J Pharmacol Sci **111**, 269 – 275 (2009)

Journal of Pharmacological Sciences

Full Paper

Proteinase-Activated Receptor-2–Triggered Prostaglandin E_2 Release, but Not Cyclooxygenase-2 Upregulation, Requires Activation of the Phosphatidylinositol 3–Kinase / Akt / Nuclear Factor-κB Pathway in Human Alveolar Epithelial Cells

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Received May 21, 2009; Accepted September 8, 2009

Abstract. Proteinase-activated receptor-2 (PAR2) triggers upregulation of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) formation in human alveolar epithelial A549 cells. This COX-2 upregulation appears to involve the Src / epidermal growth factor (EGF) receptor / p38 MAP kinase (p38MAPK) pathway and also the cAMP-response element–binding protein (CREB) pathway. Here, we investigated the roles of nuclear factor-κB (NF-κB)–related signals in the PAR2-triggered PGE_2 release / COX-2 upregulation in A549 cells. The PAR2-triggered PGE_2 release was clearly blocked by an inhibitor of the NF-κB pathway. Stimulation of PAR2 actually caused phosphorylation of inhibitor-κB, an indicator of NF-κB activation, an effect being blocked by inhibitors of MEK, phosphatidylinositol 3–kinase (PI3-kinase), and Akt, but little or not by inhibitors of p38MAPK and JNK. Stimulation of PAR2 also caused phosphorylation of Akt, an effect suppressed by inhibitors of PI3-kinase and MEK. Nonetheless, the PAR2-triggered upregulation of COX-2 was resistant to inhibitors of NF-κB, PI3-kinase, and Akt, but was attenuated by inhibitors of MEK and JNK. Stimulation of PAR2 induced phosphorylation of CREB, an effect abolished by an inhibitor of MEK but not inhibitors of p38MAPK and EGF receptor. These findings demonstrate that the MEK / ERK / PI3-kinase / Akt / NF-κB pathway is involved in PAR2-triggered PGE_2 formation, but not upregulation of COX-2 that is dependent on activation of ERK/CREB and JNK in addition to p38MAPK.

Keywords: proteinase-activated receptor-2 (PAR2), nuclear factor-κB (NF-κB), prostaglandin E_2 (PGE_2), cyclooxygenase-2 (COX-2), human alveolar epithelial cell line A549

Introduction

Proteinase-activated receptors (PARs), a family of G protein–coupled seven trans-membrane receptors consisting of PAR1 to PAR4, are activated by proteolytic cleavage of the extracellular N-terminal of the receptor followed by generation of a tethered ligand that interacts with the extracellular loop 2 of the receptor (1). PAR1, PAR3, and PAR4 are the receptors for thrombin, whereas PAR2 is insensitive to thrombin, but can be activated by trypsin, mast cell tryptase, coagulation factors VIIa and Xa, acrosin, human tissue kallikrein, and so on (2 – 7). PAR1, PAR2, and PAR4, but not PAR3, can also be selectively activated by short synthetic peptides based on the tethered ligand sequence (8).

PAR2 is considered to be a primarily pro-inflammatory molecule, and intriguingly responds to certain exogenous proteinases such as mite allergens, Der p1, Der p3 and Der p9; serine proteinases in cockroach extract; purified salmon trypsin; and so on (5, 6, 9). Particularly, in the respiratory system, PAR2 is abundantly expressed in the epithelial cells, and its activation by the endogenous and exogenous proteinases leads to release of pro-inflammatory mediators including cyto-

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Published online in J-STAGE on October 31, 2009 (in advance)
doi: 10.1254/jphs.09155FP

kines and prostanoids (10 – 12). It is also to be noted that the pro-inflammatory role of PAR2 has been well described in the gastrointestinal tract (13), kidney (14), skin (15), synovial joints (16), and so on.

In human alveolar epithelial A549 cells, PAR2 stimulation causes prostaglandin E_2 (PGE_2) formation accompanied by upregulation of cyclooxygenase-2 (COX-2) and also release of pro-inflammatory cytokines (17 – 20). Our signal transduction studies have shown that PAR2-triggered PGE_2 production involves formation of arachidonic acid via the MEK / ERK / cytosolic phospholipase A_2 ($cPLA_2$) pathway and upregulation of COX-2 via the Src / epidermal growth factor (EGF) receptor / p38 MAP kinase (p38MAPK) pathway in A549 cells (18, 21). An independent study has also suggested possible involvement of the cAMP-response element–binding protein (CREB) pathway in the PAR2-mediated upregulation of COX-2 in the same cell line (22).

Nuclear factor-κB (NF-κB) regulates the transcription of a diverse number of inflammatory gene products such as inducible nitric oxide synthase (iNOS), COX-2, tumor necrosis factor α, interleukin-8, ICAM-1, and so on (23, 24). In A549 cells, PAR2 stimulation actually causes activation of the NF-κB pathway (9, 20), whereas it has yet to be examined whether the NF-κB pathway is involved in the PAR2-mediated pro-inflammatory responses. In the present study, we thus evaluated the roles of the NF-κB pathway in the PAR2-triggered PGE_2 release and upregulation of COX-2 in A549 cells and also analyzed the upstream and downstream signals of the NF-κB activation.

Materials and Methods

Materials

The PAR2-activating peptide Ser-Leu-Ile-Gly-Arg-Leu-amide (SLIGRL-NH_2) was synthesized and purified by high-performance liquid chromatography (HPLC), and its composition and purity were determined by HPLC or mass spectrometry. U0126 and pyrrolidine-dithiocarbamate (PDTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA); and SB203580, SP600125, MG-132, BAY11-7082, and PD153035 were from Calbiochem (Darstadt, Germany). Perifosine was obtained from Cayman Chemical (Ann Arbor, MI, USA), and LY294002 was from Tocris Bioscience (Ellisville, MO, USA). SLIGRL-NH_2 was dissolved in saline, and PDTC was dissolved in distilled water. Perifosine was first dissolved in ethanol (Wako, Osaka) and all other inhibitors were dissolved in DMSO (Sigma-Aldrich). The final concentration of ethanol and DMSO was 0.1%.

Cell culture

A549 cells, derived from a human alveolar type II-like adenocarcinoma, were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplied with 10% heat-inactivated fetal bovine serum (FBS; Thermo Electron Corporation, Waltham, MA, USA) and 50 mg/L kanamycin (Meiji Seika, Tokyo). The cells were grown in the above medium for 24 h and then cultured in the serum-free medium overnight followed by stimulation with the PAR2-activating peptide SLIGRL-NH_2.

Determination of PGE_2 release

A549 cells (1.5×10^5 cells/well), cultured in 6-well culture plates, were stimulated with SLIGRL-NH_2 for 3 h, and the supernatant in a small volume (6 μL) was collected for the assay of PGE_2. The supernatant was diluted 10-fold with the enzyme immunoassay (EIA) buffer, and the amount of PGE_2 in the diluted supernatant was determined using a PGE_2 EIA kit (Cayman Chemical). The released PGE_2 was represented as the difference between the amounts of PGE_2 before and after stimulation. PDTC and LY294002 were added to the culture medium 30 min before and perifosine was added 10 min before the stimulation.

Western blotting

After the stimulation with SLIGRL-NH_2, A549 cells were washed with cold PBS, and then lysed with the lysis buffer containing 2% sodium dodecyl sulfate (SDS), 62.5 mM Tris-HCl (pH 6.8), and 10% glycerol. Protein samples (10 μg protein/well for GAPDH; 30 μg protein/well for COX-2; 50 μg protein/well for Akt, phospho-Akt, phospho-I-κB, and phospho-CREB) were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA, USA). The following antibodies were used: anti-phospho-I-κB-α (Ser32) (1:500), anti-phospho-Akt (Ser473) (1:100), and anti-Akt antibodies (1:1000) (Cell Signaling Technology, Beverly, MA, USA); anti-COX-2 (1:200) and anti-GAPDH (FL-335) (1:5000) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phospho-ATF1 (Ser63) / CREB (Ser133) (1:500) antibody (Upstate, Lake Placid, NY, USA). Anti-rabbit and anti-goat horseradish peroxidase (HRP)–linked IgG antibodies (Cell Signaling Technology) were used as secondary antibodies. Positive bands were developed by enhanced chemiluminescence detection (ECL, Western blotting detection reagent; Amersham Biosciences, Little Chalfont, UK).

Statistics

Data are expressed as the means ± S.E.M. Statistical significance was analyzed by ANOVA followed by Tukey's test for multiple comparisons. Significance was set at the $P<0.05$ level.

Results

Involvement of the NF-κB pathway and its upstream MAP kinase signaling in the PGE_2 release caused by PAR2 activation in A549 cells

SLIGRL-NH_2, the PAR2-activating peptide, at 100 μM caused a significant increase in PGE_2 release in A549 cells, and this effect was clearly blocked by PDTC, an inhibitor of NF-κB, at 50 μM (Fig. 1A). To ask whether PAR2 triggers NF-κB signaling, phosphorylation of I-κB, an indicator of NF-κB activation, was determined by Western blotting. PAR2 stimulation with SLIGRL-NH_2 for 30 – 120 min caused phosphorylation of I-κB (Fig. 1B). An inhibitor of MEK (U0126), but not of JNK (SP600125), abolished the phosphorylation of I-κB induced by SLIGRL-NH_2 (Fig. 1C). An inhibitor of p38MAPK (SB203580) slightly suppressed the PAR2-triggered phosphorylation of I-κB (Fig. 1C), but the effect was not statistically significant when quantified by densitometry; the quantified value for SB203580 + SLIGRL-NH_2 was 81.2 ± 22.4% (n = 5) (as the percentage of the value for SLIGRL-NH_2 alone).

Involvement of the phosphatidylinositol 3–kinase (PI3K) / Akt pathway in the PAR2-triggered NF-κB activation and PGE_2 release

The PI3K / Akt pathway is capable of activating I-κB kinase α (IKKα), followed by phosphorylation of I-κB and activation of NF-κB (25). Therefore, we next asked if the PI3K / Akt pathway is involved in the PAR2-triggered NF-κB signaling and PGE_2 release in A549 cells. LY294002, an inhibitor of PI3K, and perifosine, an inhibitor of Akt, clearly decreased the phosphorylation of I-κB caused by SLIGRL-NH_2 at 100 μM (Fig. 2A). LY294002 and perifosine also significantly attenuated the PAR2-triggered PGE_2 release (Fig. 2B). Stimulation of PAR2 with SLIGRL-NH_2 indeed evoked transient phosphorylation of Akt at 5 min (Fig. 2C), and this effect was blocked by LY294002 (Fig. 2D). In addition, the PAR2-triggered phosphorylation of Akt was partially suppressed by the MEK inhibitor U0126 (Fig. 2D).

Intracellular signaling underlying the PAR2-triggered upregulation of COX-2 protein

There is much in vitro and in vivo evidence that the NF-κB pathway contributes to upregulation of COX-2 in various cells and/or organs (26 – 28). In this context, we examined whether activation of the NF-κB pathway is involved in the PAR2-triggered upregulation of COX-2 in A549 cells. Surprisingly, distinct inhibitors of NF-κB, PDTC, MG-132, and BAY11-7082, failed to attenuate the increased expression of COX-2 protein caused by PAR2 stimulation (Fig. 3A). In addition, the COX-2 upregulation was also resistant to inhibitors of PI3K and Akt, LY294002 and perifosine, respectively (Fig. 3B), that blocked the PAR2-triggered phosphorylation of I-κB and PGE_2 formation (see Fig. 2A). Furthermore, it should be noted that inhibitors of MEK and

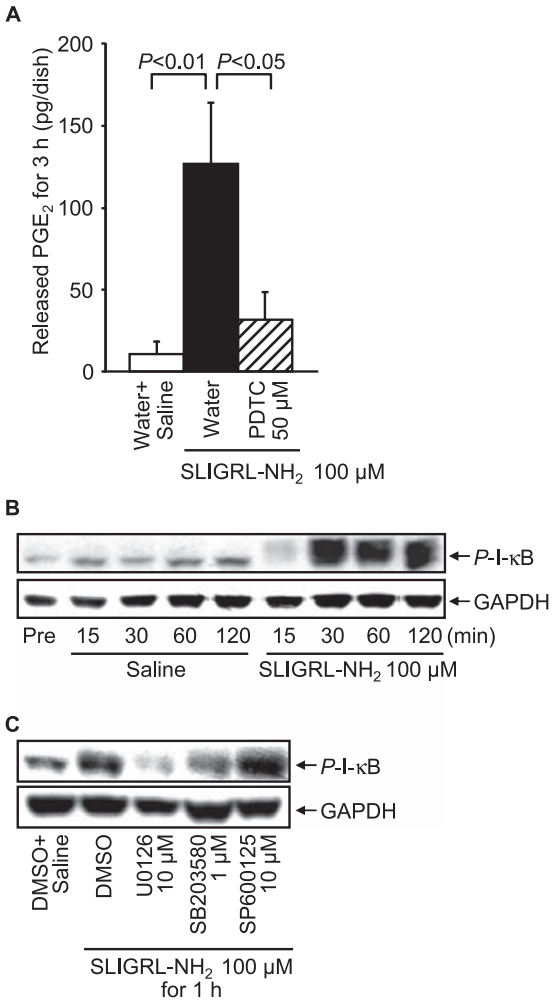

Fig. 1. The NF-κB pathway contributes to the PAR2-triggered PGE_2 release in A549 cells. A) Inhibitory effect of PDTC, an inhibitor of NF-κB, on the increase in PGE_2 release caused by stimulation with SLIGRL-NH_2, a PAR2-activating peptide, at 100 μM for 3 h. Data show the mean ± S.E.M. from 8 experiments. B) Time course of phosphorylation of I-κB caused by stimulation with SLIGRL-NH_2 at 100 μM. C) Effects of inhibitors of MEK (U0126), p38MAPK (SB203580), and JNK (SP600125) on the PAR2-triggered phosphorylation of I-κB at 1 h. Each inhibitor was added 30 min before stimulation with SLIGRL-NH_2. *P*-I-κB, phosphorylated I-κB.

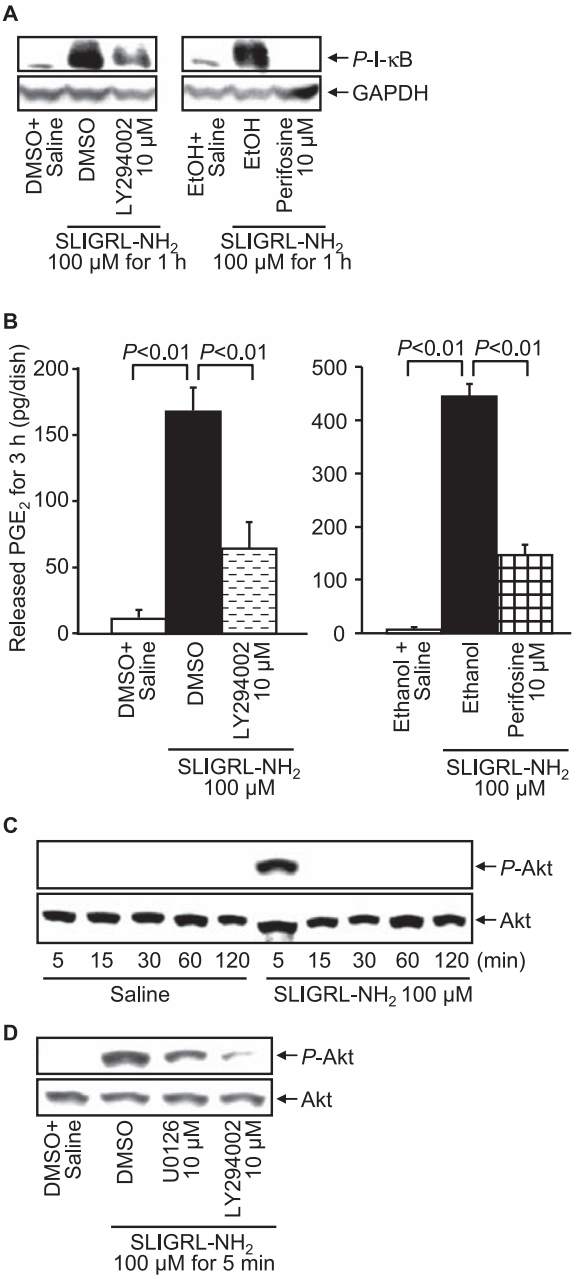

Fig. 2. The PI3-kinase / Akt pathway is necessary for the PAR2-triggered activation of the NF-κB pathway and release of PGE_2 in A549 cells. A) Inhibitory effects of inhibitors of PI3-kinase, LY294002, and Akt, perifosine, on the PAR2-triggered phosphorylation of I-κB at 1 h. B) Inhibitory effect of LY294002 and perifosine on the PAR2-triggered release of PGE_2 for 3 h. Data show the mean ± S.E.M. from 4 experiments. C) Time course of phosphorylation of Akt caused by stimulation with SLIGRL-NH_2 at 100 μM. D) Inhibitory effects of inhibitors of MEK (U0126) and PI3-kinase (LY294002) on the PAR2-triggered phosphorylation of Akt. Perifosine was added 3 h before, and other inhibitors were added 30 min before stimulation with SLIGRL-NH_2. *P*-Akt, phosphorylated Akt.

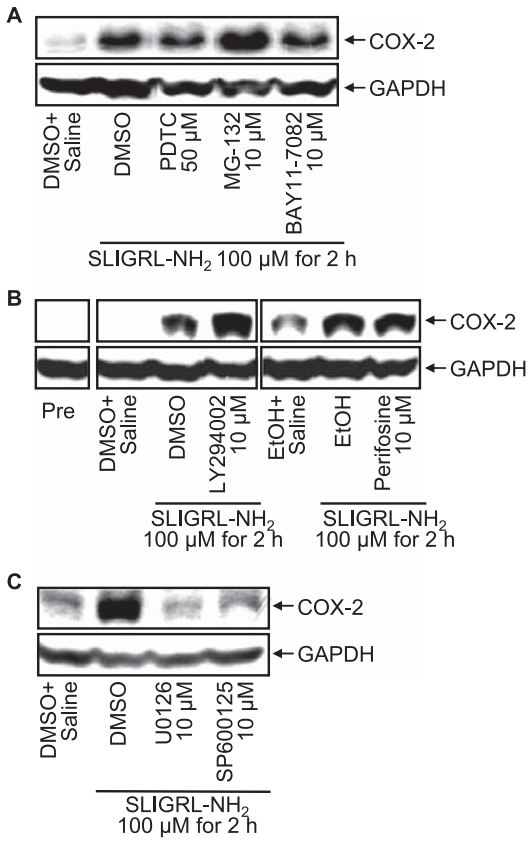

Fig. 3. The PI3-kinase / Akt/NF-κB pathway does not contribute to the PAR2-triggered upregulation of COX-2 protein in A549 cells. Inhibitors of NF-B (PDTC, MG-132, and BAY11-7082), PI3-kinase (LY294002), MEK (U0126), and JNK (SP600125) were added 30 min before and an inhibitor of Akt (perifosine) was added 3 h before stimulation with SLIGRL-NH_2 for 2 h.

JNK, U0126 and SP600125, respectively, suppressed the PAR2-triggered upregulation of COX-2 (Fig. 3C), as an inhibitor of p38MAPK did in our previous study (18).

Apart from NF-κB, CREB has been shown to be downstream of ERK or p38MAPK and mediate upregulation of COX-2 in various cells including A549 cells (22, 29, 30). In our experimental conditions, SLIGRL-NH_2 at 100 μM for 20 min caused phosphorylation of CREB, an effect suppressed by the MEK inhibitor U0126, but not by the p38MAPK inhibitor SB203580 (Fig. 4). PD153035, an inhibitor of EGF receptor–tyrosine kinase that is trans-activated by PAR2 signaling and activates the downstream p38MAPK in A549 cells (18), did not affect the PAR2-triggered phosphorylation of CREB (Fig. 4).

Discussion

We have previously shown that PAR2-triggered PGE_2 release in A549 cells involves two major signaling pathways: 1) arachidonic acid formation via the MEK

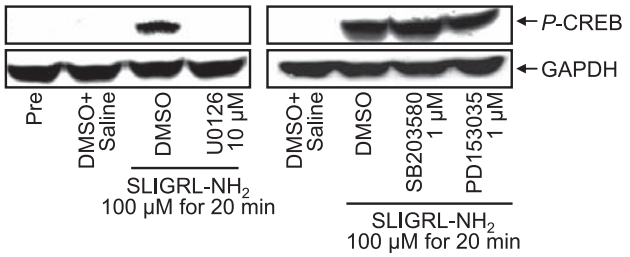

Fig. 4. Activation of PAR2 induces phosphorylation of CREB via MEK / ERK, but not p38MAPK or EGF receptor-tyrosine kinase, in A549 cells. Phosphorylation of CREB was observed after stimulation with SLIGRL-NH_2 for 20 min in the absence or presence of inhibitors of MEK (U0126), p38MAPK (SB203580), and EGF receptor-tyrosine kinase (PD153035). Each inhibitor was added 30 min before SLIGRL-NH_2. *P*-CREB, phosphorylated CREB.

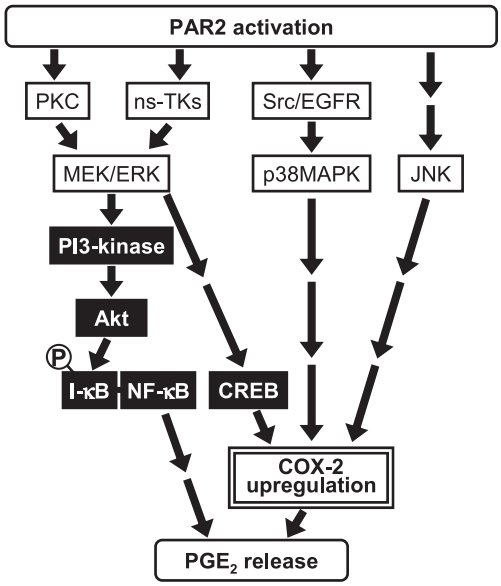

Fig. 5. Possible signal transduction mechanisms for the PAR2-triggered PGE_2 formation in A549 cells. PKC, protein kinase C; ns-TKs, non-Src genistein–sensitive tyrosine kinases; EGFR, epidermal growth factor receptor; PI3-kinase, phosphatidylinositol 3–kinase; NF-κB, nuclear factor-κB; I-κB, inhibitor of NF-κB; CREB, cAMP-response element–binding protein.

/ ERK / $cPLA_2$ pathway downstream of protein kinase C (PKC) and non-Src genistein–sensitive tyrosine kinases and 2) upregulation of COX-2 via the Src / EGF receptor / p38MAPK pathway (18). The present data add the NF-κB pathway to the signaling mechanisms underlying the PAR2-triggered PGE_2 release, but not upregulation of COX-2, in A549 cells.

Our previous studies have demonstrated that stimulation of PAR2 causes phosphorylation of all three MAP kinases, ERK, p38MAPK, and JNK, in A549 cells (18, 19). The data from inhibition experiments in the present study suggest that the PAR2-triggered NF-κB activation is mainly mediated by the MEK / ERK pathway, but not p38MAPK or JNK (see Fig. 1C). Our data provide novel evidence for involvement of PI3K and Akt in the PAR2-triggered PGE_2 formation (see Fig. 2B) and interestingly suggest that NF-κB is downstream of PI3K and Akt (see Fig. 2A). It is also clear that Akt is downstream of PI3-kinase and that the PI3-kinase / Akt pathway is downstream of the MEK / ERK pathway, considering that the transient phosphorylation of Akt induced by PAR2 stimulation was blocked by inhibitors of PI3-kinase and MEK (see Fig. 2: C and D). Together, it is suggested that the MEK / ERK / PI3-kinase / Akt pathway mediates the PAR2-triggered activation of NF-κB in A549 cells (Fig. 5).

Many papers suggest that activation of NF-κB contributes to upregulation of COX-2 in response to various pro-inflammatory stimuli (23, 24, 27, 28). There is evidence that an inhibitor of NF-κB, PG490, blocks the upregulation of COX-2 caused by a PAR2-activating peptide in human umbilical vein endothelial cells (HUVEC) (26). Therefore, we were surprised that the increased expression of COX-2 protein caused by PAR2 stimulation was resistant to specific inhibitors of NF-κB and also to inhibition of PI3-kinase and Akt in A549 cells (see Fig. 3: A and B). Thus, the PAR2-triggered activation of NF-κB downstream of the PI3-kinase / Akt pathway appears not to be involved in the upregulation of COX-2. In addition to COX-2, PLA_2 and PGE synthase (PGES) are considered as enzymes responsible for production of PGE_2. Among various isozymes of PLA_2, type IIA secretary PLA_2 ($sPLA_2$-IIA) has been shown to be upregulated in endotoxin-induced lung injury (31). There is evidence that upregulation of $sPLA_2$-IIA is mediated by the NF-κB pathway (32). Although we have previously shown that the PAR2-triggered PGE_2 production involves activation of $cPLA_2$ (18), it has yet to be clarified whether $sPLA_2$ contributes to the PGE_2 production. Thus, it is likely that the NF-κB pathway might induce upregulation of $sPLA_2$-IIA, contributing to the PAR2-triggered PGE_2 production in A549 cells. On the other hand, our previous study has shown that activation of PAR2 induces upregulation of microsomal PGES-1 (mPGES-1) through the MEK / ERK pathway (18, 21). Given the present finding that the MEK / ERK pathway was upstream of the NF-κB pathway, it may also be hypothesized that the activation of NF-κB pathway might contribute to the PAR2-triggered mPGES-1 upregulation in A549 cells.

It has been demonstrated that activation of CREB downstream of ERK or p38MAPK might contribute to upregulation of COX-2 in certain cells including A549 cells (22, 29, 30). These reports are supported by our present findings that PAR2 stimulation actually induced

phosphorylation of CREB in a manner dependent on the MEK / ERK pathway, but not the EGF receptor / p38MAPK pathway (see Fig. 4). In this context, the PAR2-triggered COX-2 upregulation is attributable to activation of two major signaling cascades, that is, the MEK / ERK / CREB pathway in addition to the Src / EGF receptor / p38MAPK pathway as shown in our previous study (18). Unfortunately, in the present study, we could not obtain direct evidence showing that CREB is upstream of the PAR2-triggered COX-2 upregulation. However, Wang et al. have reported that in A549 cells, the same cell line of ours, COX-2 upregulation caused by activation of PAR2 was suppressed by knockdown of CREB with the siRNA (22), supporting possible involvement of the MEK / ERK / CREB pathway in the PAR2-triggered COX-2 upregulation in A549 cells. Considering the blockade of COX-2 induction by an inhibitor of JNK (see Fig. 3C), it is hypothesized that all three MAP kinase pathways might synergistically induce the upregulation of COX-2 (Fig. 5). Such cooperative actions of three MAP kinase (ERK, p38MAPK, and JNK) pathways have also been reported in the LPS-induced COX-2 upregulation (33), suggesting that activation of CREB is mediated by ERK and p38MAPK, but not by JNK, while activation of activator protein-1 (AP-1) is mediated by ERK and JNK.

It has been reported that in rat alveolar type II cells, stimulation with PGE_2 enhanced surfactant secretion via EP_1 receptor (34). Lung surfactant forms a lipid monolayer at the interface of liquid and air and reduces the surface tension along the alveolar, leading to lowering the work of breathing and preventing alveolar collapse at expiration (35). In addition, it is well known that PGE_2 induces relaxation in the airways (8). Considering that PAR2 could be activated by tryptase and trypsin-like proteinases possibly released under the inflammatory condition (6), the PAR2-triggered PGE_2 release in the alveolar type II cell line might thus play a protective role in the lung during inflammation.

In summary, PAR2 activation causes MEK / ERK-dependent activation of the PI3-kinase / Akt pathway followed by NF-κB activation in A549 cells, which contributes to PGE_2 release, but not COX-2 upregulation. The PAR2-triggered upregulation of COX-2 appears to require activation of all three MAP kinase pathways, the MEK / ERK / CREB, Src / EGF receptor / p38MAPK, and also JNK pathways (Fig. 5).

Acknowledgment

This work was financially supported in part by the "Antiaging Center Project" for Private Universities from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, 2008-2012.

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