containing the test agents. At the end of the incubation period, the transfected cells were washed once with phosphate-buffered saline, and total proteins were
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lysed in passive lysis buffer (100 μl/well) provided by the Dual-Luciferase Reporter Assay system (Promega). Firefly and Renilla luciferase activities were measured from the lysate by the Dual-Luciferase Reporter Assay system, and luminescence was measured with a DCR-1 luminometer (DGENE Corp., Gaithersburg, MD). Firefly luciferase values were normalized to Renilla luciferase values to control for transfection efficiency.

FIGURE 1. Expression of COX and PGES enzymes and synthesis of PGE2 in gastric adenocarcinoma cell lines. A, TMK-1 and MKN-28 cells were incubated with or without PMA (10 ng/ml) for 24 h, after which they were incubated further with AA (10 μM) for 20 min. PGE2 was measured from AA medium from two separate experiments (n = 6) by enzyme immunoassay. Results are means ± S.E. *, p < 0.05 versus control. B, TMK-1 cells were preincubated with or without NS-398 (5 μM) for 2 h and then further with or without PMA for 6 h, after which the cells were treated with AA. PGE2 was measured from AA medium (n = 6). Inhibition was 89.3% for control and 89.6% under PMA-induced conditions. C, TMK-1 cells were incubated with PMA (10 ng/ml) for the indicated time periods, after which either total RNA or protein was extracted. PMA stimulated expression of COX-2 mRNA and protein as detected by Northern blot and Western blot analysis, respectively. Ribosomal 28 S RNA and β-actin protein served as loading controls. D, expression of COX-1 and PGES enzymes in TMK-1 cells. TMK-1 cells incubated with or without PMA (10 ng/ml) for 6–48 h and expression of cytosolic PGES, mPGES-1, mPGES-2, COX-1, and β-actin were measured by Western blot analysis.

RESULTS

We have recently characterized the expression of COX and PGES enzymes in human gastric cancer cells of the MKN series (12). We chose the MKN-28 cell line for further studies because >90% of its COX activity could be contributed to COX-2 and because PMA induced this MKN cell type the highest induction of COX-2 protein expression and PGE2 synthesis. Here we describe another gastric cancer cell line, TMK-1, that produced relatively high basal PGE2 levels, which were further increased by PMA (Fig. 1A). The effects of PMA appeared to be independent of phospholipase A2 activation, because it induced a 12.9-fold increase in PGE2 production during a 24-h incubation period, which was comparable with a 10.8-fold induction when these cells were exposed to exogenous AA for 20 min after the initial 24-h incubation period. Furthermore, because the selective COX-2 inhibitor NS-398 (5 μM) inhibited ~90% of AA-induced PGE2 release (Fig. 1B), our results show that the principal COX isoenzyme in TMK-1 cells is COX-2. PMA stimulated expression of COX-2 mRNA and protein in TMK-1 cells, and the peak steady-state level of the transcript was reached earlier (2–6 h) than that of the protein (6–24 h) (Fig. 1C). However, PMA did not modulate expression of COX-1 or any of the PGES enzymes tested (Fig. 1D, cytosolic PGES, mPGES-1, and mPGES-2). This suggests that COX-2 is the key enzyme in production of PGE2 in TMK-1 cells in both basal and PMA-induced conditions.

Next, we investigated signal transduction pathways that could lead to COX-2 expression in gastric cancer cells. The PI3K inhibitor LY294002 suppressed both basal and PMA-induced expression of COX-2 protein in TMK-1 and MKN-28 cells (Fig. 2, A and B). As shown in Fig. 2C, LY294002 also inhibited synthesis of PGE2, confirming that not only steady-state levels of the COX-2 protein but also the biologically active pool of COX-2 enzyme is reduced by treatment with the PI3K inhibitor. Consistent with our data suggesting that induction of COX-2 protein
expression is dependent on modulation of COX-2 transcript levels (Fig. 1C), we found that PMA-induced COX-2 mRNA expression was inhibited by LY294002 in TMK-1 (2.7-fold) and MKN-28 (4.6-fold) cells as detected by real-time RT-PCR (Fig. 2, D and E). However, the basal COX-2 expression was affected only in MKN-28 cells (1.8-fold reduction; Fig. 2E). As PMA is known to exert its effects through protein kinase C activation, the protein kinase C inhibitor bisindolylmaleimide I was used. In contrast to the effect observed with PMA, LY294002, bisindolylmaleimide I inhibited only the PMA-induced COX-2 expression but did not suppress basal COX-2 expression (Fig. 3A). We also examined the contribution of mitogen-activated protein kinase kinase signaling and found that inhibition of JNK by JNK Inhibitor II did not inhibit COX-2 expression (Fig. 3B). Furthermore, inhibition of MEK 1/2 by UO126 (or PD98059, data not shown) and p38 (SB203580, 10 μM) had no effect on COX-2 expression. C, inhibitors for MEK 1/2 (UO126, 10 μM) and p38 (SB203580, 10 μM) or a combination of these two inhibitors did not reduce basal or PMA-induced COX-2 expression.

To better define the PI3K/Akt pathway in TMK-1 cells, we investigated the phosphorylation status and activity of Akt at basal conditions in these cells. We observed constitutive phosphorylation of Akt at Ser-473 and Thr-308, and Ser-473 phosphorylation was inhibited by LY294002 (20 μM; Fig. 4A). We also measured the ability of immunoprecipitated Akt to phosphorylate an Akt substrate, glycogen synthase kinase (GSK)-3α, and found that Akt activity was inhibited by LY294002 (Fig. 4B). Furthermore, we found that the expression of phosphatase and tensin homologue, an inhibitor of the PI3K/Akt pathway, was almost undetectable in TMK-1 cells (Fig. 4C), which could explain the high basal levels of pAkt as shown in Fig. 4A.

As the PI3K/Akt signaling pathway seems to play a pivotal role in COX-2 expression in gastric cancer cells, we next investigated downstream factors of this pathway (Fig. 5). The GSK-3β inhibitor SB415286 (20 μM) stimulated both basal and PMA-induced COX-2 protein expression in TMK-1 and MKN-28 cells (Fig. 6A). Rapamycin, which inhibits the mammalian target of rapamycin (mTOR), did not alter basal or PMA-induced COX-2 protein expression in either cell line (30 nM; Fig. 6B). Furthermore, the induction of COX-2 protein expression by SB415286 was dose dependent (Fig. 7A), and two additional GSK-3β inhibitors, SB216763 (20 μM) and lithium chloride (30 mM), enhanced COX-2 protein expression (Fig. 7, B and C). In agreement with the
protein expression data, basal mRNA levels of COX-2 increased following treatment with SB415286 by 1.9-fold in TMK-1 cells and by 4.4-fold in MKN-28 cells (Fig. 8, A and B). Similarly, SB415286 enhanced PMA-induced COX-2 mRNA expression in TMK-1 and MKN-28 cells by 2.2- and 2.8-fold, respectively. As shown in Fig. 8, C and D, basal and PMA-induced PGE2 production were also induced after incubation with SB415286 (by 3.3- and 1.5-fold in TMK-1 cells and by 2.9- and 1.9-fold in MKN-28 cells, respectively).

Expression of COX-2 can be regulated at both transcriptional and post-transcriptional mRNA stability levels (22–25). To address the aspect of GSK-3β-mediated signaling on COX-2 transcriptional regulation, we investigated the effect of SB415286 on expression of the COX-2 promoter reporter. Our results show that SB415286 led to a relatively modest (1.6 ± 0.14-fold) increase in COX-2 promoter activity, whereas PMA increased it by 4.5 ± 0.58-fold (Fig. 9A). The combination of SB415286 and PMA did not further induce the COX-2 promoter when compared with PMA alone (p = 0.26). The COX-2 3′-untranslated region contains elements related to the stability of the COX-2 transcript (22, 23, 25, 30). To determine whether GSK-3β induced COX-2 expression through a post-transcriptional mRNA stability mechanism, we investigated the effect of SB415286 on decay of the COX-2 mRNA. As shown in Fig. 9B, inhibition of GSK-3β led to stabilization of the COX-2 transcript, whereas PMA slightly accelerated the decay of the transcript.

**DISCUSSION**

COX-2 expression, but not that of COX-1 or PGES enzymes, is responsible for PMA-induced PGE2 synthesis in TMK-1 gastric cancer cells. In addition, the great majority (90%) of basal COX enzyme activity was due to COX-2 in this cell type. Here we studied several putative signaling pathways that could lead to up-regulation of COX-2 expression. Our results indicate that the mitogen-activated protein kinase pathways that are frequently activated in colorectal cancer cells and that can induce COX-2 expression (25, 31) did not have an effect on COX-2 expression in gastric cancer cells. Furthermore, although PMA induction was blocked by the protein kinase C inhibitor, it did not have any effect on the relatively high basal COX-2 expression in TMK-1 cells. In contrast, inhibition of GSK-3β played a central role in COX-2 expression in both diffuse-type (TMK-1) and intestinal-type (MKN-28) gastric cancer cells. This conclusion is supported by our data that show that three distinct inhibitors of GSK-3β induced COX-2 expression and that suppression of the PI3K activity led to an opposite effect.

Contradictory data exist as to the role of the PI3K/Akt pathway in COX-2 expression. Similar to our data, in Ras-transformed rat intestinal epithelial cells and in ultraviolet B radiation-treated human keratinocytes, inhibition of PI3K/Akt pathway led to regulation of COX-2 expression. Similar to our data, in Ras-transformed rat intestinal epithelial cells and in ultraviolet B radiation-treated human keratinocytes, inhibition of PI3K/Akt pathway led to...
transcription of a COX-2 promoter (construct length human keratinocytes and in colon cancer cells (construct length expression, we could conclude that mTOR is not involved. Another downstream target of Akt in addition to GSK-3 

Because the PI3K inhibitor LY294002 can also inhibit mTOR (36), induction-dependent differences in regulation of COX-2 expression, we could conclude that mTOR is not involved. Consistent with our findings, inhibition of GSK-3β led to up-regulation of COX-2 in human keratinocytes and in renal medullary interstitial cells (33, 37, 38). However, in certain conditions inhibition of GSK-3β can lead to inhibition of COX-2 expression, because fibroblasts that lack GSK-3β lose their responsiveness to tumor necrosis factor-α in respect to NF-κB-mediated COX-2 induction (39). Furthermore, GSK-3β inhibitors induced transcription of a COX-2 promoter (construct length -327/+59) in human keratinocytes and in colon cancer cells (construct length -1432/+59) (33, 40). In contrast to these previous studies, inhibition of GSK-3β in gastric cancer cells induced only a modest (1.6-fold) increase in COX-2 transcription (construct length -1840/+123), which cannot fully explain the increase found in COX-2 mRNA and protein levels. To this end, we performed mRNA stability studies. In TMK-1 gastric cancer cells the untreated COX-2 mRNA did not reach its half-life after 60 min of incubation with actinomycin D, which is in agreement with colon cancer cells or with serum-stimulated human lung fibroblasts, where the half-life of COX-2 ranged from 80 min to >2 h (23, 25). Furthermore, our studies showed COX-2 mRNA to be stabilized by the GSK-3β inhibitor treatment. In fact, a 2-h incubation with SB415286 almost completely blocked degradation of the COX-2 transcript. To our knowledge this is the first time that modulation of GSK-3β activity has been linked to regulation of mRNA stability. In mammary cancer cells, serum withdrawal induces post-transcriptional stabilization of COX-2 mRNA involving the p38 pathway (24), and the PI3K/Akt pathway in concert with the MEK/extracellular signal-regulated kinase pathway acts similarly in rat intestinal epithelial cells (32).

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