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Journal of Cellular Biochemistry 112:909–915 (2011)

Journal of Cellular Biochemistry

Delayed Production of Arachidonic Acid Contributes to the Delay of Proteinase-Activated Receptor-1 (PAR1)-Triggered Prostaglandin E_2 Release in Rat Gastric Epithelial RGM1 Cells

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ABSTRACT

Proteinase-activated receptor-1 (PAR1), upon activation, exerts prostanoid-dependent gastroprotection, and increases prostaglandin E_2 (PGE_2) release through cyclooxygenase-2 (COX-2) upregulation in rat gastric mucosal epithelial RGM1 cells. However, there is a big time lag between the PAR1-triggered PGE_2 release and COX-2 upregulation in RGM1 cells; that is, the former event takes 18 h to occur, while the latter rapidly develops and reaches a plateau in 6 h. The present study thus aimed at clarifying mechanisms for the delay of PGE_2 release after PAR1 activation in RGM1 cells. Although a PAR1-activating peptide, TFLLR-NH_2, alone caused PGE_2 release at 18 h, but not 6 h, TFLLR-NH_2 in combination with arachidonic acid dramatically enhanced PGE_2 release even for 1–6 h. TFLLR-NH_2 plus linoleic acid caused a similar rapid response. CP-24879, a Δ^5/Δ^6-desaturase inhibitor, abolished the PGE_2 release induced by TFLLR-NH_2 plus linoleic acid, but not by TFLLR-NH_2 alone. The TFLLR-NH_2-induced PGE_2 release was not affected by inhibitors of cytosolic phospholipase A_2 ($cPLA_2$), Ca^{2+}-independent PLA_2 ($cPLA_2$) or secretory PLA_2 ($sPLA_2$), but was abolished by their mixture or a pan-PLA_2 inhibitor. Among PLA_2 isozymes, mRNA of group IIA $sPLA_2$ ($sPLA_2$-IIA) was upregulated following PAR1 stimulation for 6–18 h, whereas protein levels of PGE synthases were unchanged. These data suggest that the delay of PGE_2 release after COX-2 upregulation triggered by PAR1 is due to the poor supply of free arachidonic acid at the early stage in RGM1 cells, and that plural isozymes of PLA_2 including $sPLA_2$-IIA may complementarily contribute to the liberation of free arachidonic acid. J. Cell. Biochem. 112: 909–915, 2011.

KEY WORDS: PROTEINASE-ACTIVATED RECEPTOR-1 (PAR1); PGE_2; COX-2; RAT GASTRIC EPITHELIAL MUCOSAL RGM1 CELLS; PHOSPHOLIPASE A_2 (PLA_2)

Prostaglandins (PGs), especially PGE_2 and PGI_2, are released continuously from the gastric mucosa, and crucially contribute to maintenance of gastric integrity [Brzozowski et al., 2005; Laine et al., 2008]. Release of PGs has been shown to be enhanced by oral administration of mild irritants [Robert et al., 1983] and short episodes of gastric ischemia [Pajdo et al., 2001]. In the stomach with mucosal injury caused by the irritants, ischemia, stress, etc., the increased release of PGE_2 by inducible cyclooxygenase-2 (COX-2), in addition to constitutive COX-1, is important for protection and healing of gastric mucosa [Mizuno et al., 1997; Brzozowski et al., 2005; Laine et al., 2008]. The increase in PGE_2 release has been reported to involve upregulation of not only COX-2, but also microsomal PGE synthase-1 (mPGES-1) [Gudis and Sakamoto, 2005; Kawao et al., 2005; Nagataki et al., 2008] and cytosolic or secretory phospholipase A_2 ($cPLA_2$ and $sPLA_2$) [Akiba et al., 2000; Murakami et al., 2000; Balsinde et al., 2002]. In addition, changes in expression and activity of Δ^6- and Δ^5-desaturase that contributes to formation of arachidonic acid from linoleic acid, are also considered to affect the release of PGs [Ramanadham et al., 2002; Nakamura and Nara, 2003; Das, 2007].

Proteinase-activated receptors (PARs), a family of G-protein-coupled receptors, are activated by proteolytic unmasking of the cryptic tethered ligand present in the extracellular N-terminal domain by various proteinases [Kawabata, 2002; Sekiguchi and Kawabata, 2004; Steinhoff et al., 2005]. Among the four members of PARs, PAR1, PAR2, and PAR4, but not PAR3, are non-enzymatically activated by the synthetic PAR-activating peptides (PAR-APs), for example, TFLLR-NH_2 for PAR1, SLIGRL-NH_2 for

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology, Japan, 2008–2012; Grant number: S0801975.

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Received 26 May 2010; Accepted 14 December 2010 • DOI 10.1002/jcb.23005 • © 2010 Wiley-Liss, Inc.
Published online 29 December 2010 in Wiley Online Library (wileyonlinelibrary.com).

PAR2, and AYPGKF-NH_2 for PAR4 [Kawabata, 2002; Sekiguchi and Kawabata, 2004; Steinhoff et al., 2005]. We have previously reported that PAR1 and PAR2 play a protective role in the gastric mucosa, and that the protective effect of PAR1, but not PAR2, is dependent on endogenous PGs [Kawabata et al., 2001; Kawabata et al., 2004]. Because PAR1 is enzymatically activated by thrombin and various proteinases released under inflammatory conditions [Kawabata, 2002; Sekiguchi and Kawabata, 2004; Steinhoff et al., 2005; Sekiguchi et al., 2007b; Kawabata et al., 2008], the PG-dependent gastroprotection by PAR1 activation is considered critically important in the diseased stomach with bleeding and inflammation.

We have reported that stimulation of PAR1 with thrombin or the PAR1-AP actually increases production of PGE_2 in the normal rat gastric mucosal epithelial cell line, RGM1, and that the underlying mechanisms involve upregulation of COX-2 mediated by transactivation of EGF receptors and subsequent activation of the MEK/ERK pathways [Sekiguchi et al., 2007a; Takaoka et al., 2010]. A problem to be solved is the considerable time lag between the PAR1-triggered release of PGE_2 and up-regulation of COX-2; that is, the former event takes 18 h to occur, while the latter develops and reaches a plateau within 1–6 h [Sekiguchi et al., 2007a]. In the present study, we thus attempted to clarify the mechanisms for the delay of PGE_2 release induced by activation of PAR1 in RGM1 cells.

MATERIALS AND METHODS

CHEMICALS

A PAR1-activating peptide (PAR1-AP), TFLLR-NH_2, was synthesized and purified by high-performance liquid chromatography (HPLC), and the concentration and purity were determined by HPLC or mass spectrometry. Arachidonic acid, linoleic acid, bromoenol lactone (BEL), CP-24879, quinacrine, and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). Thioetheramide-PC (Thio-PC) was obtained from Cayman Chemical (Ann Arbor, MI), and arachidonyl trifluoromethyl ketone ($AACOCF_3$) was from Calbiochem (Darmstadt, Germany). TFLLR-NH_2 was dissolved in saline, and arachidonic acid and Thio-PC were in ethanol. Linoleic acid and quinacrine were dissolved in distilled water, and CP-24879, $AACOCF_3$, and BEL were in DMSO.

CELL CULTURE

RGM1, the normal rat gastric mucosal epithelial cell line [Kobayashi et al., 1996], was provided by Riken Cell Bank (Tsukuba, Japan). The cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma–Aldrich) supplemented with 20% fetal bovine serum (FBS) (Thermo, Melbourne, Australia) and 50 mg/L kanamycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan).

DETERMINATION OF PGE_2 PRODUCTION

RGM1 cells (1.5×10^5 cells/well of 6-well plate) were grown in the 20% FBS-containing medium for 24 h, and then cultured in the 1% FBS-containing medium overnight. Small volume (6 μl) of the culture medium was repeatedly collected before and after stimulation with TFLLR-NH_2. The amount of released PGE_2 was determined using an EIA kit (Cayman Chemical), and calculated by subtracting the basal value at time 0 (before stimulation) from that at each time point. Arachidonic acid or linoleic acid was added simultaneously with the PAR1-AP, TFLLR-NH_2. Inhibitors were applied 30 min before the stimulation with TFLLR-NH_2.

LACTATE DEHYDROGENASE (LDH) RELEASE ASSAY

RGM1 cells were cultured in the 1% FBS-containing medium overnight and then stimulated with TFLLR-NH_2 alone or in combination with arachidonic acid or linoleic acid for 18 h. After the stimulation, activity of LDH released into the medium (released LDH) and collected from the cells lysed with 1% Triton X-100 (total LDH) was measured with an LDH cytotoxicity detection kit (Takara Bio, Shiga, Japan). The percentage of the released LDH to the total LDH was calculated.

REVERSE-TRANSCRIBED-POLYMERASE CHAIN REACTION (RT-PCR)

RGM1 cells, after pre-cultured overnight in the 1% FBS-containing medium, were stimulated with TFLLR-NH_2 at 100 μM for 1, 3, 6, 12, and 18 h, and then lysed in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA extracted from the cell lysate 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′-AAG GCC AAG TGA CAC CAG CC-3′ and 5′-GAA ACA GAG CAA CGA GAT GGG-3′ for rat $cPLA_2$; 5′-AAG TGA ACG TGT TCG AGA GG-3′ and 5′-GAT GAT GCG GCT GTG ATG G-3′ for Ca^{2+}-independent PLA_2 ($iPLA_2$); 5′-TTG CCA TTG TGG TGT GGG TGG-3′ and 5′-CAA CTG GGC GTC TTC CCT TTG C-3′ for group IIA secretary PLA_2 ($sPLA_2$-IIA); 5′-CCC TAA GGA TGG CAC TGA TTG G-3′ and 5′-CCG GTC ACA AGC ACA AAG CC-3′ for group V $sPLA_2$ ($sPLA_2$-V). PCR cycles were 25 for GAPDH, or 35 for $cPLA_2$, $iPLA_2$, $sPLA_2$-IIA, and $sPLA_2$-V. The PCR products (452 bp for GAPDH, 453 bp for $cPLA_2$, 322 bp for $iPLA_2$, 300 bp for $sPLA_2$-IIA, and 172 bp for $sPLA_2$-V) were visualized by 2% agarose gel electrophoresis followed by the ethidium bromide staining.

REAL-TIME PCR

Real-time PCR was performed using Light Cycler 480 (Roche Applied Sci., Basel, Switzerland). The volume of each reaction solution was 20 μl, containing 10 μl of 2× Power SYBR Green PCR Master Mix (Applied Biosystems Japan Ltd., Tokyo, Japan), 50 ng cDNA and 0.1 μM each of forward and reverse primers. The cycling conditions of PCR were: preincubation at 50°C for 2 min followed by 95°C for 10 min; then 45 cycles of 95°C for 15 s and 60°C for 60 s. The PCR primers employed for real-time PCR were: 5′-ACA GCA TGA AGG TCC TCC TGT T-3′ and 5′-GGC TCC CCT GGA CCT GAA-3′ ($sPLA_2$-IIA), 5′-CTG GGA AGA ATG CCG TAA AGA ACT-3′ and 5′-ATC AGT GCC ATC CTT AGG GGT C-3′ ($sPLA_2$-V); and 5′-GAT GGT GAA GGT CGG TGT GAA C-3′ and 5′-TGA CTG TGC CGT TGA ACT TGC-3′ (GAPDH). Product size was 72, 86, and 176 bp for $sPLA_2$-IIA, $sPLA_2$-V, and GAPDH, respectively.

DETECTION OF PROTEIN EXPRESSION OF COX-2 AND PGE SYNTHASES BY WESTERN BLOTTING

RGM1 cells were stimulated with TFLLR-NH_2 at 100 μM for 1, 3, 6, 12, or 18 h in the 1% FBS-containing medium, and then lysed in sodium dodecyl sulfate (SDS) buffer (2% SDS, 62.5 mM Tris–HCl, and 10% glycerol, pH 6.8). Protein samples (10 μg for GAPDH, 30 μg for other proteins) were separated by electrophoresis on 5–20% gradient SDS–polyacrylamide gel (Wako Pure Chemicals, Osaka, Japan), and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was blocked with a blocking buffer (5% skim milk, 137 mM NaCl, 0.1% Tween-20 and 20 mM Tris–HCl, pH 7.6) for 1 h at room temperature, and then incubated with each primary antibody overnight at 4°C. The primary antibodies used in the present study were: the anti-COX-2 (1:200) and anti-GAPDH (FL-335) (1:5,000) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); the anti-mPGES-1 (1:100), anti-mPGES-2 (1:200), and anti-cytosolic PGES (cPGES) (1:200) antibodies (Cayman Chemical). Anti-rabbit and anti-goat horseradish peroxidase (HRP)-linked IgG antibodies (Cell Signaling Technology, Beverly, MA) were used as secondary antibodies. Positive bands were developed by enhanced chemiluminescence detection (ECL, Western blotting detection reagent; Amersham Biosciences, Little Chalfont, UK). The resulting films were scanned and quantified using densitometric software (Scion Image downloaded from www.microsoft.com/DirectX/).

STATISTICS

Data are shown as mean ± SEM. 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.

RESULTS

DISTINCT TIME-COURSES OF COX-2 UPREGULATION AND PGE_2 RELEASE CAUSED BY PAR1 ACTIVATION IN RGM1 CELLS

In RGM1 cells, stimulation with the PAR1-AP, TFLLR-NH_2, at 100 μM induced upregulation of COX-2 protein in 1 h, an effect increasing thereafter and reaching a plateau at 6 h (Fig. 1A). Regardless of the rapid expression of COX-2 protein, PGE_2 release hardly increased even after 6 h stimulation with TFLLR-NH_2, and its significant increase was detected at 18 h (Fig. 1B). Thus, there was a considerable time lag between PAR1-triggered COX-2 upregulation and PGE_2 release, in agreement with our previous paper [Sekiguchi et al., 2007a].

ACCELERATION OF THE PAR1-TRIGGERED PGE_2 RELEASE BY ARACHIDONIC ACID OR LINOLEIC ACID IN RGM1 CELLS

Although arachidonic acid at 1 μM itself constantly and slowly increased PGE_2 release, arachidonic acid at the same concentration in combination with TFLLR-NH_2 at 100 μM caused rapid and dramatic increase in PGE_2 release even at 1 h, an effect further developing thereafter (Fig. 2A). This time course of PGE_2 release resembles that of COX-2 upregulation following PAR1 stimulation (see Fig. 1A). Similarly, linoleic acid, a source for production of arachidonic acid by Δ^5/Δ^6-desaturase, at 30 μM in combination with TFLLR-NH_2 at 100 μM dramatically accelerated

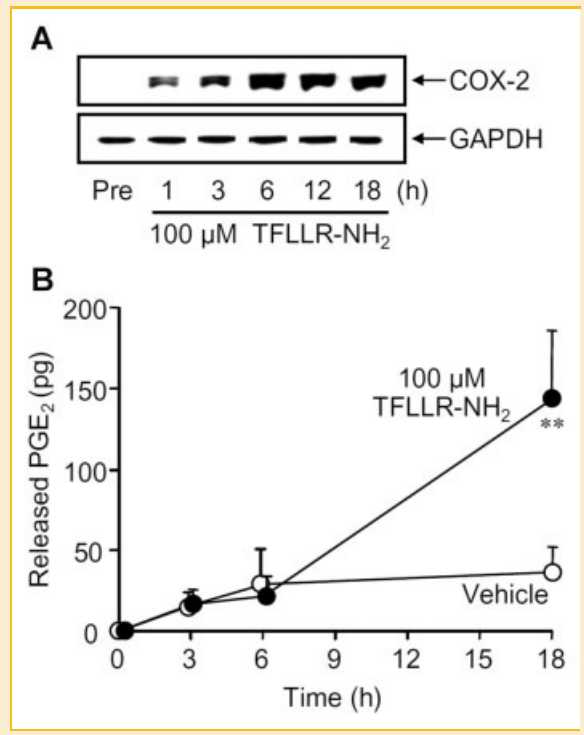

Fig. 1. Time course of COX-2 upregulation (A) and PGE_2 release (B) caused by stimulation with the PAR1-activating peptide, TFLLR-NH_2, in RGM1 cells. Note the lack of PGE_2 release at 6 h, regardless of the rapid induction of COX-2 protein in 1–6 h. $^{**}P < 0.01$ versus vehicle, n = 4.

the PAR1-triggered increase in PGE_2 release (Fig. 2B), mimicking the time course of PGE_2 release caused by arachidonic acid plus TFLLR-NH_2 (see Fig. 2A). It is to be noted that arachidonic acid and linoleic acid in concentration ranges up to 10 and 30 μM, respectively, in the presence and absence of TFLLR-NH_2 at 100 μM, did not increase LDH release at 18 h in RGM1 cells (data not shown), indicating that the PGE_2 release shown in Figure 2 was not due to the cytotoxicity of those compounds.

EVALUATION OF INVOLVEMENT OF ARACHIDONIC ACID PRODUCTION BY Δ^5/Δ^6-DESATURASE IN THE PAR1-TRIGGERED PGE_2 RELEASE IN RGM1 CELLS

As expected, an inhibitor of Δ^5/Δ^6-desaturase, CP-14879, blocked the accelerating effect of linoleic acid on the TFLLR-NH_2-induced PGE_2 release (the left panel of Fig. 3). On the other hand, the PGE_2 release caused by TFLLR-NH_2 alone was not significantly reduced by CP-14879 (the right panel of Fig. 3), suggesting that the PAR1-triggered PGE_2 release is independent of the supply of arachidonic acid via the Δ^5/Δ^6-desaturase pathway.

EFFECTS OF INHIBITORS OF PLA_2 ON THE PAR1-TRIGGERED PGE_2 RELEASE IN RGM1 CELLS

The PAR1-triggered PGE_2 release in RGM1 cells was resistant to inhibitors of $cPLA_2$ ($AACOCF_3$) (Fig. 4A) and $iPLA_2$ (BEL) (Fig. 4B), in agreement with our previous paper [Sekiguchi et al., 2007a]. An inhibitor of $sPLA_2$, thioetheramide-PC (Thio-PC), also failed to affect

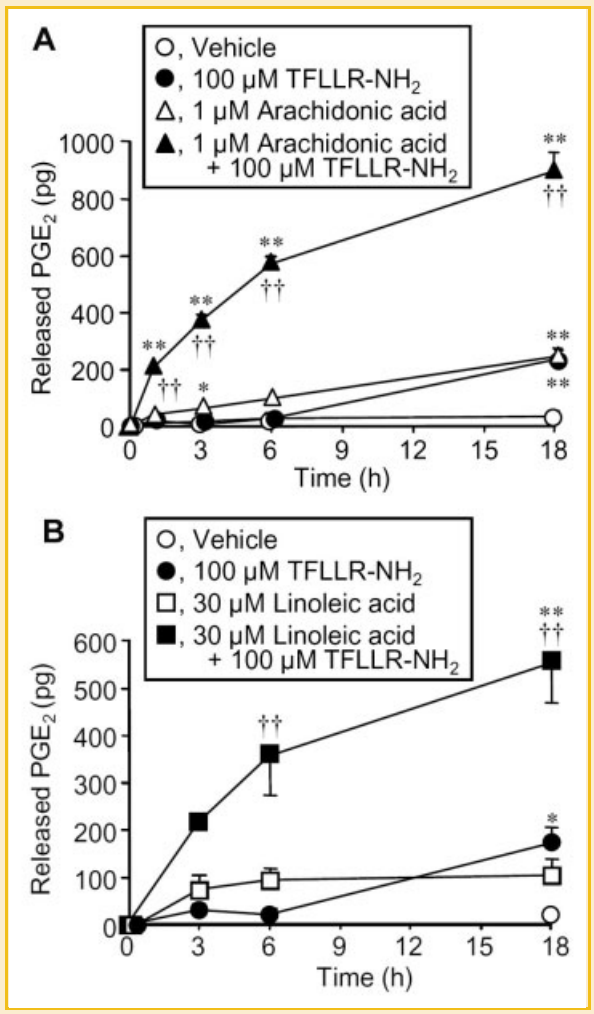

Fig. 2. Effect of arachidonic acid (A) or linoleic acid (B) on the time course of PGE_2 release caused by stimulation with the PAR1-activating peptide, TFLLR-NH_2 in RGM1 cells. Arachidonic acid or linoleic acid was applied simultaneously with TFLLR-NH_2. $^{*}P<0.05$, $^{**}P<0.01$ versus vehicle; $^{\dagger\dagger}P<0.01$ versus TFLLR-NH_2, n = 4 (A) or n = 4–12 (B).

the PGE_2 release caused by TFLLR-NH_2 (Fig. 4C). However, a mixture of inhibitors of $cPLA_2$, $iPLA_2$, and $sPLA_2$ ($AACOCF_3$, BEL, and Thio-PC) or a pan-PLA_2 inhibitor, quinacrine, completely suppressed the PGE_2 release caused by TFLLR-NH_2 (Fig. 4D,E), suggesting that multiple isozymes of PLA_2 may contribute to the PAR1-triggered delayed PGE_2 release in a complementary manner.

EFFECT OF PAR1 ACTIVATION ON EXPRESSION OF mRNA FOR ISOZYMES OF PLA_2 IN RGM1 CELLS

In the RT-PCR analysis, the levels of mRNA for $sPLA_2$-IIA and $sPLA_2$-V tended to elevate after PAR1 stimulation for 1–3 and 6–18 h, respectively, whereas mRNA of $cPLA_2$ and $iPLA_2$ did not change after the stimulation (Fig. 5A). The quantitative analysis of mRNA levels by the real-time PCR method showed that mRNA levels of $sPLA_2$-IIA, but not $sPLA_2$-V, significantly elevated after PAR1 stimulation for 6–18 h (Fig. 5B).

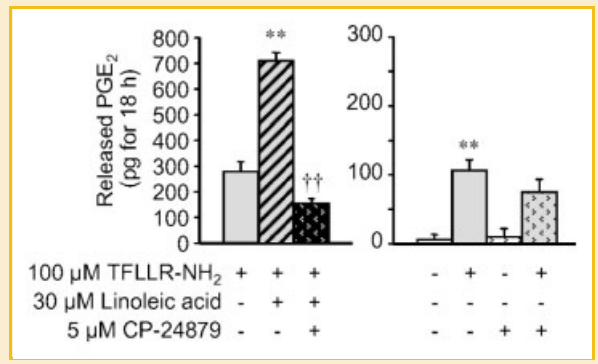

Fig. 3. Effect of CP-24879, an inhibitor of Δ^5/Δ^6-desaturase, on the PGE_2 release caused by stimulation with the PAR1-activating peptide, TFLLR-NH_2, in combination with linoleic acid (left panel), or alone (right panel) in RGM1 cells. Note that CP-24879 blocked the increase in PGE_2 release caused by stimulation with a combination of TFLLR-NH_2 and linoleic acid (A), but not by TFLLR-NH_2 alone (B). $^{**}P<0.01$ versus TFLLR-NH_2 (left panel) or versus vehicle (right panel); $^{\dagger\dagger}P<0.01$ versus TFLLR-NH_2 + linoleic acid, n = 4.

LACK OF EFFECT OF PAR1 ACTIVATION ON EXPRESSION OF PGE SYNTHASES IN RGM1 CELLS

Finally, we determined protein levels of PGE synthases following PAR1 stimulation by Western blotting. Expression levels of mPGES-

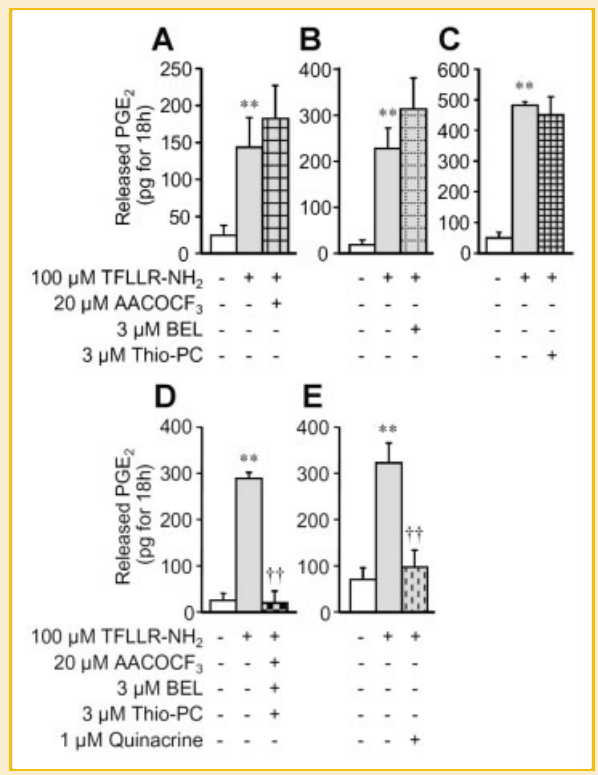

Fig. 4. Effect of inhibition of three isozymes of PLA_2 on the PAR1-triggered PGE_2 release in RGM1 cells. Inhibitors of $cPLA_2$ ($AACOCF_3$) (A), $iPLA_2$ (BEL) (B) and $sPLA_2$ (Thio-PC) (C), their mixture (Thio-PC plus $AACOCF_3$ plus BEL) (D), or a pan-PLA_2 inhibitor (quinacrine) (E) were applied 30 min before stimulation with TFLLR-NH_2. $^{**}P<0.01$ versus vehicle; $^{\dagger\dagger}P<0.01$ versus TFLLR-NH_2, n = 4.

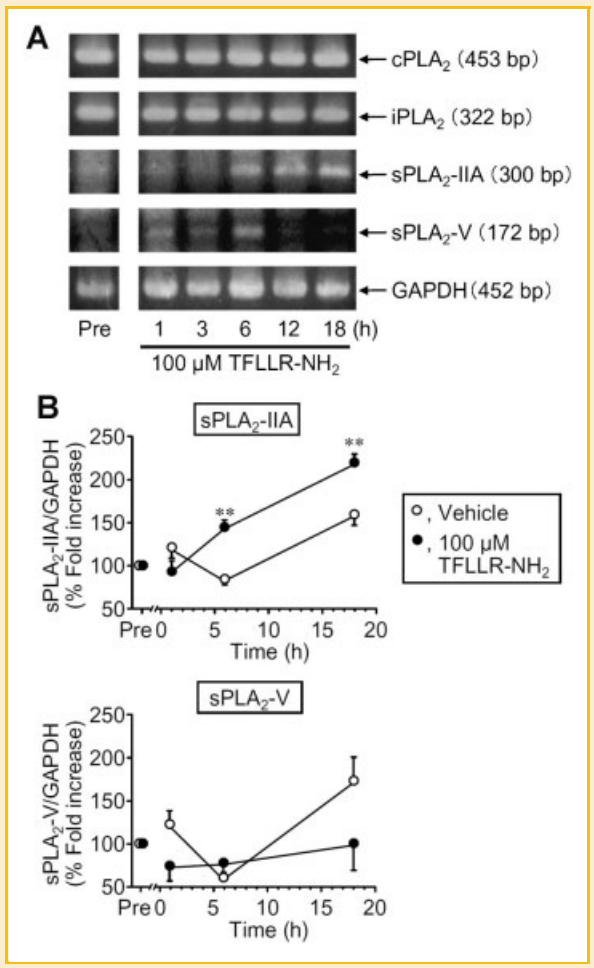

Fig. 5. Effect of PAR1 activation on expression of mRNA of PLA_2 in RGM1 cells. A: Detection of mRNA by gel electrophoresis. Parentheses show the size of PCR products. PCR cycles were 25 for GAPDH and 35 for others. $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; $sPLA_2$-IIA, group IIA secretory PLA_2; $sPLA_2$-V, group V $sPLA_2$; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. B: Real-time PCR analysis of $sPLA_2$-IIA and $sPLA_2$-V. $^{**}P < 0.01$ versus vehicle, n = 5–6.

1, mPGES-2, and cPGES proteins did not change after stimulation with TFLLR-NH_2 for 1–18 h (Fig. 6A,B).

DISCUSSION

From the present study, it appears that the delayed onset of PGE_2 release after the upregulation of COX-2 following PAR1 stimulation is due to slowly developing liberation of free arachidonic acid from the nuclear or plasma membrane in normal rat gastric mucosal epithelial RGM1 cells. The results from the standard RT-PCR and quantitative real-time PCR analyses suggest that the delayed upregulation of $sPLA_2$-IIA might be responsible for the delayed liberation of free arachidonic acid, although the data from inhibition experiments imply that multiple PLA_2 isozymes should complementarily contribute to the arachidonic acid release. Further, our data show that neither Δ^6/Δ^5-desaturase activity nor expression levels of mPGES-1, mPGES-2, and cPGES are associated with the delay of the PAR1-triggered PGE_2 release in RGM1 cells.

In the previous paper, we have shown that the MEK/ERK pathway and transactivation of EGF receptors are essential for the PAR1-triggered COX-2 upregulation and PGE_2 formation in RGM1 cells [Sekiguchi et al., 2007a]. However, as shown in Figure 1, COX-2 upregulation was observed 1 h after stimulation with the PAR1-AP, albeit PGE_2 release did not occur even 6 h after the stimulation. Liberation of free arachidonic acid from the nuclear and/or plasma membrane is one of the rate limiting steps for PGE_2 production. Actually, in the present study, when arachidonic acid or linoleic acid, a source of arachidonic acid, was added into the culture medium, PGE_2 release almost paralleled COX-2 upregulation following PAR1 activation (see Figs. 1A and 2). These results clearly demonstrate that the delayed supply of free arachidonic acid is the main cause for the time lag between the PAR1-triggered COX-2 upregulation and PGE_2 formation in RGM1 cells. It is known that intracellular levels of PGs, short-lived lipid messengers, are not detectable in most cells, since PGs are rapidly de novo synthesized in response to various stimuli, and then immediately diffuse across the plasma membrane [Legler et al., 2010]. Thus, it is not likely that internal storage of PGE_2 is associated with the delayed release of PGE_2 in the present study.

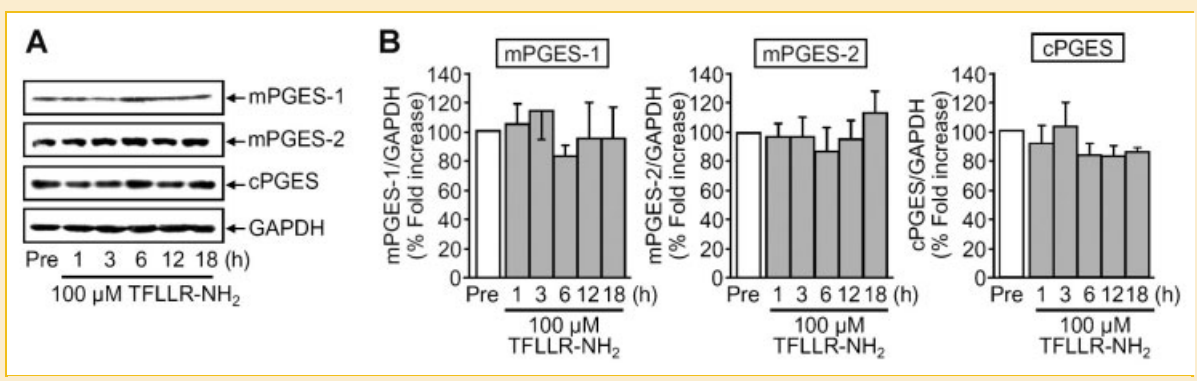

Fig. 6. Lack of effect of PAR1 activation on the protein levels of various PGE synthases in RGM1 cells. A: Representative photographs of Western blotting. mPGES, microsomal PGE synthase; cPGES, cytosolic PGES; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. B: Quantified protein levels of mPGES-1, mPGES-2, and cPGES, n = 4.

Two possibilities may be considered to interpret the poor supply of free arachidonic acid in RGM1 cells; that is, (1) the amount of membrane-bound arachidonic acid may be poor in RGM1 cells, and (2) PLA_2s that liberate free arachidonic acid from the membrane may be poor in a resting state and need to be upregulated for the supply of free arachidonic acid after PAR1 stimulation. Our findings that linoleic acid, like arachidonic acid, when applied in combination with the PAR1-AP, accelerated the PGE_2 release in a manner dependent on Δ^6/Δ^5-desaturase (see Figs. 2B and 3A), suggest that the lipid metabolism for the formation of membrane-bound arachidonic acid from linoleic acid may normally function in RGM1 cells. In addition, the activity of Δ^6/Δ^5-desaturase is not considered to be associated with the delayed PGE_2 release, because the PGE_2 release caused by stimulation with PAR1-AP alone for 18 h was not suppressed by an inhibitor of Δ^6/Δ^5-desaturase (CP-24879) (see Fig. 3). Multiple isozymes of PLA_2 appear to complementarily contribute to the liberation of free arachidonic acid from the cell membrane in RGM1 cells, since quinacrine, a pan-PLA_2 inhibitor, as well as the mixture, but not each, of selective inhibitors of $cPLA_2$, $iPLA_2$, and $sPLA_2$, suppressed the PAR1-triggered PGE_2 release (see Fig. 4). It is to be noted that quinacrine, a pan-PLA_2 inhibitor, also inhibits acetylcholine receptors, glibenclamide-sensitive K^+-currents, and monoamine oxidase (MAO) [Sakuta and Yoneda, 1994; Holscher, 1995]. Nonetheless, they do not appear to be directly associated with the inhibitory effect on the PAR1-triggered PGE_2 production in RGM1 cells, since it is unlikely that acetylcholine, catecholamines, and K^+-currents are involved in the PGE_2 production in RGM1 cells.

Among PLA_2 isozymes, $sPLA_2$-IIA and $sPLA_2$-V are upregulated in concordance with induction of COX-2 after proinflammatory stimuli [Kuwata et al., 1998; Shinohara et al., 1999; Murakami et al., 2000]. In the present study, mRNA for $sPLA_2$-IIA was significantly upregulated by activation of PAR1. The delayed liberation of free arachidonic acid following PAR1 activation may be explained by the delayed upregulation of $sPLA_2$-IIA in RGM1 cells, since the time course of the upregulation of $sPLA_2$-IIA mRNA nearly paralleled with the PAR1-triggered PGE_2 production (see Figs. 1 and 5). The mechanisms for the PAR1-triggered $sPLA_2$-IIA upregulation remain to be clarified by future studies. It has been reported that the promoter region of $sPLA_2$-IIA gene contains TATA and CCAAT boxes as well as several elements required for binding of transcription factors such as activator protein-1 (AP-1), CCAAT/enhancer-binding proteins (C/EBPs), cyclic AMP response element-binding protein (CREB), nuclear factor-κB (NF-κB), signal transducer and activator of transcription (STAT), and peroxisome proliferator-activated receptor-γ (PPARγ) [Murakami and Kudo, 2004]. The NF-κB pathway might be involved in the PAR1-triggered $sPLA_2$-IIA upregulation in RGM1 cells, since we recently found that an inhibitor of NF-κB, pyrrolidine dithiocarbamate (PDTC), suppressed the PGE_2 release, but not the upregulation of COX-2 expression, caused by PAR1 activation (data not shown). This possibility has yet to be examined by future inhibition experiments. It is not consistent that $sPLA_2$-IIA was upregulated, while Thio-PC, a selective $sPLA_2$ inhibitor, had no effect on the PGE_2 production in the present study (see Fig. 4). We also tested $sPLA_2$-IIA inhibitor I as an alternative specific inhibitor for $sPLA_2$-IIA, but found that $sPLA_2$-IIA inhibitor I, like Thio-PC, exerted no inhibitory effect (data not shown). Considering the significant inhibitory effects of a pan-PLA_2 inhibitor (quinacrine) or a mixture of three PLA_2 inhibitors ($AACOCF_3$, BEL, and Thio-PC), it can be speculated that inhibition of one isozyme of PLA_2 might result in upregulation of other isozymes of PLA_2, leading to the lack of effect of each inhibitor. Therefore, it would be particularly of interest to determine expression levels of $cPLA_2$ and $iPLA_2$ in the presence of the $sPLA_2$ inhibitor in future studies. Silencing of $sPLA_2$-IIA by siRNA or shRNA strategies is considered an effective method to determine the involvement of $sPLA_2$-IIA in the PAR1-triggered PGE_2 production. However, RGM1 cells were too vulnerable to transfection reagents, to be used for gene silencing experiments. Distinct transfection reagents that we tested easily made the cells detached from the bottom of culture dishes. Thus, it seems critical to select a transfection reagent with low toxicity, in order to achieve effective silencing of $sPLA_2$-IIA in RGM1 cells.

We have shown that in the human pulmonary type II-like adenocarcinoma A549 cells, the PAR2-triggered PGE_2 is associated with upregulation of mPGES-1 in addition to COX-2 [Kawao et al., 2005; Nagataki et al., 2008]. However, in the present study, protein levels of mPGES-1, mPGES-2, and cPGES were not altered by PAR1 activation. It is known that cPGES and mPGES-2 are constitutively expressed, whereas mPGES-1, like COX-2, is upregulated under inflammatory conditions or in cancer development, and metabolically couples with COX-2, leading to a large amount of PGE_2 production [Samuelsson et al., 2007]. However, it has also been reported that mPGES-1, but not COX-2, is constitutively expressed in some organs including the stomach, spleen, kidney, and lung in mice [Boulet et al., 2004], where mPGES-1 expression might not necessarily parallel with COX-2 expression. This notion might interpret why mPGES-1 expression was not coupled to upregulation of COX-2 after PAR1 stimulation in the present study, considering that mPGES-1 was constitutively expressed in RGM1 cells (see Fig. 6).

It is of interest that the mechanism for PGE_2 release caused by activation of PAR1 in RGM1 cells, as shown in the present and previous studies [Sekiguchi et al., 2007a], are greatly different from that for PAR2-triggered PGE_2 release in A549 cells [Kawao et al., 2005], despite that PAR1 and PAR2 share a common G-protein, Gq [Steinhoff et al., 2005]. Thus, the data shown in the present study imply that the release of free arachidonic acid is a rate limiting step in the PAR1-triggered, COX-2-dependent PGE_2 release in RGM1 cells, and provide a novel aspect for intracellular signals following activation of PAR1, which might be useful for understanding of the gastric functions of PAR1 in health and disease.

ACKNOWLEDGMENTS

This work was supported by Antiaging Center Project for Private Universities from Ministry of Education, Culture, Sports, Science and Technology, Japan, 2008–2012 S0801975.

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