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Mechanisms for prostaglandin E_2 formation caused by proteinase-activated receptor-1 activation in rat gastric mucosal epithelial cells

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Abbreviations:
$AACOCF_3$, arachidonyl trifluoromethyl ketone
ADAMs, a disintegrin and metalloproteinases
BEL, bromoenol lactone
COX, cyclooxygenase
$cPLA_2$, cytosolic Ca^{2+}-dependent phospholipase A_2
EGF, epidermal growth factor
EGFR-TK, EGF receptor-tyrosine kinase
ERK, extracellular signal-regulated kinase

ABSTRACT

Proteinase-activated receptor-1 (PAR1), a thrombin receptor, plays a protective role in gastric mucosa via prostanoid formation. Thus, we studied effects of PAR1 stimulation on prostaglandin E_2 (PGE_2) formation in rat normal gastric mucosal epithelial RGM1 cells and analyzed the underlying signal transduction mechanisms. The PAR1-activating peptide (PAR1-AP) and thrombin increased PGE_2 release from RGM1 cells for 18 h, an effect being suppressed by inhibitors of COX-1, COX-2, MEK, p38 MAP kinase (p38 MAPK), protein kinase C (PKC), Src and EGF receptor-tyrosine kinase (EGFR-TK), but not JNK and matrix metalloproteinase (MMP)/a disintegrin and metalloproteinases (ADAMs). PAR1-AP caused persistent (6 h or more) and transient (5 min) phosphorylation of ERK and p38 MAPK, respectively, followed by delayed reinforcement at 18 h. PAR1-AP up-regulated COX-2 in a manner dependent on MEK and EGFR-TK, but not p38 MAPK. The PAR1-mediated persistent ERK phosphorylation was reduced by inhibitors of Src and EGFR-TK. PAR1-AP actually phosphorylated EGF receptors and up-regulated mRNA for heparin-binding-EGF (HB-EGF), the latter effect being blocked by inhibitors of Src, EGFR-TK and MEK. Heparin, an inhibitor for HB-EGF, suppressed PAR1-mediated PGE_2 formation and persistent ERK phosphorylation. These results suggest that PAR1 up-regulates COX-2 via persistent activation of MEK/ERK that is dependent on EGFR-TK activation following induction of HB-EGF, leading to PGE_2 formation. In addition, our data also indicate involvement of COX-1, PKC and p38 MAPK in PAR1-triggered PGE_2 formation. PAR1, thus stimulates complex multiple signaling pathways responsible for PGE_2 formation in RGM1 cells.

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GAPDH, glyceraldehyde 3-phosphate dehydrogenase
HB-EGF, heparin-binding-EGF
$iPLA_2$, Ca^{2+}-independent phospholipase A_2
JNK, c-Jun NH_2-terminal kinase
MAPK, mitogen-activated protein kinase
MEK, MAPK/ERK kinase
MMP, matrix metalloproteinase
PAR, proteinase-activated receptor
PAR1-AP, PAR1-activating peptide
PGE_2, prostaglandin E_2
PI3-kinase, phosphatidyl inositol 3-kinase
PKC, protein kinase C
SLIGRL-NH_2, Ser-Leu-Ile-Gly-Arg-Leu-amide
TFLLR-NH_2, Thr-Phe-Leu-Leu-Arg-amide
TGF-α, transforming growth factor-α

1. Introduction

Proteinase-activated receptors (PARs) belong to a large superfamily of G-protein-coupled seven-transmembrane domain receptors, consisting of four family members, PAR1 to PAR4 [1–5]. PAR1, PAR3 and PAR4 are thrombin receptors, whereas PAR2 is a receptor activated by trypsin, mast cell tryptase, coagulation factors VIIa and Xa, and some other specific proteinases [6–9]. PARs are activated by proteolytic unmasking of the cryptic tethered ligand present in the extracellular N-terminal domain [7]. PARs, except PAR3, can also be activated non-enzymatically by synthetic peptides as short as five to six amino acids based on the tethered ligand sequences [6–9]. PARs couple to multiple signaling pathways and can regulate various cellular functions. A common signaling pathway for PARs is activation of $G_{q/11}$ protein/phospholipase C, resulting in formation of inositol triphosphate and diacylglycerol, which cause cytosolic Ca^{2+} mobilization and activation of protein kinase C (PKC), respectively [7].

PAR1 and PAR2 are widely distributed in the mammalian body, especially in the alimentary system, and their activation causes various physiological/pathophysiological reactions [6–10]. In the gastric mucosa, PAR1 and PAR2 play multiple roles, being primarily protective, whereas the underlying mechanisms are greatly different [10–16]. The gastric mucosal protection exerted by PAR2 agonists is mediated by activation of capsaicin-sensitive sensory neurons, but independent of endogenous prostanoids [12,16]. On the other hand, the protective effect of PAR1 agonists is dependent on endogenous prostanoids but independent of capsaicin-sensitive sensory neurons [11]. It has also been shown that the endogenous prostanoids formed following PAR1 activation contributes to suppression of acid secretion in the stomach and, in part, increase in gastric mucosal blood flow [11]. In the isolated rat gastric artery, the PAR1-activating peptide (PAR1-AP) causes endothelium-dependent relaxation, which is mediated by prostanoids in addition to nitric oxide and endothelium-derived hyperpolarizing factor (EDHF) [17].

Prostanoids play critical roles in a broad range of physiological/pathophysiological processes, including glandular secretion, cytoprotection, motility, pain transmission, inflammation, angiogenesis, cancer, allergic diseases and so on [18,19]. In the stomach, among various prostanoids, prostaglandin E_2 (PGE_2) is particularly important in regulating secretion of acid, pepsinogen and mucus, as well as motility of smooth muscle [20,21], being protective [22,23]. RGM1, a normal rat gastric mucosal epithelial cell line [24], is useful to study functions of non-cancer gastric mucosal epithelial cells. In RGM1 cells, transforming growth factor-α (TGF-α), a ligand for EGF receptors, is capable of producing PGE_2 [25]. Given recent evidence that PGE_2 release increases after stimulation of PAR1 for 3 days in RGM1 [26], the present study performed careful time-course and concentration-dependence experiments for PAR1-triggered PGE_2 formation in RGM1 cells, and then investigated the underlying cell signaling mechanisms in detail.

2. Materials and methods

2.1. Major chemicals

A PAR1-activating peptide (PAR1-AP) TFLLR-NH_2, a PAR1-inactive control peptide FTLLR-NH_2, and a PAR2-AP SLIGRL-NH_2, were synthesized and purified by high-performance liquid chromatography (HPLC), and the concentration and purity were determined by HPLC or mass spectrometry. Human thrombin, bromoenol lactone (BEL), genistein,

heparin, dimethyl sulfoxide (DMSO) and U0126 were purchased from Sigma (St. Louis, MO, USA), and wortmannin was from Wako Pure Chem. (Osaka, Japan). SC-560, NS-398 and nimesulide were obtained from Cayman Chemical (Ann Arbor, MI, USA), and arachidonyl trifluoromethyl ketone ($AACOCF_3$), PD98059, SB203580, SP600125, GF109203X, PP2, PD1538035, GM6001, and BAPTA-AM were from Calbiochem (Darmstadt, Germany). RHC-80267 was obtained from Biomol Research Laboratories (Plymouth Meeting, PA).

2.2. Cell culture

Normal rat gastric epithelial cell line (RGM1) [24] was provided by Riken Cell Bank (Tsukuba, Japan). Cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma) supplemented with 20% heat-inactivated fetal bovine serum (Thermo, Melbourne, Australia) and 50 μg/ml kanamycin. The cells were grown to 90% confluence in a CO_2 incubator maintained at 5% CO_2 and 37 °C, then harvested by a brief exposure to 0.025% trypsin-0.02% EDTA and passaged after 2–3 days.

2.3. Determination of PGE_2 formation with enzyme immunoassays (EIA)

RGM1 cells were grown in the above medium for 24 h, and then cultured in serum-free medium overnight. Samples were repeatedly collected from the supernatant of the culture medium before and after stimulation for 3, 6 and 18 h with TFLLR-NH_2, FTLLR-NH_2, SLIGRL-NH_2 or thrombin. Various inhibitors or vehicle were added to the culture medium 0.5–1 h before stimulation. The amount of PGE_2 was determined using an EIA kit (Cayman Chemical Company), and calculated by subtracting the basal value at time 0 (before stimulation) from the value at each time point.

2.4. Reverse-transcribed-polymerase chain reaction (RT-PCR)

RGM1 cells were stimulated with TFLLR-NH_2 at 100 μM after pre-culture in serum-free medium overnight, then lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at 1, 3 and 6 h after the stimulation. Total RNA extracted from the cell lysate in the TRIzol reagent was reverse-transcribed and then amplified by PCR using the RNA LA PCR kit (AMV) version 1.1 (Takara, Otsu, Japan). The PCR primers employed were: 5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′ for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH); 5′-TGC TGC TGA GAA GGG AGT TCA TTC-3′ and 5′-CAA GTC ACA CAC ACG GTT ATG CTC-3′ for rat COX-1; 5′-ACA CTC TAT CAC TGG CAT CC-3′ and 5′-GAA GGG ACA CCC TTT CAC AT-3′ for rat COX-2; 5′-GAC AAC TCC CCT AAG GCT TA-3′ and 5′-CAT GCA CAC GCC ACC ATT GA-3′ for rat epidermal growth factor (EGF); 5′-TCC CAC TGG AAC CAC AAA CCA G-3′ and 5′-CCC ACG ATG ACA AGA AGA CAG AC-3′ for rat heparin-binding EGF (HB-EGF). The PCR products (452 bp for GAPDH, 403 bp for COX-1, 584 bp for COX-2, 567 bp for EGF and 414 bp for HB-EGF) were visualized by 2% agarose gel electrophoresis followed by the ethidium bromide staining.

2.5. Assay of cytosolic Ca^{2+} mobilization

RGM1 cells were grown on round glass coverslips (13.2 mm in diameter) coated with collagen (Cellmatrix Type I-A, Nitta Gelatin Inc., Osaka, Japan). The cells were loaded with 10 μM of Fura-2/AM (Dojindo, Kumamoto, Japan) for 1 h at room temperature in a HEPES buffer of the following composition: NaCl, 150 mM; KCl, 3 mM; $CaCl_2$, 1.5 mM; $MgCl_2$, 1.0 mM; HEPES, 20 mM; D-glucose, 10 mM. After wash the cells with a fresh HEPES buffer, the cytosolic Ca^{2+} level of the cells was measured by an Intracellular Ion Analyzer (CAF-110, Japan Spectroscopic Co., Tokyo, Japan) at 25 °C. The maximal increase in cytosolic Ca^{2+} levels was observed by adding 20 μM ionomycin. Changes in cytosolic Ca^{2+} levels caused by various stimuli are expressed as the percentage of the response to ionomycin.

2.6. Detection of phosphorylation of ERK, p38 MAPK, JNK and EGF receptors, and expression of COX-2 protein by Western blot analysis

RGM1 cells were stimulated with TFLLR-NH_2 at 100 μM for 5 min, 0.5, 1, 3, 6 or 18 h in the serum-free medium. Inhibitors were added 0.5–1 h before the stimulation. For detection of ERK, p38 MAPK, JNK and their phosphorylated forms, the cells were lysed in sodium dodecyl sulfate (SDS) buffer (2% SDS, 62.5 mM Tris–HCl and 10% glycerol, pH 6.8). For determination of EGF receptor phosphorylation and COX-2 expression, the cells were lysed in a RIPA buffer (PBS, 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenyl methyl sulfonyl fluoride, 0.15 U/ml aprotinin and 1 mM sodium orthovanadate). Protein samples (10–30 μg) were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan), and transferred onto polyvinylidene difluoride (PVDE) membrane (Immobilon™-P, Millipore, Bedford, MA, USA). The primary antibodies employed were: rabbit anti-p44/42 MAPK antibody, rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody, rabbit anti-p38 MAPK antibody, rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) antibody, rabbit anti-SAPK/JNK antibody, rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody, rabbit anti-phospho-EGF receptor (Thr1068) antibody (Cell Signaling Tech., Beverly, MA, USA), sheep anti-EGFR antibody (Upstate Cell Signaling Solution, Lake Placid, NY, USA) and goat anti-COX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.7. Statistics

Data are shown as mean ± S.E.M. Statistical analysis was performed by Student's *t*-test for two-group data and Tukey's test for multiple comparisons. Significance was set at a $P < 0.05$ level.

3. Results

3.1. Effects of PAR1 and PAR2 agonists on cytosolic Ca^{2+} levels and PGE_2 formation in RGM1 cells

Both TFLLR-NH_2, a PAR1-AP, and SLIGRL-NH_2, a PAR2-AP, at 1–100 μM caused increase in cytosolic Ca^{2+} levels in RGM1 cells.

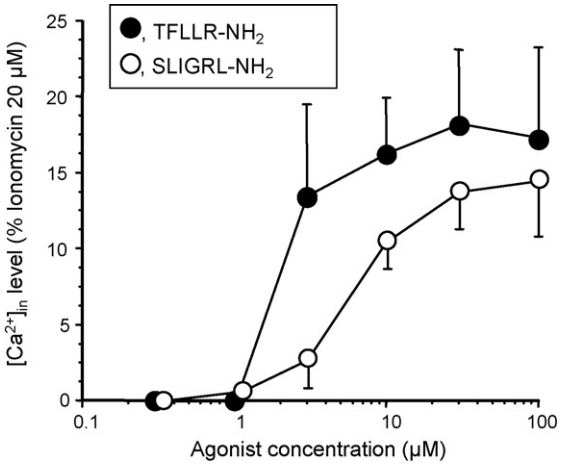

Fig. 1 – Cytosolic Ca^{2+} mobilization caused by stimulation of PARs in RGM1 cells. Maximal increase in cytosolic Ca^{2+} levels ($[Ca^{2+}]_{in}$) caused by the PAR1-AP, TFLLR-NH_2, or the PAR2-AP, SLIGRL-NH_2, in fura-2-loaded RGM1 cells (n = 5–7).

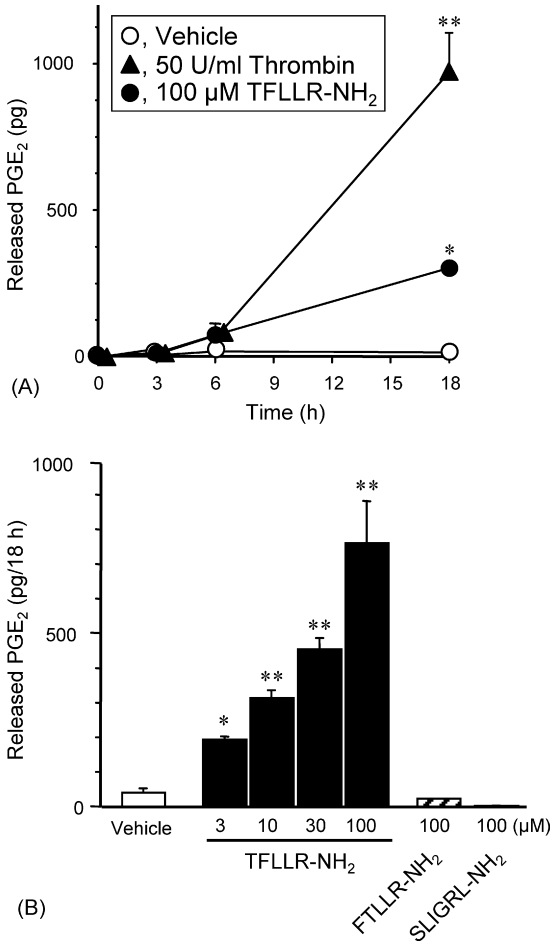

Fig. 2 – PGE_2 formation caused by PAR1 agonists in RGM1 cells. (A) Time course of PGE_2 formation caused by the endogenous PAR1-activating proteinase thrombin or the PAR1-AP, TFLLR-NH_2. (B) Concentration-dependent PGE_2 formation caused by TFLLR-NH_2, but not a PAR1-inactive control peptide, FTLLR-NH_2, or the PAR2-AP, SLIGRL-NH_2, in RGM1 cells. The baseline level of PGE_2 in the medium before stimulation was 14.2 ± 1.9 pg (n = 28). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle (n = 4).

The sensitivity to TFLLR-NH_2 was higher than that to SLIGRL-NH_2 (Fig. 1). Stimulation of PAR1 with TFLLR-NH_2 at 100 μM and thrombin, an endogenous PAR1-activating proteinase, at 50 U/ml for 18 h, but not 0–6 h, produced significant increase in PGE_2 formation (Fig. 2A). Of note is that prolonged incubation (24 h or more) of the cells with the PAR1-AP caused further increase in PGE_2 release, but was accompanied by over-confluency and detachment of the cells (data not shown). The effect of TFLLR-NH_2 on PGE_2 formation was concentration-dependent in a range of 3–100 μM, while a PAR1-inactive control peptide, FTLLR-NH_2, and the PAR2-AP, SLIGRL-NH_2, were inactive (Fig. 2B).

3.2. *Effects of inhibitors on the increase in PGE_2 formation caused by the PAR1-AP, TFLLR-NH_2*

The PAR1-AP-caused PGE_2 formation was completely suppressed by a COX-1 inhibitor, SC-560, or COX-2 inhibitors, NS-398 and nimesulide, but not by inhibitors of cytosolic Ca^{2+}-dependent phospholipase A_2 (cPLA$_2$; AACOCF$_3$), Ca^{2+}-independent phospholipase A_2 (iPLA$_2$; BEL) and diacylglycerol lipase (RHC-80267) (Fig. 3A). Two distinct MEK inhibitors, PD98059 and U0126, and a p38 MAPK inhibitor, SB203580, but not a c-Jun NH_2-terminal kinase (JNK) inhibitor, SP600125, abolished the TFLLR-NH_2-caused PGE_2 formation (Fig. 3B). Further, a protein kinase C (PKC) inhibitor, GF109203X, a Src inhibitor, PP2, and an inhibitor of pan-tyrosine kinases, genistein, also reduced the PGE_2 formation (Fig. 3C). Interestingly, an EGF receptor tyrosine kinase (EGFR-TK) inhibitor, PD153035, completely blocked the PAR1-AP-caused PGE_2 formation, suggesting involvement of trans-activation of EGF receptors. Nonetheless, GM6001, a broad range inhibitor for matrix metalloproteinase (MMP)/a disintegrin and metalloproteinase (ADAMs) [27], exhibited no inhibitory effect (Fig. 3C). Another MMP inhibitor, ONO-4817, known to also inhibit ADAMs [28], at 10 μM, when applied 12 h before PAR1 stimulation, also failed to suppress the PAR1-mediated PGE_2 formation (data not shown). Furthermore, our inhibition study showed that the PAR1-AP-caused PGE_2 formation was partially blocked by an intracellular Ca^{2+} chelator, BAPTA/AM, and unaffected by an inhibitor of phosphatidyl inositol 3-kinase (PI3 kinase), wortmannin (Fig. 3C).

3.3. *Effects of the PAR1-AP, TFLLR-NH_2, on phosphorylation of ERK, p38 MAPK and JNK*

Given evidence for involvement of MAP kinase pathways in the PAR1-mediated PGE_2 formation (see Fig. 3B), we determined effect of the PAR1-AP, TFLLR-NH_2, on phosphorylation of ERK, p38 MAPK and JNK. The PAR1-AP caused prompt phosphorylation of ERK at 5 min, an effect persisting for 6 h or more. Most interestingly, the phosphorylation of ERK was reinforced at 18 h (Fig. 4A). On the other hand, the PAR2-AP, SLIGRL-NH_2, caused only transient phosphorylation of ERK

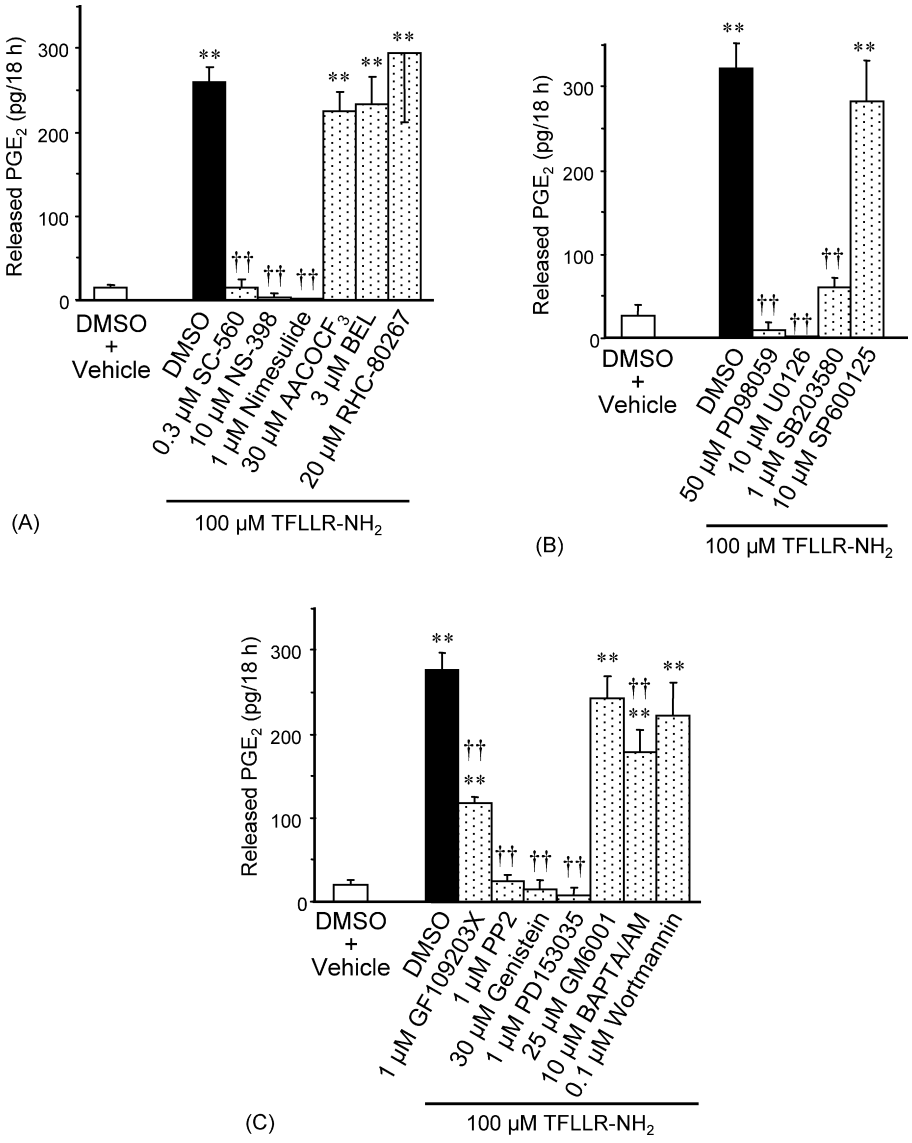

Fig. 3 – Effect of inhibitors on the increased PGE_2 formation caused by the PAR1-AP, TFLLR-NH_2, in RGM1 cells. (A) Effects of inhibitors of COX-1 (SC-560), COX-2 (NS-398 and nimesulide), $cPLA_2$ ($AACOCF_3$), $iPLA_2$ (bromoenol lactone; BEL) and diacylglycerol lipase (RHC-80267). (B) Effects of inhibitors of MEK1/2 (PD98059 and U0126), p38 MAP kinase (SB203580), and JNK (SP600125). (C) Effects of inhibitors of PKC (GF109203X), Src (PP2), pan-tyrosine kinases (genistein), EGFR-TK (PD153035), MMPs (GM6001) and PI3 kinase (wortmannin), and a cytosolic Ca^{2+} chelator (BAPTA/AM). All inhibitors were added 30 min before addition of TFLLR-NH_2. $^{**}P < 0.01$ vs. DMSO + vehicle; $^{††}P < 0.01$ vs. DMSO + TFLLR-NH_2 (n = 8–16) (DMSO + vehicle, DMSO + TFLLR-NH_2) or 4–6 (inhibitors).

for 5 min (Fig. 4B). The PAR1-AP also caused phosphorylation of p38 MAPK at 5 min, which rapidly declined for 0.5 h, disappeared at 1 h, and interestingly, revived at 18 h (Fig. 4C). The PAR1-AP cause only slight and transient phosphorylation of JNK at 5 min (Fig. 4D).

3.4. *Effect of the PAR1-AP, TFLLR-NH_2, on COX-1 or COX-2 expression*

Since the PAR1-triggered PGE_2 release required long-term stimulation (18 h) of PAR1 (see Fig. 2A) and involved COX-1 and COX-2 activity (see Fig. 3A), we evaluated effects of PAR1 activation on COX-1 and COX-2 expression. The PAR1-AP, TFLLR-NH_2, caused prompt up-regulation of COX-2 mRNA for 1 h, which lasted at least for 6 h, whereas COX-1 mRNA was detected even before stimulation and not affected by TFLLR-NH_2 (Fig. 5A). The PAR1-AP also gradually increased COX-2 at protein levels—an effect peaking at 6 h and persisting even at 18 h (Fig. 5B). The PAR1-triggered up-regulation of COX-2 protein was blocked by the MEK inhibitor U0126 and the EGFR-TK inhibitor PD153035, but not by the p38 MAPK inhibitor SB203580 (Fig. 5C). Thus, activation of the MEK/ERK pathway and EGF receptors, but not p38 MAPK, is considered upstream of the COX-2 up-regulation caused by PAR1 stimulation.

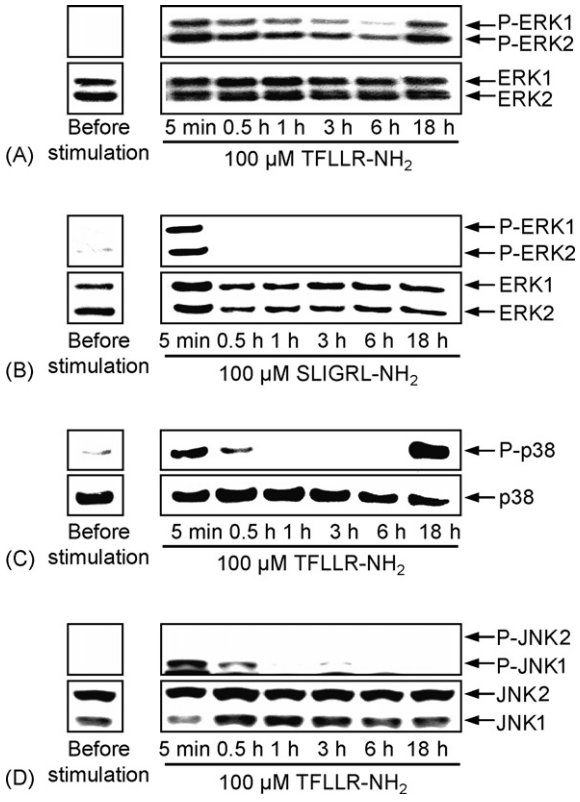

Fig. 4 – Time course of phosphorylation of ERK1/2 and/or p38 MAPK caused by PAR1 or PAR2 activation in RGM1 cells. (A and B) Phosphorylation of ERK1/2 caused by the PAR1-AP, TFLLR-NH_2 (A), and by the PAR2-AP, SLIGRL-NH_2 (B). (C and D) Phosphorylation of p38 MAPK (C) and JNK (D) caused by the PAR1-AP, TFLLR-NH_2. P-ERK, phosphorylated-ERK; P-p38, phosphorylated-p38 MAP kinase, P-JNK, phosphorylated JNK. Data show the representatives of two to three experiments.

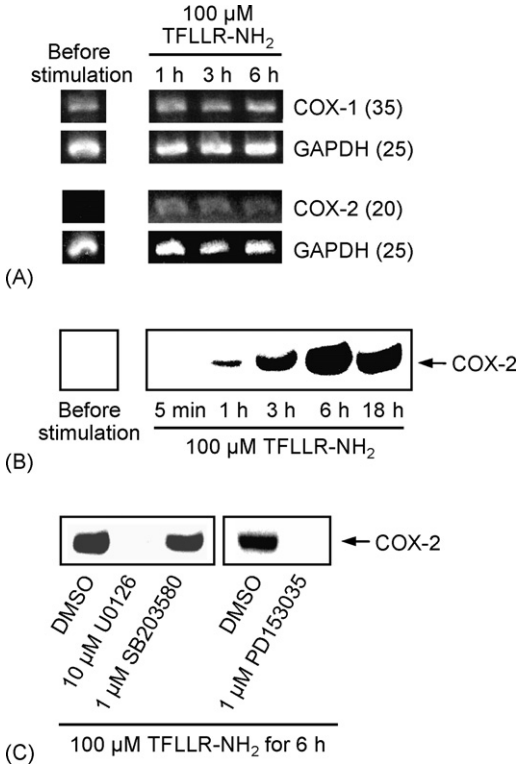

Fig. 5 – Up-regulation of COX-2 caused by PAR1 activation in RGM1 cells, and effects of inhibitors of MEK, p38 MAPK and EGFR-TK. (A) Effect of stimulation with the PAR1-AP, TFLLR-NH_2, for 1, 3 and 6 h on mRNA levels for COX-1 and COX-2, as determined by the RT-PCR analysis. Figures in parentheses show the number of PCR cycles. (B) Time-related up-regulation of COX-2 protein caused by TFLLR-NH_2. (C) Effects of inhibitors of MEK (U0126), p38 MAPK (SB203580) and EGFR-TK (PD153035) on PAR1-triggered COX-2 up-regulation. All inhibitors were added 30 min before TFLLR-NH_2 at 100 μM. None of these inhibitors affected COX-2 expression by themselves. Data show the representatives of two to three experiments.

3.5. *Effects of kinase inhibitors on the phosphorylation of ERK and p38 MAPK caused by the PAR1-AP, TFLLR-NH_2*

We next evaluated upstream mechanisms for PAR1-triggered activation of ERK and p38 MAPK. We first confirmed complete inhibition of the TFLLR-NH_2-evoked ERK phosphorylation by the MEK inhibitor U0126 (data not shown). The prompt phosphorylation of ERK caused by 5-min stimulation of PAR1 was resistant to the Src inhibitor PP2 and the PKC inhibitor GF109203X, and reduced only slightly by the EGFR-TK inhibitor PD153035 (Fig. 6). Nevertheless, the persistent phase of the ERK phosphorylation caused by 3-h stimulation of PAR1 was markedly suppressed by either PP2 or PD153035, but not by GF109203X (Fig. 6), suggesting involvement of upstream activation of Src and EGF receptors for the long-lasting (3 h) phosphorylation of ERK that was responsible for COX-2 up-regulation. Of note is that the delayed reviving phosphorylation of ERK caused by 18-h stimulation of PAR1 was also abolished by PD153035 (data not shown).

The prompt phosphorylation of p38 MAPK following 5-min stimulation of PAR1 (early phase) was only slightly suppressed by PD153035, and unaffected by U0126 (Fig. 7). However, those inhibitors blocked the delayed reviving phase of phosphorylation of p38 MAPK following 18-h stimulation of PAR1 (Fig. 7). Thus, activation of the MEK/ERK pathway and EGF receptors is upstream of the delayed (18 h), but not early (5 min), phosphorylation of p38 MAPK.

3.6. *Phosphorylation of EGF receptors and up-regulation of HB-EGF caused by the PAR1-AP, TFLLR-NH_2*

We found that the stimulation of PAR1 for 3 h with TFLLR-NH_2 at 100 μM actually caused phosphorylation of EGF receptors (Fig. 8A). The RT-PCR analysis showed that stimulation with the PAR1-AP for 1, 3 and 6 h clearly up-regulated mRNA levels for HB-EGF (Fig. 8B) known to mediate trans-activation of EGF receptors by G protein-coupled receptors [29], while mRNA for EGF was present in the non-stimulated cells and unaffected by PAR1 stimulation (Fig. 8B). The PAR1-triggered up-regulation of HB-EGF mRNA was strongly suppressed by PP2, a Src

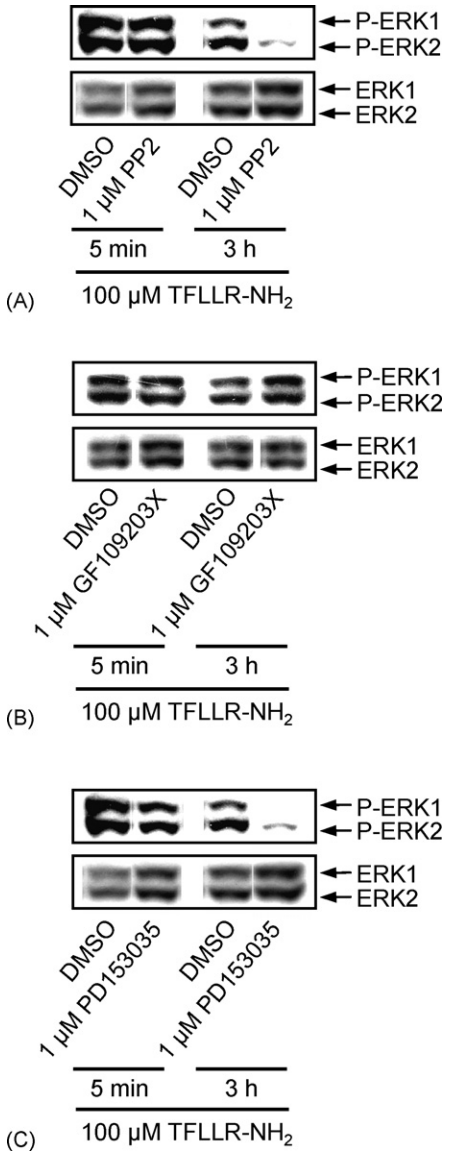

Fig. 6 – Effect of inhibitors of Src, PKC and EGFR-TK on prompt (5 min) and persistent (3 h) phosphorylation of ERK caused by the PAR1-AP, TFLLR-NH_2, in RGM1 cells. A Src inhibitor (PP2) (A), a PKC inhibitor (GF109203X) (B) and an EGFR-TK inhibitor (PD153035) (C) were added to 30 min before stimulation with TFLLR-NH_2 at 100 μM. None of these inhibitors affected ERK phosphorylation by themselves. Data show the representatives of two to three experiments.

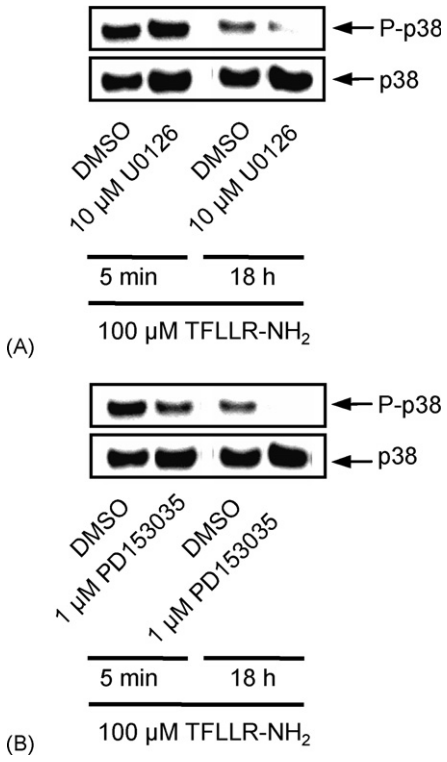

Fig. 7 – Effect of inhibitors of MEK and EGFR-TK on prompt (5 min) and delayed (18 h) phosphorylation of p38 MAPK caused by the PAR1-AP, TFLLR-NH_2, in RGM1 cells. A MEK inhibitor (U0126) (A) and an EGRF-TK inhibitor (PD153035) (B) were added 30 min before stimulation with TFLLR-NH_2 at 100 μM. None of these inhibitors affected p38 MAPK phosphorylation by themselves. Data show the representatives of two to three experiments.

inhibitor, U0126, a MEK inhibitor, and PD153035, an EGFR-TK inhibitor (Fig. 8C). To evaluate if HB-EGF could mediate the trans-activation of EGF receptors by PAR1 stimulation, we examined effect of heparin that is known to block effect of HB-EGF on EGF receptors [30]. As expected, heparin significantly suppressed the PGE_2 formation at 18 h (Fig. 8D) and abolished the persistent ERK phosphorylation at 3 h caused by the PAR1-AP (Fig. 8E). It is of note that CRM197 at 20 μg/ml, known as an antagonist for human HB-EGF, when given 1 h before addition of TFLLR-NH_2 at 100 μM, failed to exert inhibitory effect in RGM1 cells that originate from rat stomach (data not shown), in agreement with the early study showing lack of effect of CRM197 on rodent HB-EGF [31].

3.7. *Effect of COX inhibitors on delayed phosphorylation of ERK and p38 MAPK and up-regulation of COX-2 caused by the PAR1-AP, TFLLR-NH_2*

We next determined whether endogenous prostaglandins themselves would contribute to the delayed phosphorylation of ERK and p38 MAPK and/or induction of COX-2 protein caused by the PAR1-AP, TFLLR-NH_2 (Fig. 9). The ERK phosphorylation following PAR1 stimulation for 5 min and 18 h was resistant to either COX-1 or COX-2 inhibitor (Fig. 9A). In contrast, the p38 MAPK phosphorylation caused by PAR1 stimulation for 18 h, but not 5 min, was completely blocked by either COX-1 or COX-2 inhibitor (Fig. 9B), suggesting involvement of endogenous prostaglandins. Neither COX-1 nor COX-2 inhibitor modified the induction of COX-2 protein caused by PAR1 stimulation for 6 h (Fig. 9C).

4. Discussion

The present study shows that activation of PAR1 causes delayed (18 h later) PGE_2 formation through up-regulation of

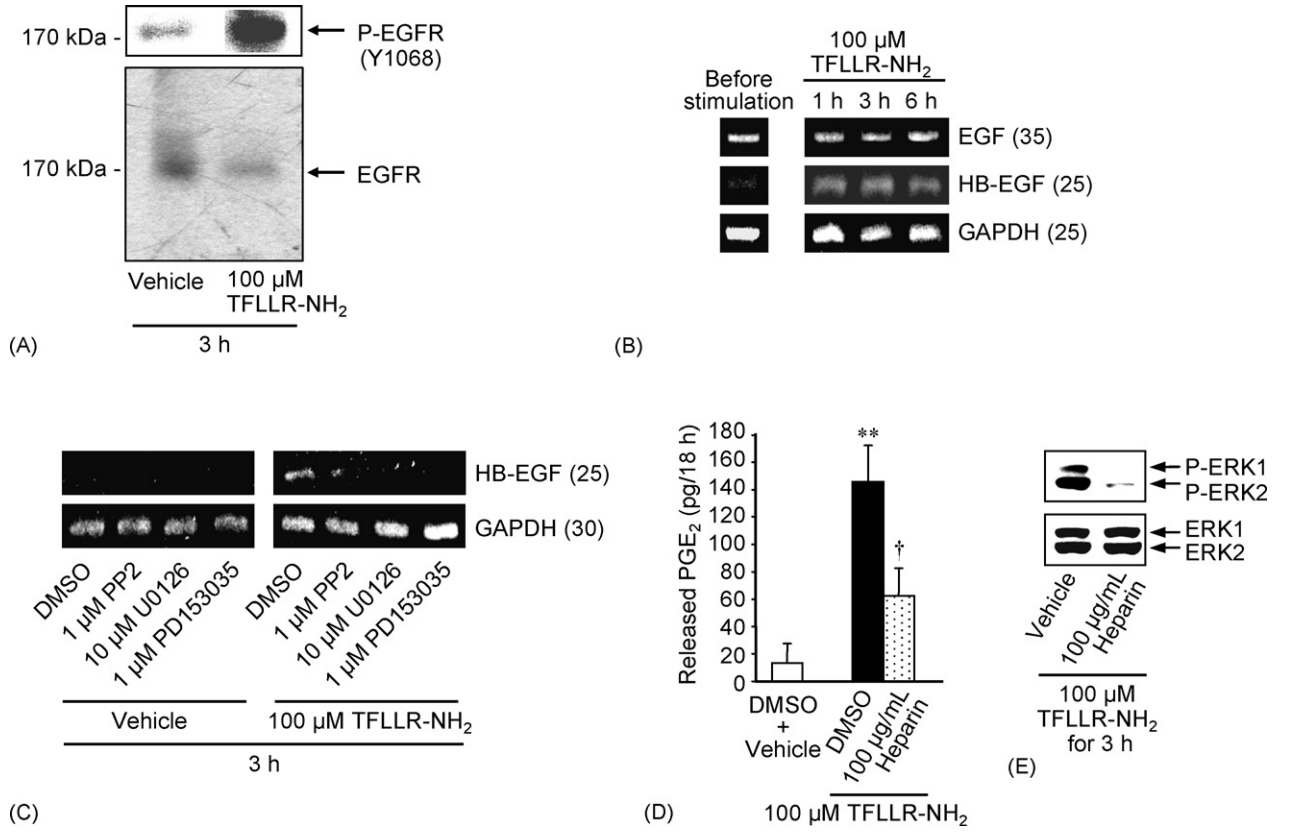

Fig. 8 – Trans-activation of EGF receptors and up-regulation of HB-EGF caused by PAR1 activation in RGM1 cells. (A) Phosphorylation of EGF receptors (Tyr 1068) caused by 3-h stimulation with the PAR1-AP, TFLLR-NH_2 at 100 μM. (B) TFLLR-NH_2-evoked prompt up-regulation of mRNA for HB-EGF. Expression of mRNAs for HB-EGF and EGF following PAR1 stimulation were determined by the RT-PCR method. (C) Effect of inhibitors of Src (PP2), MEK (U0126) and EGFR-TK (PD153035) on up-regulation of HB-EGF mRNA following 3-h stimulation with TFLLR-NH_2 at 100 μM. Figures in parentheses indicate the number of PCR cycles. (D and E) Inhibitory effects of heparin, an antagonist of HB-EGF, on PGE_2 formation (for 18 h) (D) and persistent (3 h) ERK phosphorylation (E) caused by PAR1-AP. All inhibitors including heparin were added 30 min before stimulation with TFLLR-NH_2. Heparin did not affect ERK phosphorylation by itself. Data show the representatives of two to three experiments. $^{**}P < 0.01$ vs. DMSO + vehicle; $^{\dagger}P < 0.05$ vs. DMSO + TFLLR-NH_2 ($n = 8$).

COX-2 in RGM1 cells. The PAR1-triggered COX-2 induction appears to involve persistent activation of MEK/ERK downstream of MMP/ADAMs-independent trans-activation of EGF receptors. Up-regulation of HB-EGF following PAR1 activation is considered responsible for the trans-activation of EGF receptors. In contrast, PAR2 stimulation failed to cause PGE_2 formation, although the PAR2-AP caused relatively weak increase in cytosolic Ca^{2+} levels and transient phosphorylation of ERK in RGM1 cells (see Figs. 1 and 4B). This may be simply attributable to poor expression of PAR2 in RGM1 cells, since expression of PAR2 mRNA detected by the RT-PCR analysis was lower than that of PAR1 mRNA (data not shown). These findings concerning the difference between PAR1 and PAR2 might be consistent with our previous in vivo evidence that the gastric mucosal protection exerted by the PAR1-AP, but not the PAR2-AP, is dependent on endogenous prostaglandin formation [11,12,16].

There have been many papers showing that MAP kinases contribute to up-regulation of COX-2 expression [32–36]. It is clear that PAR1 stimulation triggers prompt and persistent activation of the MEK/ERK pathway that contributes to COX-2 up-regulation and subsequent PGE_2 formation in RGM1 cells. In contrast, the PAR1-triggered transient activation of the p38 MAPK pathway appears to contribute to PGE_2 formation, but not COX-2 up-regulation, in RGM1 cells. The downstream signaling for prompt (5 min) activation of p38 MAPK caused by PAR1 activation has yet to be investigated in detail, while the upstream signaling might be partially mediated by prompt activation of EGF receptors (see Fig. 7B). On the other hand, the delayed activation of p38 MAPK observed after 18-h stimulation with the PAR1-AP is considered to be mediated by endogenous prostanoids themselves, as suggested by its inhibition by COX inhibitors (see Fig. 9B). Nonetheless, the long-lasting activation of MEK/ERK pathway observed after 18-h stimulation of PAR1 appears to be independent of endogenous prostanoids (see Fig. 9A). It is particularly interesting that activation of ERK and EGF receptors is essential for COX-2 up-regulation (see Fig. 5C), and activation of Src and EGF receptors is upstream of persistent (3 h), but not rapid (5 min), activation of ERK (see Fig. 6A and C) caused by PAR1 activation in RGM1 cells. JNK is not considered to contribute to the PAR1-triggered PGE_2 formation, since the JNK inhibitor did not affect

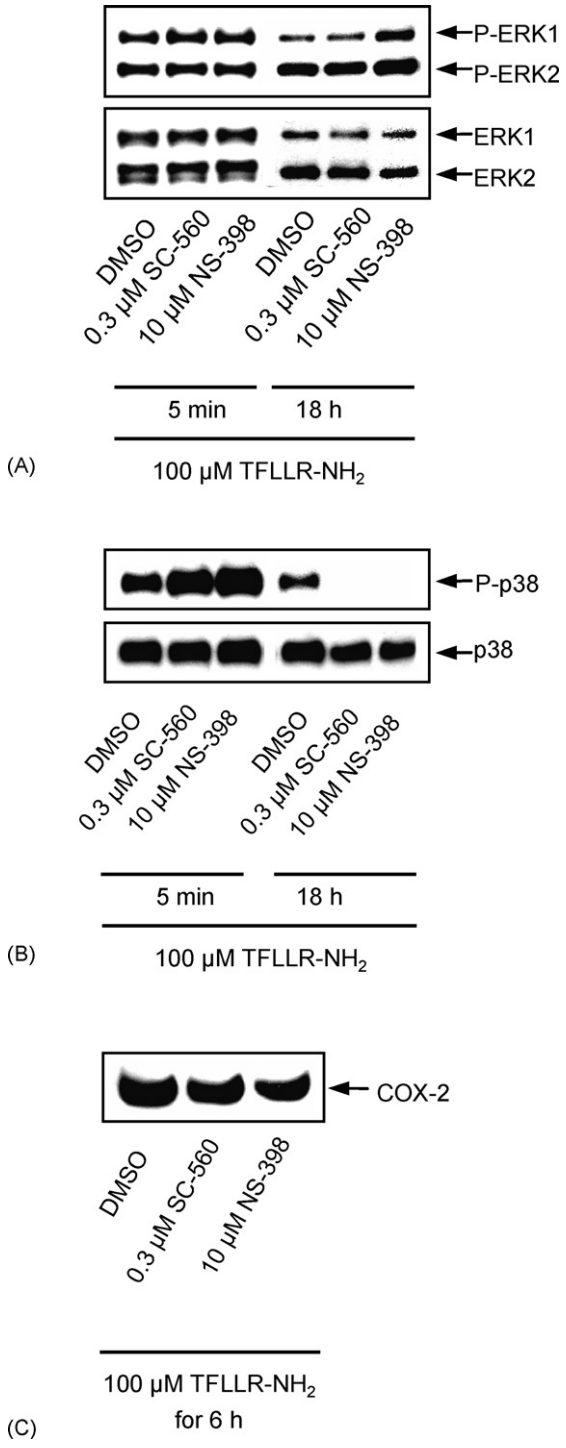

Fig. 9 – Effects of selective inhibitors for COX-1 and COX-2 on prompt (5 min) and delayed (18 h) phosphorylation of ERK1/2 (A) and p38 MAPK (B), and up-regulation of COX-2 protein (C) caused by PAR1 activation in RGM1 cell. The COX-1 inhibitor SC-560 and the COX-2 inhibitor NS-398 were added 30 min before stimulation with TFLLR-NH_2 at 100 μM. None of these inhibitors affected phosphorylation of ERK and p38 MAPK, and COX-2 expression by themselves. Data show the representatives of two to three experiments.

the formation and JNK phosphorylation caused by the PAR1 agonist was weak and transient (see Figs. 3B and 4D).

It has been reported that a number of G protein-coupled receptors including PARs are capable of trans-activating EGF receptors through MMP- or ADAMs-dependent release of EGF receptor ligands such as HB-EGF and TGF-α [27,29,37–40]. Although, in the present study, trans-activation of EGF receptors by PAR1 activation is considered essential for persistent activation of the MEK/ERK pathway, up-regulation of COX-2 and subsequent formation of PGE_2 (see Figs. 3C, 5C and 6C), PAR1-triggered PGE_2 formation is independent of MMP/ADAMs, as indicated by lack of effects of broad-range MMP/ADAMs inhibitors such as GM6001 at 25 μM (Fig. 3C) and ONO-4817 at 10 μM (data not shown). It is of note that the K_i value of GM6001 is 0.4, 20 and 20 nM against collagenase, thermolysin and elastase, respectively [41]. Similar metalloproteinases-independent mechanisms have been described for trans-activation of EGF receptors by PAR2 or lysophosphatidic acid in distinct cells [36,42]. HB-EGF might play a role in PAR1-triggered PGE_2 formation in RGM1 cells, because PAR1 stimulation rapidly up-regulated mRNA for HB-EGF within 1 h (see Fig. 8B), and heparin, known to block the binding of HB-EGF to EGF receptors [30], suppressed both PGE_2 formation and persistent ERK phosphorylation following PAR1 activation (see Fig. 8D and E). These results suggest that PAR1-mediated up-regulation of HB-EGF contributes, at least in part, to phosphorylation of EGF receptors followed by persistent activation of ERK, leading to COX-2 up-regulation. The upstream signaling for PAR1-triggered up-regulation of HB-EGF might involve activation of Src, ERK and also EGF receptors, considering the results from inhibition experiments (see Fig. 8C). We have reported that PAR2 stimulation triggers prompt Src-dependent, but MMP-independent, activation of EGF receptors in human lung epithelial cells [36]. Miyazaki et al. [43] have shown that oxidative or osmotic stress causes prompt activation of EGF receptors within 2 min, leading to up-regulation of HB-EGF in RGM1 cells. Taken together with the slight inhibitory effect of the EGFR-TK inhibitor on prompt (5 min) phosphorylation of ERK (see Fig. 6C), it might be hypothesized that PAR1-triggered prompt activation of the Src-EGF receptor-ERK pathway would up-regulate HB-EGF, resulting in stronger and more persistent activation of EGF receptors responsible for persistent activation of ERK and up-regulation of COX-2. Nevertheless, the complete inhibition of the expression of mRNA for HB-EGF by the EGFR-TK inhibitor (Fig. 8C) is inconsistent with the finding that PD153035 only partially inhibited the prompt (5 min) activation of ERK (see Fig. 6C). One possibility is that very strong activation of ERK might be necessary for up-regulation of HB-EGF, and therefore even partial inhibition of ERK phosphorylation by PD153035 might cause complete inhibition of HB-EGF up-regulation. Another possibility is that unknown pathways in addition to MEK-ERK downstream of EGF receptors might also contribute to up-regulation of HB-EGF. The precise mechanisms for the PAR1-triggered HB-EGF up-regulation have yet to be analyzed in detail.

In addition to the critical role of COX-2, as described above, COX-1 activity is also essential for PAR1-triggered PGE_2 formation, since a selective inhibitor of COX-1, SC-560, at 0.3 μM also completely blocked PGE_2 formation following PAR1

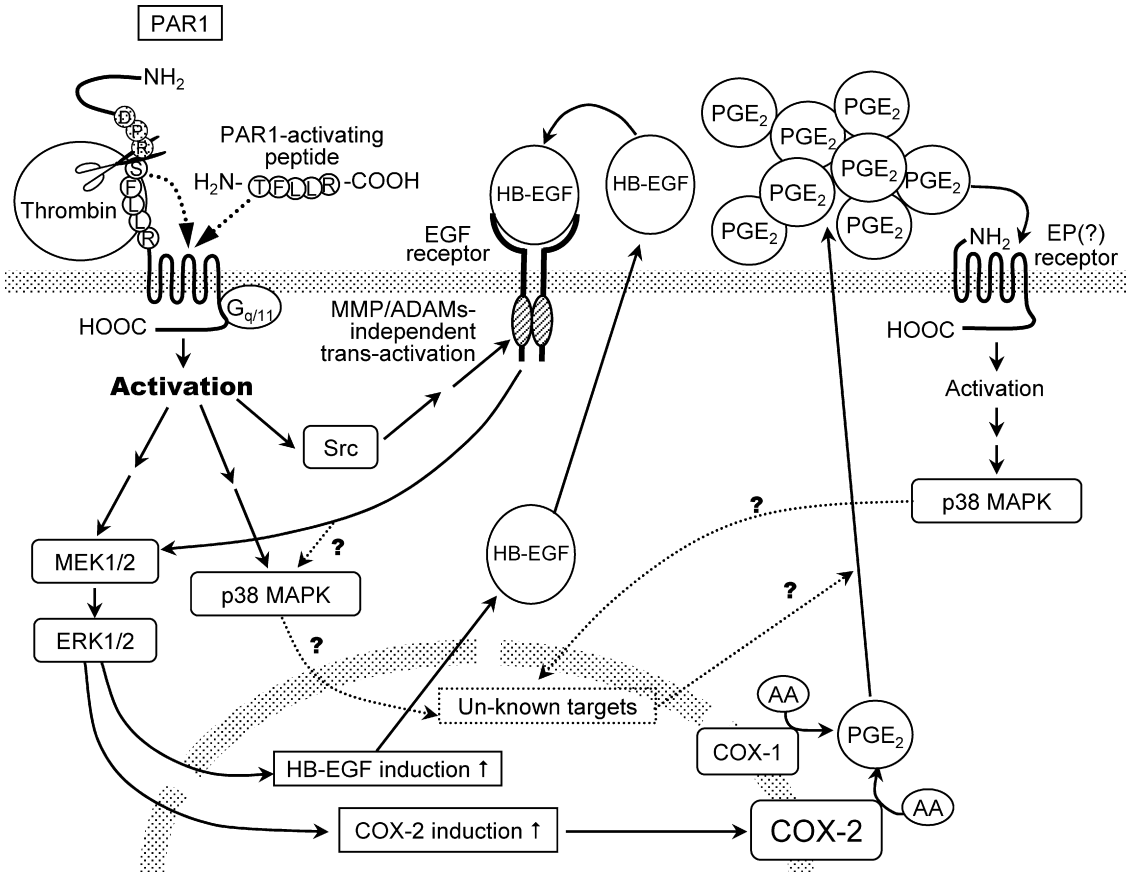

Fig. 10 – A hypothetical scheme for signal transduction mechanisms responsible for PGE_2 formation caused by PAR1 activation in RGM1 cells. AA, arachidonic acid. PAR1, upon activation, immediately induces ERK activation, which is partially mediated by Src-dependent, but MMP/ADAMs-independent, trans-activation of EGF receptors. The ERK activation up-regulates expression of HB-EGF, which causes persistent activation of EGF receptors followed by persistent activation of ERK, resulting in up-regulation of COX-2. PAR1-triggered p38 MAPK activation might stimulate unknown targets, contributing to PGE_2 formation. PGE_2 formed by COX-1 and COX-2 is involved in the delayed p38 MAPK activation.

stimulation. It is unlikely that SC-560 at the concentration used in the present study inhibits not only COX-1 but also COX-2, since the IC_{50} value of SC-560 are 0.0048 μM for COX-1 and 1.4 μM for COX-2 [44,45]. Recently, we have reported that PAR2 stimulation causes prompt formation of endogenous prostanoids other than PGE_2 in a COX-1-dependent manner, leading to rapid up-regulation of microsomal PGE synthase-1 (mPGES-1) in the human lung epithelial cell line, A549 [36]. A study to test possible involvement of similar mechanisms is now in progress in our laboratory. PKC is also considered to be partially involved in PAR1-triggered PGE_2 formation (see Fig. 3C), whereas its downstream signaling has yet to be investigated. Further, cell signaling for formation of arachidonic acid is also still open to question.

Among various endogenous pathways known to cause up-regulation of COX-2 and subsequent PGE_2 formation, the thrombin-PAR1 pathway might play an important role particularly in the gastric mucosal injury accompanied by hemorrhage where thrombin would become available and accessible to the mucosal epithelium. We have previously shown that immunoreactive PAR1 is abundant in the muscularis mucosae and blood vessels in both human and rat gastric mucosae [11], predicting possible roles for PAR1 in human stomach. In this context, we are now analyzing functions of PAR1 and regulation of its expression, particularly in inflammatory conditions, in a human gastric carcinoma cell line, KATO III, where we detected abundant PAR1 at protein levels by Western blotting (unpublished observation). Clinically, oral thrombin is useful to stanch gastric mucosal hemorrhage in patients with advanced gastric ulcer. Together with our previous in vivo study [11], the present data suggest that stimulation of PAR1 with thrombin or PAR1-activating peptides in the gastric luminal surface might bring about additional therapeutic benefit in the ulcer patients. Our ongoing study now focuses on the relationship between PAR1 and infection with *H. pylori* in cancer cell lines including KATO III and in the gastric tissues isolated from cancer patients.

Apart from some unclear signaling details, as outlined in Fig. 10, we propose that PAR1 activation causes prompt activation of Src, EGF receptors and MEK/ERK and subsequent up-regulation of HB-EGF, leading to persistent activation of EGF receptors and then ERK, followed by COX-2 up-regulation that is essential for PGE_2 formation. Activation of p38 MAPK

and COX-1 is also involved in PGE_2 formation, although the downstream targets remain to be elucidated.

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