Induced microsomal PGE synthase-1 is involved in cyclooxygenase-2-dependent PGE2 production in gastric fibroblasts

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—We have previously shown that the cyclooxygenase (COX)-2/PGE2 pathway plays a key role in VEGF production in gastric fibroblasts. Recent studies have identified three PGE synthase (PGES) isozymes: cytosolic PGES (cPGES) and microsomal PGES (mPGES)-1 and -2, but little is known regarding the expression and roles of these enzymes in gastric fibroblasts. Thus we examined IL-1β-stimulated mPGES-1 and cPGES mRNA and protein expression in gastric fibroblasts by quantitative PCR and Western blot analysis, respectively, and studied both their relationship to COX-1 and -2 and their roles in PGE2 and VEGF production in vitro. IL-1β-stimulated increases in both COX-2 and mPGES-1 mRNA and protein expression levels. However, COX-2 mRNA and protein expression were more rapidly induced than mPGES-1 mRNA and protein expression. Furthermore, MK-886, a nonselective mPGES-1 inhibitor, failed to inhibit IL-1β-induced PGE2 release at the 8-h time point, while totally inhibiting PGE2 at the later stage. However, MK-886 did inhibit IL-1β-stimulated PGE2 activity in vitro by 86.8%. N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide (NS-398), a selective COX-2 inhibitor, totally inhibited PGE2 production at both the 8-h and 24-h time points, suggesting that COX-2-dependent PGE2 generation does not depend on mPGES-1 activity at the early stage. In contrast, NS-398 did not inhibit VEGF production at 8 h, but only partially at 24 h, whereas MK-886 totally inhibited VEGF production at each time point. These results suggest that IL-1β-induced mPGES-1 protein expression preferentially coupled with COX-2 protein at late stages of PGE2 production and that IL-1β-stimulated VEGF production was totally dependent on membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) superfamily proteins, which includes mPGES-1, but was partially dependent on the COX-2/PGE2 pathway.

interleukin-1β; MK-886; MAPEG superfamily; prostaglandin E synthase activity

PROSTAGLANDINS (PGs), potent lipid mediators during pain and inflammation, are derived through the cyclooxygenase (COX)-catalyzed conversion of arachidonic acid to PGH2 (5, 9, 25, 33). PGs also play a physiological role in maintaining the integrity of the gastric mucosa, in which COX-1 is constitutively expressed (12, 30, 35). In addition, we have shown in previous studies that COX-2 is expressed in fibroblasts beneath the gastric ulcer bed in humans (20, 45) and that N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide (NS-398), a selective COX-2 inhibitor, delays gastric ulcer healing in mice (21), suggesting that COX-2 is also important for gastric mucosal integrity (7, 31). Furthermore, in a more recent study, we were able to show in vitro that gastric fibroblasts express COX-2 and produce COX-2/PGE2-dependent VEGF in response to IL-1β, a proinflammatory cytokine (20).

Recently, three different PGE synthase (PGES) isozymes have been identified: cytosolic PGES (cPGES) (44), microsomal PGES (mPGES)-1 (11, 18, 23), and mPGES-2 (22, 43). cPGES is constitutively and ubiquitously expressed and selectively coupled with COX-1 (44). mPGES-1 is induced by proinflammatory stimuli, downregulated by anti-inflammatory glucocorticoids, and functionally coupled with COX-2 in marked preference to COX-1 (11, 18, 23). mPGES-1 is a member of the membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) superfamily that includes other proteins involved in arachidonic acid metabolism, such as 5-lipoxygenase-activating protein (FLAP) and leukotriene C4 synthase (10, 11, 18, 23). Induced expression of mPGES-1 has been postulated to be associated with various pathophysiological events in which COX-2-derived PGE2 has been implicated, such as rheumatoid arthritis (38), febrile response (47), reproduction (3, 15), bone metabolism (23), and Alzheimer’s disease (36). mPGES-2 does not show homology with mPGES-1 and has a unique NH2-terminal hydrophobic domain and a glutaredoxin-like domain (22, 43), although its cellular function has yet to be addressed.

Thus in this study, to clarify the role of cPGES and mPGES-1 in PGE2 production in human gastric fibroblasts, we tested the effect of IL-1β on cPGES and mPGES-1 patterns of expression, compared with that on COX-1 and -2. We also examined the effect of these enzymes on the production of VEGF, a factor known to be involved in angiogenesis and gastric epithelial restitution in the course of ulcer repair (19, 20, 39, 40).

MATERIALS AND METHODS

Reagents. 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC-560) was purchased from Calbiochem (Darmstadt, Germany). NS-398 was kindly provided by Taisho Pharmaceutical (Tokyo, Japan). The MAPEG inhibitor 3-[1-(p-chlorobenzyl)-5-(isopropyl)-3-t-butylinomindol-2-yl]-2,2-dimethylpropanoic acid, sodium (MK-886) was obtained from Toronto Research Chemicals.

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(North York, Canada). IL-1β was from Genzyme-Technie (Minneap-
olis, MN); pepstatin A, PMSF, and 3-[(cholamidopropyl)dimethyl-
ammonio]-1-propanesulfonate (CHAPS) were from Sigma-Aldrich
(St. Louis, MO). Mouse anti-human mPGES-1 monoclonal antibody
was a generous gift from Dr. Y. Urade (Osaka Bioscience Institute;
Osaka, Japan). Commercial antibodies included rabbit anti-human
COX-1 (C24) and -2 (C31) polyclonal antibodies (ImmuNo-Bio-
logical Laboratories; Fujioka, Japan); mouse anti-human β-actin
antibody (Sigma-Aldrich); rabbit anti-human cPGES antibody (Cayman
Chemical; Ann Arbor, MI); horseradish peroxidase (HRP)-conjugated
donkey anti-rabbit IgG and sheep anti-mouse IgG (Amersham Bio-
sciences; Piscataway, NJ); and fluorescein-conjugated horse anti-
mouse IgG and Texas Red-conjugated goat anti-rabbit IgG from
Vector Laboratories (Burlingame, CA).

Cell culture and stimulation by IL-1β. Gastric fibroblasts (Hs 262.
St), derived from a gastric ulcer patient, were obtained from American
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St), derived from a gastric ulcer patient, were obtained from American
IL-1β/H9252
Primer sequences for real-time quanitative PCR
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COX2 U04636
5'-AAA TGG CTG GGA GGG TGT CT-3'
5'-cac cct GAT ACT TTC TGT ACT GCG GGT G-3'
5'-ATG CCT GTC TGG CAG TGC T-3'
5'-cag tca AAG CAG CCC CGG CCG TGA GTG-3'
5'-TTT CCT GCT GGG CCG TGT-3'
5'-gac cta TGG GGC AGG GGT TAG GTC-3'
5'-AAG ACT GGG AAG ATG ATT CAG ATG-3'
5'-gaa csa ccc TCA TCA OCA CCC CCA ATG TGT TGC-3'

All cDNA sequences were obtained from the GenBank database. Each fluorogenic LUX primer is labeled with an FAM at the 3′ end and has a short tail of
4–6 nucleotides on the 5′ end that is complementary to the 3′ end of the primer. The bold T designates the labeled base. COX, cyclooxygenase; mPGES, micromosal PGE synthase; cPGES, cytosolic PGE synthase.

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Aliquots of culture supernatants (100 μl) were harvested at indicated time points, clarified by centrifugation, and subjected to PGE2- and VEGF-specific enzyme immunoassay using commercially available kits (PGE2 from Assay Designs, Ann Arbor, MI, and VEGF from Biosource International, Camarillo, CA).

Assay of PGES enzymatic activity. PGES activity in cell lysate was measured by assessing the conversion of PGH2 to PGE2, as previously reported (23). Fibroblasts cultured in the presence or absence of 1 ng/ml IL-1β for 24 h were scraped off the dishes and disrupted by sonication (10 s, 3 times, 1-min interval) in 400 μl of 10 mM Tris-HCl buffer at pH 8.0. After sonicated centrifugation at 1,700 g of PGH2 for 30 s at 4°C, the supernatants were used as the enzyme source. An aliquot of each lysate (100-μg protein equivalents) was incubated with 2 μg of PGH2 for 10 min at 4°C, the supernatants were treated as the enzyme source. An aliquot of each lysate (100-μg protein equivalents) was incubated with 2 μg of PGH2 for 30 s at 24°C in 0.1 ml of 1 M Tris-HCl, pH 8.0, containing 2 mM glutathione. After the reaction was terminated by the addition of 100 mM FeCl2, PGE2 contents in the supernatants were quantified with the enzyme immunoassay kit, and PGES activity was expressed as nanograms of PGE2 per 30 s per 10 μg protein.

Immunohistochemical analysis. Gastric fibroblasts were cultured on a SlideFlask (Nunc) as described above. After fixing with 4% paraformaldehyde in PBS for 30 min at 4°C, the slides were washed with PBS once and incubated with 10% normal horse and goat serum (Vector Laboratories) for 20 min to block nonspecific binding of secondary antibody, and then incubated overnight with primary antibody at 4°C. The anti-human COX-2 antibody (1:10 dilution) and anti-human mPGES-1 antibody (1:1,000 dilution) were used as primary antibodies. The antibodies were allowed to react with a secondary antibody (horse anti-mouse IgG, Vector Laboratories; dilution, 1:200) labeled with FITC or a secondary antibody (goat anti-rabbit IgG, Vector Laboratories; dilution, 1:200) labeled with Texas Red. Antibody binding was detected by the immunofluorescence method and confocal laser scanning microscopy (model TCS4D/DMIRBE; Leica, Heidelberg, Germany) equipped with argon and argonkrypton laser sources. The nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma) for 15 min to facilitate identification of morphological features. Negative control immunohistochemical procedures included replacement of the primary antibody with normal mouse and rabbit IgG.

Statistical analysis of data. Statistical significance was determined using ANOVA followed by Fisher’s projected least significant difference test as a post hoc test; a P value of <0.05 was considered to indicate a statistically significant difference.

RESULTS

Expression of COX-2, mPGES-1, COX-1, and cPGES in human gastric fibroblasts. First, to determine time-dependent COX and PGES expression, mRNA expression levels for cPGES, mPGES-1, and COX-1, and -2 in cultured gastric fibroblasts were tested at selected time points by real-time quantitative PCR, after a 2-h IL-1β pulse stimulus at 10 ng/ml (Fig. 1). IL-1β stimulated COX-2 mRNA levels up to 63-fold at the 2-h time point, and whereas mRNA levels decreased thereafter, their levels remained significantly higher at the 8-h time point, compared with levels in the absence of stimulus (Fig. 1A). The initial increase in mPGES-1 mRNA levels at the 2-h time point was not significant but thereafter increased significantly up to a 17.5-fold maximum at the 8-h time point and then decreased at 24 h (Fig. 1C).

In contrast, there was no change in COX-1 mRNA expression levels in response to IL-1β throughout the 24-h time course (Fig. 1B). Likewise, IL-1β had no effect on cPGES mRNA levels at any time point (Fig. 1D).

We also examined the effect of IL-1β pulse stimulation on COX and PGES protein expression in gastric fibroblasts at selected time points by Western blot analysis. COX-2 protein expression was first seen at 2 h, peaked at 8 h, and then decreased at 24 h. In contrast, at no point did IL-1β stimulation affect COX-1 protein expression levels. mPGES-1 protein expression was first seen at the 8-h time point and further increased at 24 h, in contrast to cPGES protein expression, which failed to respond to IL-1β stimulation at all time points (Fig. 2). When protein expression levels were normalized to α-actin, statistically significant differences were observed at each time point.
their respective β-actin levels and calculated as relative expression to basal unstimulated levels, maximal COX-2 and mPGES-1 protein expression were seen at the 8-h and 24-h time point, respectively (Fig. 3). By contrast, COX-1 and cPGES protein expression did not change over time (data not shown).

**Effect of SC-560, NS-398, and MK-886 on PGE2 release.** We then examined the effect of inhibitors SC-560, NS-398, and MK-886 on PGE2 release induced by IL-1β. IL-1β significantly stimulated PGE2 release at 8 h. This induced PGE2 release, while totally inhibited by NS-398, was not affected by SC-560, suggesting that in fibroblasts stimulated by IL-1β, increased PGE2 release is tied to increased COX-2 expression.

Of interest, MK-886 did not affect PGE2 release at 8 h, suggesting that COX-2-dependent PGE2 generation at this early time point might be independent of mPGES-1. At 24 h, there was a further increase in IL-1β-stimulated PGE2 release, which again was totally inhibited by NS-398, but not at all affected by SC-560. However, in contrast to PGE2 generation at the 8-h time point, at 24 h MK-886 inhibited IL-1β-stimulated PGE2 levels down to those observed at the 8-h time point (Fig. 4), suggesting that the late stage of PGE2 production is totally dependent on mPGES-1.

**Effect of MK-886 on PGES activity in gastric fibroblasts.** Because MK-886 failed to inhibit IL-1β-stimulated PGE2 release at 8 h, we then examined the effect of MK-886 on IL-1β-stimulated PGES activity in gastric fibroblasts. The basal PGES activity in crude lysates of gastric fibroblasts was 47.6 ng·30 s⁻¹·10⁻⁶ g protein⁻¹. IL-1β stimulated PGES activity up to 94.1 ng·30 s⁻¹·10⁻⁶ g protein⁻¹, which MK-886 then inhibited by 86.8%. These results suggest that MK-886 effectively inhibited IL-1β-stimulated increases in PGES activity in gastric fibroblasts (Fig. 5).
Immunohistochemical localization of COX-2 and mPGES-1. Confocal microscopic analysis of untreated and IL-1β-treated gastric fibroblasts was carried out to localize mPGES-1 and COX-2 in gastric fibroblasts. Untreated cells weakly stained for mPGES-1 but not for COX-2 immunoreactivity (data not shown). In contrast, IL-1β-treated fibroblasts showed strong perinuclear signals for both mPGES-1 and COX-2 immunoreactivity (Fig. 6, B and C). No signal was detected when preabsorbed anti-mPGES-1 or COX-2 antiserum was used (Fig. 6E). Figure 6D shows the merged image of IL-1β-treated fibroblasts after double staining for mPGES-1 and COX-2; the yellow areas in the cytoplasm of IL-1β-stimulated fibroblasts indicate colocalization of these two enzymes.

**Effect of MK-886 on IL-1β-stimulated VEGF release in gastric fibroblasts.** We then tested whether COX-1, COX-2, and mPGES-1 play a role in VEGF production in gastric fibroblasts. In the presence of IL-1β, there was a slight increase in VEGF release at the 8-h time point, which MK-886 significantly reduced to basal levels, although neither NS-398 nor SC-560 affected VEGF production. At the 24-h time point, we saw a significant increase in VEGF levels in the presence of IL-1β. In contrast to the 8-h time point, this increase in VEGF production was significantly inhibited by NS-398, although inhibition was not total. On the other hand, MK-886 totally inhibited IL-1β-stimulated VEGF production down to basal levels. SC-560 did not inhibit IL-1β-stimulated VEGF production at any time point (Fig. 7).

**DISCUSSION**

In the present study, we focused on PGES expression and activity in human gastric fibroblasts and found that mPGES-1 mRNA and protein expression followed COX-2 expression,
and whereas COX-2-derived PGE2 release did not depend on mPGES-1 expression at the early stage, it largely depended on its expression at the late stage. In a recent study (20), we showed IL-1β-stimulated increases in COX-2 expression in human gastric fibroblasts with subsequent increases in PGE2 release. NS-398, a selective COX-2 inhibitor, totally inhibited IL-1β-induced PGE2 release. In contrast, IL-1β had no effect on COX-1 expression; thus SC-560, a selective COX-1 inhibitor, could not affect IL-1β-stimulated PGE2 release, suggesting that this induced PGE2 production exclusively depended on COX-2 induction.

PGE2 released by COX-2-expressing gastric fibroblasts plays a pivotal role in VEGF production, angiogenesis, and, subsequently, the ulcer repair process in gastric tissue (20, 41). In a recent study, we showed strong COX-2 expression in human gastric fibroblasts of the ulcer bed in vivo (45) and that PGE2 released from gastric fibroblasts in vitro in turn stimulates VEGF production via a COX-2-dependent autocrine/paracrine pathway (20). In addition, we have shown VEGF and COX-2 colocalization in fibroblasts of granulation tissue just beneath the gastric ulcer bed in humans (20). Celecoxib, a selective COX-2 inhibitor, also has been shown to impair angiogenesis and subsequently delay gastric ulcer healing with a concomitant decrease in serum VEGF/endostatin ratios (17). All of these data suggest that, in the process of wound repair, COX-2 expression and consequent PGE2 release precede VEGF production in gastric fibroblasts, although the downstream mechanisms involved in PGES and VEGF production remain to be determined. In the present study, we found for the first time that mPGES-1, after COX-2 expression, is a major enzyme contributing to PGE2 generation in human gastric fibroblasts. Furthermore, VEGF production in gastric fibroblasts was found to largely depend on MAPEG superfamily proteins, which include mPGES-1. Here, VEGF production was not completely inhibited by NS-398, as we have previously reported (20), although PGE2 production was contingent on COX-2 activity. Thus our present results suggest that, in addition to COX-2-dependent products, certain MAPEG superfamily-dependent proteins also might be involved in VEGF production in gastric fibroblasts. In fact, 5-lipoxygenase, an activating protein in the MAPEG superfamily, has been connected to VEGF production in pleural mesothelial cells (34). Furthermore, although SC-560 had no significant effect on IL-1β-stimulated VEGF production, we cannot rule out the possibility that the COX-1/mPGES2 pathway might be involved in basal VEGF production, because we used the minimal effective SC-560 dose in the present study. Thus, despite the apparent role of the COX-2/mPGES1/PGE2 pathway in gastric fibroblasts, further work is clearly required to identify other factors involved in VEGF production.

Recently, three PGES have been identified, one cPGES (44) and two microsomal fractions, mPGES-1 (11, 18, 23) and mPGES-2 (22, 43). Studies of cells overexpressing these enzymes have shown that cPGES is constitutively expressed and that it is functionally coupled with COX-1 in marked preference to COX-2 (44), whereas mPGES-1 has been shown to be inducible and preferentially coupled with COX-2, causing a delayed PGE2 release response (11, 18, 23). mPGES-2 has yet to be well characterized, although, like cPGES, it seems to be constitutively expressed (22). In the present study, we found mPGES-1 enzyme induction and functional coupling with COX-2 during PGE2 generation in gastric fibroblasts, well consistent with previous reports (23). Furthermore, cPGES, like COX-1, was constitutively expressed in gastric fibroblasts, also in accord with previous studies (44).

Although mPGES-1, in response to proinflammatory cytokine stimulation, is one of the major enzymes involved in PGE2 generation in gastric fibroblasts, it appears not to play a role in PGE2 production at the initial stage. In the present study, MK-886 did not inhibit PGE2 generation at the 8-h time point, whereas, at 24 h, it inhibited PGE2 generation down to levels seen at 8 h. Although MK-886, well-established as an inhibitor of MAPEG superfamily proteins such as FLAP, is not selective for mPGES-1, it did in fact inhibit IL-1β-stimulated PGE2 activity by 86.8%. In addition, we found that mPGES-1 expression significantly increased 8 h after IL-1β stimulation, indicating that COX-2 might preferentially couple with some other enzyme to generate PGE2 production, despite mPGES-1 expression at this time point. There are two other PGES enzymes in addition to mPGES-1. In cells cotransfected with COX-2 and mPGES-1 cDNAs, PGE2 is efficiently produced, whereas in cells cotransfected with COX-2 and cPGES cDNAs, there is no PGE2 generation (23). Taking this data into consideration, it is unlikely that the cPGES enzyme was involved in COX-2-dependent PGE2 generation in IL-1β-stimulated gastric fibroblasts. On the other hand, mPGES-2 has been shown to be distributed in the cytosol with a trend to enrichment in the perinuclear region (22). It has also been shown to be expressed constitutively in various cells and to promote PGE2 production via both COX-1 and COX-2, with modest preference for COX-2 (22). To date, there are no data available concerning MK-886-inhibition of mPGES-2 activity. However, in the present study, we saw that MK-886 inhibited IL-1β-induced PGE2 activity without affecting its uninduced expression, and, because mPGES-2 has been shown to be constitutive, it would appear that MK-886 would have no effect on its activity. Thus one can speculate that, in addition to mPGES-1, mPGES-2 might also have a role in COX-2-dependent PGE2 release, although we did not examine mPGES-2 expression levels in gastric fibroblasts. It is possible, then, that mPGES-2 is involved in COX-2-dependent PGE2 generation at the initial stage in which mPGES-1 is not fully available despite COX-2 being fully expressed.

In the present study, we found that in gastric fibroblasts, peak mRNA and protein expression on IL-1β stimulation was more rapid for COX-2 than for mPGES-1. Pulse treatment with IL-1β resulted in peak COX-2 mRNA and protein expression at the 2- and 8-h time points, respectively. Another study in primary rheumatoid synovial cells has also shown that although IL-1β coordinately regulates increases in COX-2 and mPGES-1 mRNA expression (13, 14), there are differences in the specific time of their induction. In addition, in orbital fibroblasts, a COX-2 selective inhibitor has been shown to attenuate IL-1β-stimulated induction of mPGES-1 expression (8). Exogenously added arachidonate partially restored this mPGES-1 expression (8), suggesting that mPGES-1 gene expression might be partially dependent on COX-2-derived PG products. Our present results, showing that the initial PGE2 response to IL-1β stimulation is apparently independent of mPGES-1 expression, support this previous report. A recent study (46) has shown that certain gastric cancer cell lines express COX-2, but not mPGES-1 mRNA and protein, also
suggesting that COX-2 and mPGES-1 expression is not always regulated by the same pathway. Sequence analysis of the 3'-flanking region of the COX-2 gene has revealed several potential transcription regulatory sequences including a TATA box (1, 42), whereas the mPGES-1 gene promoter lacks the TATA box (4, 24). In addition, the 3' region of the mPGES-1 gene lacks the AUUUA instability sequences (3), whereas the COX-2 gene encodes the entire 3'-untranslated region containing 22 copies of the AUUUA RNA instability sequences (1, 2, 28), suggesting other possible differences in the regulatory mechanisms of these two enzymes.

In conclusion, we found in the present study that mPGES-1 preferentially couples with COX-2 at the later stage of PGE2 production, whereas a yet-to-be-identified PGES seems to couple with COX-2 to generate PGE2 at early stages in IL-1β-stimulated gastric fibroblasts. VEGF production in response to IL-1β was largely dependent on MAPEG superfamily proteins and partially dependent on the COX-2/PGE2 pathway.

REFERENCES