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Identification of *Cis*-acting Elements and Signaling Components of High Affinity IgE Receptor that Regulate the Expression of Cyclooxygenase-2

Dong-Im Cho[1], So-Young Kim[1], Mei Zheng[1], Mingli Jin[1], Hoo Kyun Choi[2], Ae-Kyung Yi[3] and Kyeong-Man Kim[1]

[1]Department of Pharmacology, College of Pharmacy, Chonnam National University, Gwang-Ju, [2]College of Pharmacy, Chosun University, Gwang-Ju, [3]Dept. of Pediatrics, University of Tennessee Health Science Center, Memphis, TN, USA

Key Words

COX-2 • *Cis*-acting elements • MAPK • Fyn • PI 3-kinase • PLC • Akt • PKC • ERK

Abstract

Allergic and inflammatory responses are functionally linked through a cascade of signaling events that connect the aggregation of the high affinity IgE receptor (FcεRI) on mast cells and the initiation of cyclooxygenase-2 (COX-2) expression. In this study, we identified the *cis*-acting elements in the *cox-2* promoter that control the expression of COX-2 in RBL-2H3 mast cells. We also investigated how the inflammatory reaction is controlled by the allergic reaction by determining the signaling components employed by FcεRI in the transcriptional regulation of *cox-2*. Among *cis*-acting components present in the *cox-2* promoter, the CREB binding site, as well as the AP-1 and proximal NF-IL6 binding sites to a lesser extent, were required for the transcriptional regulation of the *cox-2* promoter. However, NF-κB and Ets-1 binding sites exerted negative effects on the *cox-2* promoter activity. Among the signaling components of FcεRI, Fyn, PI 3-kinase, Akt, and p38 MAPK positively mediated the COX-2 expression. Conventional PKCs and atypical PKCs exerted opposite regulatory effects on the *cox-2* promoter activity. Blockade of MEK/ERK pathway inhibited the *cox-2* promoter activity and the COX-2 expression. These results reveal intricate functional interactions among different *cis*-acting elements in the transcriptional regulation of *cox-2*. Fyn→PI 3-kinase→Akt pathway directly stimulate. On the other hand, Lyn→Syk pathway exerts auxiliary or compensatory influences on COX-2 expression via PKC and MEK/ERK.

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Introduction

Allergy, an immediate hypersensitivity reaction, is initiated by the binding of allergen on the immunoglobulin E (IgE)-sensitized high affinity IgE receptor (FcεRI) on mast cells [1, 2], leading to the release of various inflammatory mediators. It is therefore difficult to discuss allergy and inflammation separately as the latter might occur in the process of the former. Thus, elucidation of the signaling pathways involved between the FcεRI

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Kyeong-Man Kim
Dept. of Pharmacology, College of Pharmacy
Chonnam National University, Gwang-Ju, 500-757 (Korea)
Tel. +82-62-530-2936, Fax +82-62-530-2949
E-Mail kmkim@jnu.ac.kr

activation and cyclooxygenase-2 (COX-2) expression is required for a clear understanding of the functional interaction between allergic and inflammatory reactions. Some mediators are pre-formed, stored in secretory granules of mast cells, and immediately released in response to FcεRI cross-linking. Other mediators such as cytokines and lipid-derived eicosanoids, including prostanoids, are generated *de novo* [3, 4].

Prostanoids are synthesized from arachidonic acid through the catalytic action of two different isotypes of COX [5, 6]. COX-1 is constitutively expressed in most mammalian cells, and is believed to perform "housekeeping" activities for normal cellular functions. On the other hand, COX-2 is an inducible enzyme whose expression is greatly elevated at sites of inflammation. In mast cells, the generation of prostanoids follows two different phases of prostaglandin D_2 (PGD_2) production. The short-lived initial phase is mediated by COX-1. The second phase, mediated by COX-2, occurs with prolonged exposure to allergen and leads to sustained production of PGD_2 [7-9].

The promoter region of *cox-2* contains a TATA box and various putative transcriptional regulatory elements such as NF-κB, C/EBP, CREB, and AP-1 binding sites [10, 11]. The expression of COX-2 varies depending on the cell type and the nature of the stimuli. In addition, various *cis*-acting regulatory elements, transcription factors, and signal transduction pathways are involved. Therefore, most studies on the molecular mechanism of COX-2 expression have focused on the determination of signaling pathways and transcription factors that converge on these *cis*-acting *cox-2* regulatory sites.

In mast cells, Lyn and Fyn initiates two independent and interdependent signaling pathways of FcεRI [12, 13], respectively through the adaptors LAT (linker of activated T cells) and Gab2 [14, 15]. Fyn and Gab2 are required for the activation of PI3K and Akt [15, 16], whereas LAT is essential for the activation of PLCγ [17]. Mast cell activation is suggested to be regulated by these two integrated signaling pathways. It would be interesting to understand whether each pathway independently or in collaboration regulate COX-2 expression in mast cells.

In this study, we utilized mutants of the *cox-2* promoter in which extensive mutations were introduced into putative binding sites for transcriptional factors. We also used various dominant negative mutants, constitutively active mutants, or pharmacological inhibitors of transcriptional factors or signaling components of FcεRI to explore the functional relationship between allergic and inflammatory responses in mast cells.

Materials and Methods

Materials

BAY11-7082 (NF-κB inhibitor) was purchased from Biomol (Plymouth, PA, USA). Antibodies to COX-2 were purchased from BD Bioscience (Franklin Lakes, NY, USA). SP600125 (JNK inhibitor), wortmannin (PI 3-kinase inhibitor), PMA, PP2 (Src inhibitor), piceatannol (Syk inhibitor), and U-73122 (PLC inhibitor) were purchased from Sigma/Aldrich Chemical Co (St. Louis, MO, USA). Triciribine (Akt inhibitor), Gö6976, and Gö6983 (PKC inhibitors) were purchased from EMD Biosciences (Sand Diego, CA, USA). SB 202190 HCl (p38 MAPK inhibitor) was purchased from LC labs (Woburn, MA, USA). U0126 (MEK inhibitor) was purchased from Cell Signaling (Danvers, MA, USA).

DNA constructs

The full-length mouse *cox-2* promoter region (963 bp) was cloned into the pGL3 basic luciferase expression vector (Promega, Madison, WI) [11]. Deletion mutants derived from full-length *cox-2* promoter, -720F, -320F, and -52F, contained the corresponding lengths of *cox-2* promoter (720/1, 320/1, 52/1). Point mutants were created on the following putative *cis*-acting element response sites in the *cox-2* promoter: C/EBP(3)-(900/895), Ets-1-(800/794), NF-κB-(402/392), NF-IL6(2) or C/EBP(2)-(135/130), NF-IL6(1) or C/EBP(1)-(90/86), AP-1-(65/60), and CRE-(60/56). Detailed information on these mutant constructs is available from the previous study [11]. N17 Ras (DN-Ras), ΔN Raf (DN-Raf), MEK-LIDA (DN-MEK), MEK-EE (CA-MEK), DN-p38 MAPK, and DN-ERK, were provided by Dr. KY Lee (Chonnam National University, Korea). Constitutively active Fyn (Fyn-Y531F) and kinase-dead Fyn (Fyn-K299M) cDNA were provided by Dr. T. Yamamoto (University of Tokyo).

Cells and cell culture

Rat basophilic leukemia (RBL-2H3) cells (ATCC) were maintained as monolayer cultures in Eagle's Minimum Essential Medium containing 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA). Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to the culture, and the cells were maintained at 37°C in an atmosphere of 5% CO_2 in air.

Reporter gene assays

RBL-2H3 cells were plated into 100-mm cell culture plates and then incubated overnight to 70% confluence. Cells were transfected using Lipofectamine plus reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol or by electroporation. The next day, the cells were dispensed into 24-well plates and treated with anti-DNP-specific mouse IgE (0.5 μg/ml) overnight at 37°C. In some experiments, transfected cells were pretreated with vehicle or pharmacological inhibitors for 30 min before treatment with DNP-BSA (30-1,000 ng/ml) for 4 hr. The luciferase activity was measured using the Dual Luciferase Reporter System (Promega, Madison, WI, USA).

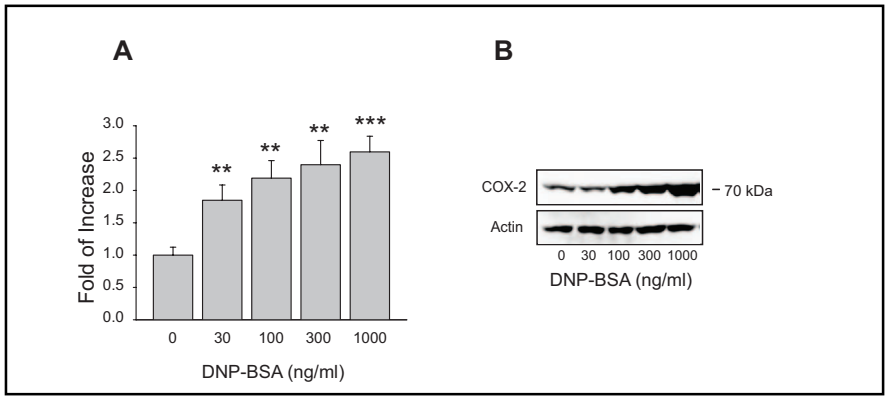

Fig. 1. Characterization of FcεRI-dependent induction of *cox-2* promoter activity in mast cells. RBL-2H3 cells were transiently co-transfected with pRL-TK-luciferase (1 μg) and *cox-2* promoter-luciferase reporter (4 μg). Transfected cells were pooled and washed 3 times with culture media. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized to pRL-TK-luciferase activity (*Renilla* luciferase activity) in each sample. Data represent the mean ± S.E. of triplicate experiments. The experiments were conducted three times with similar results. (A) Dose-response of DNP-BSA and *cox-2* promoter activity. Cells were stimulated with various concentrations of DNP-BSA for 4 hrs. (B) Cells were treated with increasing concentrations of DNP-BSA for 8 hrs. Cell lysates were analyzed by SDS-PAGE and blotted with antibodies against COX-2 and actin. Data are representative of three independent experiments.

Degranulation assays

The methodology for the degranulation assays was reported elsewhere [18]. RBL-2H3 cells were treated with IgE (0.5 μg/mL) overnight; the next morning, the cells were washed and pre-incubated in PIPES buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH, 5.6 mM glucose, 1 mM $CaCl_2$, 0.1% BSA) for 10 min at 37°C. The cells were treated with the antigen (DNP-BSA, 1 μg/ml) for 10 min at 37°C. The supernatants were added into 96-well plates and incubated with substrate (1 mM p-nitrophenyl-N-acetyl-D-glucosaminide) for 1hr. Stop solution (0.1 M Na_2CO_3/$NaHCO_3$) was added, and the absorbance at 405 nm was measured using an ELISA reader.

Statistical analysis

All of the results are expressed as mean±SEM. Comparisons between groups were performed using Student's *t* test.

Results

Characterization of allergen-mediated induction of COX-2 in mast cells

The promoter activity of *cox-2* in mast cells consists of basal and FcεRI-induced components. For example, certain levels of *cox-2* promoter activity were observed in resting RBL-2H3 cells and were further increased by stimulation with DNP-BSA. The expression of COX-2 showed similar patterns to the *cox-2* promoter activities (Fig.1A&B). A dose-dependent increase in the *cox-2* promoter activity and expression of COX-2 was observed in response to antigenic stimulation of FcεRI.

CREB, NF-IL6, and AP-1 binding sites have stimulatory effects on the transcriptional regulation of cox-2 promoter

The *cox-2* promoter (-963 to +1) contains various consensus *cis*-acting elements that bind to various transcription factors such as C/EBP, Ets-1, NF-κB, distal and proximal NF-IL6 [IL6(2) and IL6(1), respectively, in Fig.2], AP-1, CRE, and a TATA box, which are putative transcriptional response elements in the *cox-2* promoter. A previous study in MMC-34 mast cells showed that specific mutation of CRE and NF-IL6 sites inhibited the activity of *cox-2* promoter [19]. However, these studies used only a limited number of *cox-2* promoter mutants to completely understand the roles of each site, for example, it was not determined which NF-IL6 binding site is important.

As an initial step to determine which *cis*-acting elements are involved in the transcriptional regulation of *cox-2* induction, *cox-2* promoter mutants in which the 5'-region was consecutively deleted were utilized (Fig.2A). In 720F-*cox-2* promoter, the C/EBP and Ets-1 binding sites were deleted; in 320F, the NF-κB-binding site was additionally deleted; 52F contained only the TATA

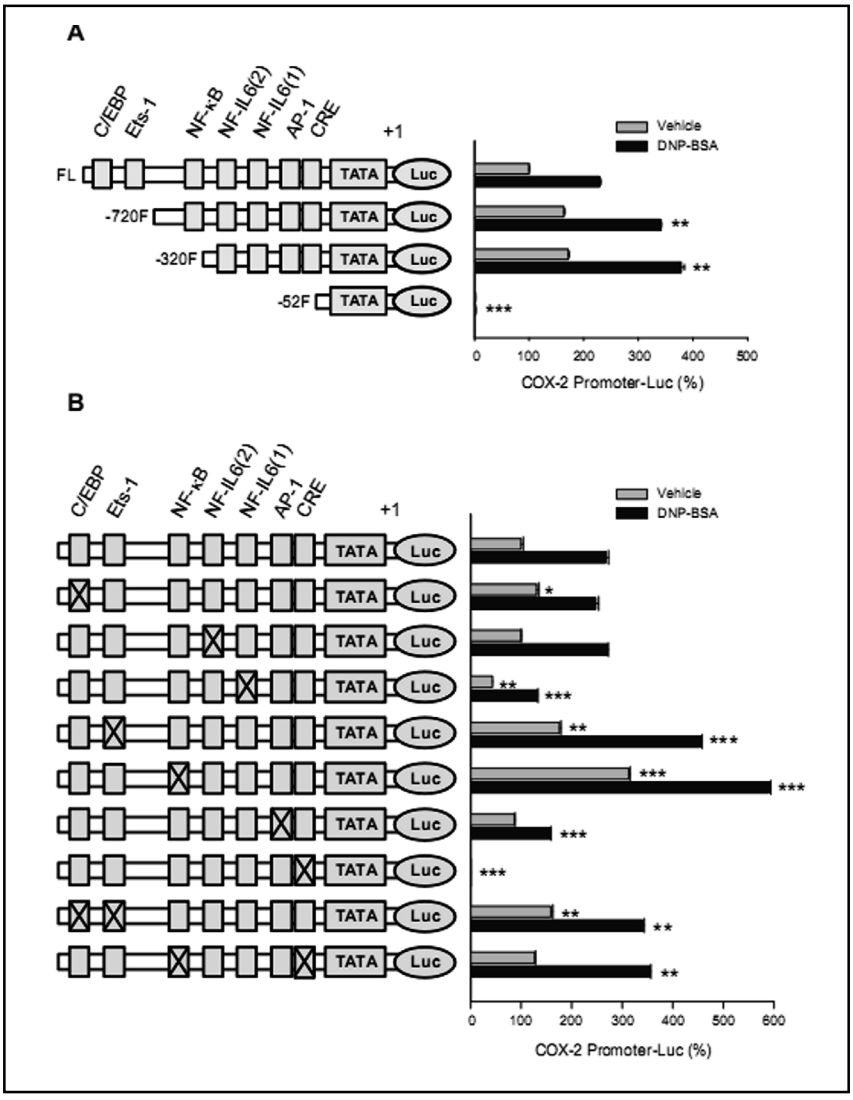

Fig. 2. Identification of *cis*-acting elements responsible for the transcriptional regulation of *cox-2* promoter in mast cells. (A) RBL-2H3 cells transfected with 4 μg of full-length or 5'-deletion mutants (-720, -320, or -52) were stimulated with DNP-BSA (1 μg/ml) for 4 hrs. **:p<0.01, ***:p<0.001 when the FcεRI-mediated *cox-2* promoter activity of corresponding deletion mutants was compared with the full-length *cox-2* promoter activity (FL). Basal *cox-2* promoter activity of each corresponding deletion mutant was significantly different from that of full-length *cox-2* promoter at p<0.001. (B) RBL-2H3 cells were transfected with the corresponding point mutants of *cox-2* promoter constructs. Reporter assay was conducted as in Fig.2A. *:p<0.05, **:p<0.01, ***:p<0.001 when the *cox-2* promoter activity of the corresponding mutants was compared with that of wild-type *cox-2* promoter.

box. As shown in Fig.2A, the *cox-2* promoter activity of the -720F and -320F deletion mutants gradually and significantly increased but was completely abolished in -52F mutant. These results suggest that proximal *cis*-acting elements between positions 1 and 320 have critical roles in *cox-2* promoter activity and that the three distal binding sites for transcription factors (C/EBP, Ets-1, NF-κB) might have inhibitory effects on *cox-2* promoter activity.

To determine the specific roles of each *cis*-acting element on the *cox-2* promoter, each consensus site was individually mutated. As shown in Fig.2B, mutation of the CREB binding sites completely abolished *cox-2* promoter activity. Mutations of the AP-1 binding site and the proximal NF-IL6 binding site also significantly inhibited the *cox-2* promoter activity. The importance of the NF-IL6 site was also confirmed with the rat *cox-2* promoter. Unlike the murine *cox-2* promoter used in this study, the rat *cox-2* promoter contains a single NF-IL6 site. Mutation of NF-IL6 site in rat *cox-2* promoter produced similar inhibition as mutation of the proximal NF-IL6 site in the murine *cox-2* promoter (Fig.3A).

NF-κB binding site has an inhibitory effect on the transcriptional activity of cox-2 promoter in mast cells

Successive deletions of the 5'-terminus of the *cox-2* promoter resulted in gradual increases in promoter activity (Fig.2A). As expected from the results with the deletion mutants, mutation of the Ets-1 or NF-κB site resulted in increased *cox-2* promoter activity. NF-κB is essential for the transcription of pro-inflammatory molecules such as IL-1, IL-6, and TNF-α [20]. In this sense, it is unexpected that an NF-κB binding site would negatively influence *cox-2* promoter activity. However, similar results were reported from MMC-34 mast cells: the promoter activity of *cox-2* was markedly elevated

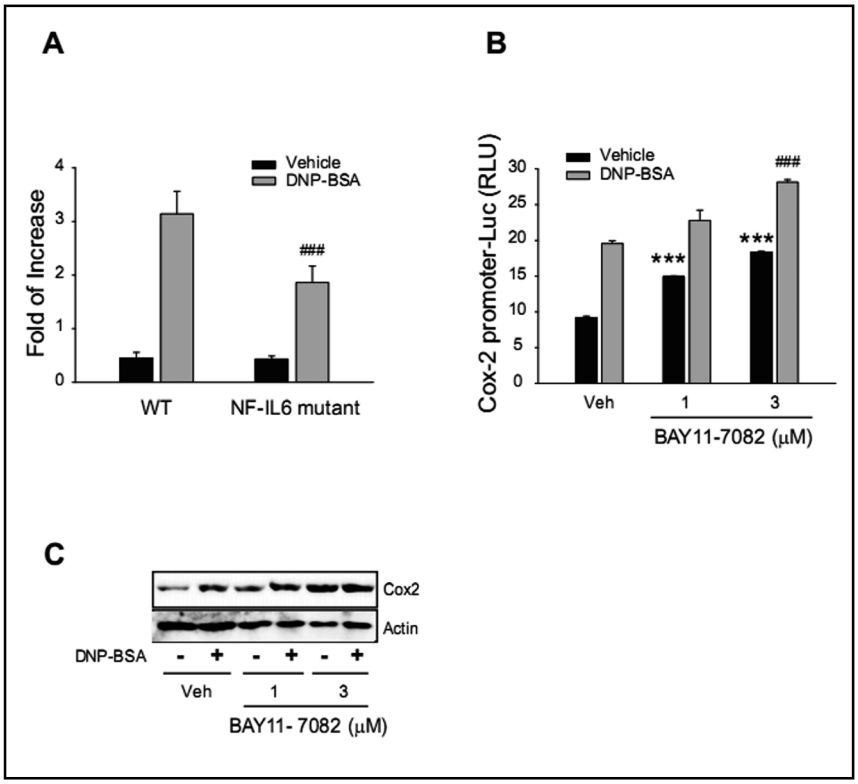

Fig. 3. Roles of binding sites for NF-IL6 and NF-κB in the transcriptional activity of *cox-2* promoter in mast cells. (A) Role of NF-IL6 binding site in *cox-2* promoter activity. Cells were transfected with wild-type rat *cox-2* or mutant rat *cox-2* promoter in the NF-IL6 binding site. ###:$p<0.001$ compared with WT/DNP-BSA group. (B) Role of NF-κB binding site in the *cox-2* promoter activity. Cells transfected with 4 μg of *cox-2* promoter-luciferase reporter and 1 μg of pRL-TK were treated with 0-3 μM BAY11-7082, a NF-κB inhibitor, for 1 hr before antigenic stimulation. ***, ###: $p<0.001$ when each experimental group was compared with the vehicle group in the resting and activated cells, respectively. RLU represents relative luminescence units. (C) Effects of BAY11-7082 on COX-2 expression. Cells were treated with increasing concentrations of BAY11-7082 for 1 hr, followed by treatment with 1 μg/ml DNP-BSA for 8 hrs. Cell lysates were analyzed by SDS-PAGE and blotted with antibodies against COX-2 and actin. Data are representative of three independent experiments.

both in resting and activated mast cells when the NF-κB binding site was mutated, even though they did not emphasize it [19]. Simultaneous mutations of the NF-κB site and of the CREB site rescued *cox-2* promoter activity that was completely abolished in the CREB mutant (the last construct in Fig. 2B). It is possible that the increase in *cox-2* promoter activity was caused by conformational changes of promoter region which were introduced by mutations in the NF-κB binding sites rather than by abolishment of NF-κB binding. To confirm these results, cells were treated with BAY11-7082 to inactivate NF-κB. As shown in Fig.3B, treatment of RBL-2H3 cells with NF-κB inhibitor increased the *cox-2* promoter activity in a dose-dependent manner. The levels of COX-2 were also increased when cells were treated with BAY11-7082 (Fig.3C). Therefore, it could be concluded that the binding of NF-κB exerts negative regulatory activities on the *cox-2* promoter in mast cells.

Roles of FcεRI signaling components in the transcriptional control of cox-2 promoter activity and protein levels in mast cells

With respect to the generation and release of PGs in mast cells, immediate release of inflammatory mediators stored in granules occurs in response to allergenic stimulation of FcεRI. Subsequent production of PGD_2 occurs with prolonged exposure to allergen. Antigenic aggregation of FcεRIs activates the receptor-associated tyrosine kinases, Lyn and Fyn, and causes tyrosine phosphorylation of multiple substrates. As previously reported [12, 14], Lyn mediates a series of signaling cascades including activation of phospholipase C (PLC) and the Ras/Raf/MEK cascade. On the other hand, Fyn mediates a different set of signaling that involves Gab2/PI 3-kinase/Akt. Therefore, it was postulated that FcεRI-mediated mast cell degranulation is mediated through collaboration between two pathways initiated by Lyn and Fyn [14, 21].

We wanted to determine how these signaling components regulate the COX-2 expression. As shown in Fig.4A, inhibition of the signaling components of FcεRI, such as Src, Syk, and PI 3-kinase, inhibited the mast cell degranulation. Antigen-induced increases in *cox-2* promoter activity were also significantly inhibited in cells treated with PP2 (a Src inhibitor), wortmannin (a PI 3-kinase inhibitor), and piceatannol (a Syk inhibitor) (Fig.4B). Since PI 3-kinase is oppositely regulated by Lyn and Fyn, the effects of the PI 3-kinase inhibitor on COX-2 expression were further studied. As shown in Fig.4C, increased COX-2 levels by DNP-BSA stimulation were

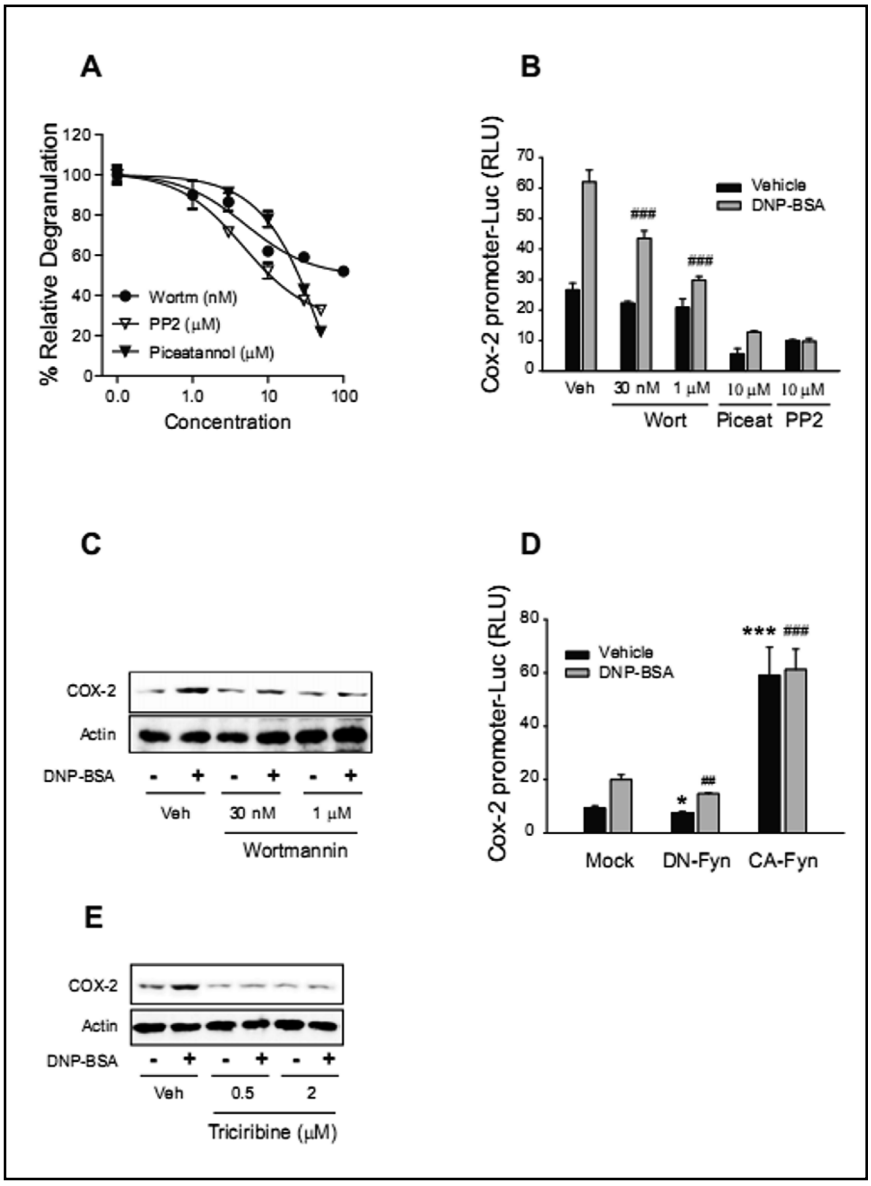

Fig. 4. Differential involvement of FcεRI signaling components in *cox-2* promoter activity in mast cells. (A) Effects of inhibitors of FcεRI signaling components on mast cell degranulation. Cells transfected with 4 μg of *cox-2* promoter-luciferase reporter and 1 μg of pRL-TK were treated with 0-100 nM wortmannin; 0-30 μM PP2 or piceatannol for 20 min. Mast cell degranulation was determined as described in Materials and methods. The 100% relative mast degranulation corresponds to 38% absolute degranulation. (B) Effects of inhibition of FcεRI signaling components on the transcriptional regulation of *cox-2* promoter in mast cells. RBL-2H3 cells transfected with 4 μg of *cox-2* promoter-luciferase reporter and 1 μg of pRL-TK were treated with wortmannin, piceatamol, and PP2 for 20 min prior to antigenic stimulation. ###:$p<0.001$ compared with Veh/DNP-BSA group. The *cox-2* promoter activities were significantly inhibited both in vehicle- and in DNP-BSA-treated groups when cells were pre-treated with 10 μM piceatannol or PP2 ($p<0.001$). (C) Effects of wortmannin on the COX-2 expression. Cells were treated with 30 nM and 1 μM wortmannin for 20 min, followed by treatment with 1 μg/ml DNP-BSA for 8 hrs. Cell lysates were analyzed by SDS-PAGE and blotted with antibodies against COX-2 and actin. Data are representative of three independent experiments. (D) Involvement of Fyn in the transcriptional regulation of *cox-2* promoter in mast cells. Cells were transfected with 4 μg of *cox-2* promoter-luciferase reporter, 1 μg of pRL-TK, and 7 μg of dominant negative or constitutively active Fyn. *:$p<0.05$, ***:$p<0.001$ compared with Mock/Vehicle group. ##:$p<0.01$, ###:$p<0.001$ compared with Mock/DNP-BSA group. All other experimental groups were significantly different from their corresponding control groups at the level of $p<0.001$. (E) Effects of triciribine on the COX-2 expression. Cells were treated with 0.5 and 2 μM tricinibine for 20 min, followed by treatment with 1μg/ml DNP-BSA for 8 hrs. Cell lysates were analyzed by SDS-PAGE and blotted with antibodies against COX-2 and actin. Data are representative of three independent experiments.

abolished by wortmannin, suggesting that the Fyn/Gab2/PI3K pathway is operative in COX-2 regulation. In support of this, the constitutively active mutant of Fyn increased the *cox-2* promoter activity while the dominant negative mutant of Fyn inhibited it (Fig.4D). An inhibitor of Akt (triciribine) also inhibited COX-2 expression (Fig.4E). These results suggest that the Fyn-initiated signaling pathway of FcεRI, which is linked to PI 3-kinase and the Akt pathway [14], constitutes a route to regulate COX-2 expression.

Roles of different MAPK cascades in the regulation of COX-2 expression in mast cells

ERK, JNK, and p38 MAPK are activated in response to aggregation of FcεRIs [22]. To determine the specific MAPK subtype required for transcriptional

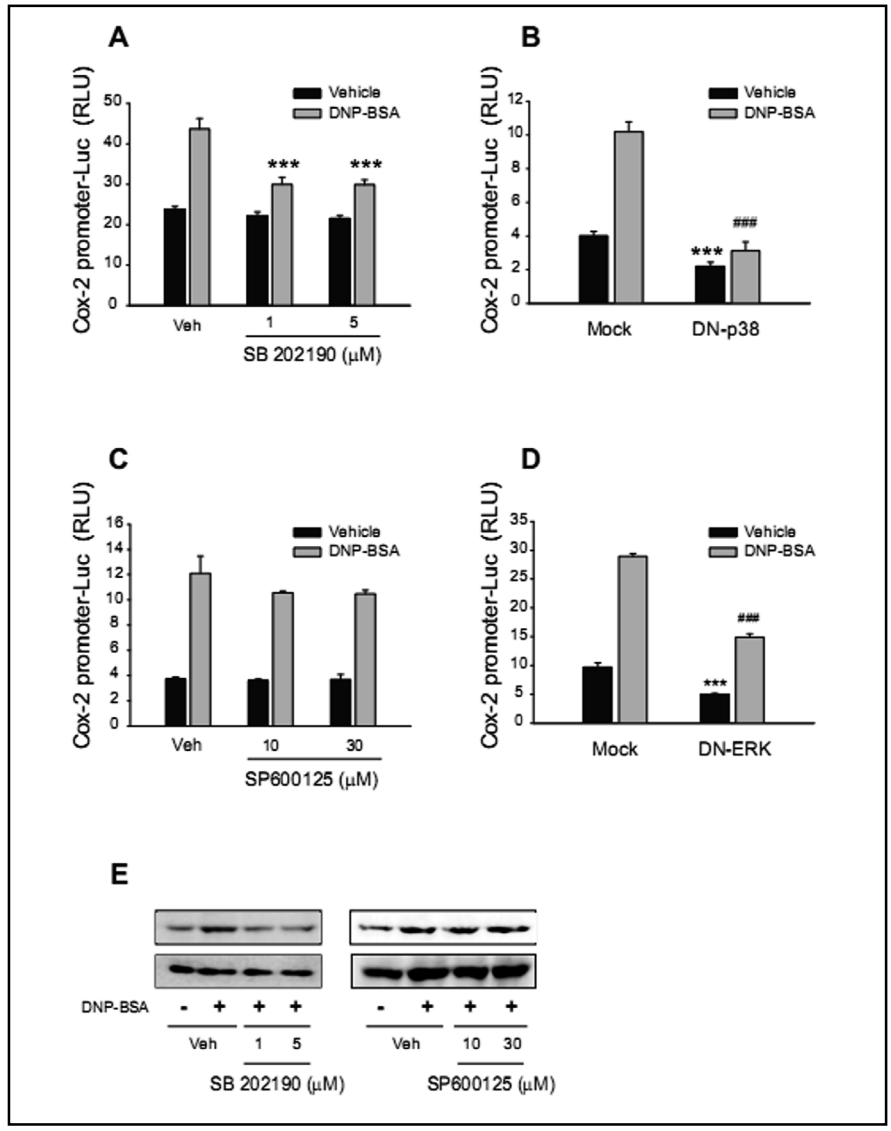

Fig. 5. Roles of different MAPKs in the regulation of COX-2 expression in mast cells. (A) Cells transfected with 4 μg of *cox-2* promoter-luciferase reporter and 1 μg of pRL-TK were treated with 1 and 5 μM SB 202190, a p38 MAPK inhibitor, 20 min before treatment with 1 μg/ml DNP-BSA. ***:$p<0.001$ compared with vehicle/DNP-BSA group. (B) Cells were co-transfected with 4 μg of *cox-2* promoter-luciferase reporter, 1 μg of pRL-TK, and 7 μg of DN-p38 MAPK. ***, ###: $p<0.001$ compared with Mock/vehicle and Mock/DNP-BSA group, respectively. (C) Cells were transfected as in Fig.5A, and treated with 10 and 30 μM SP600125, a JNK MAPK inhibitor. (D) Cells were transfected with 4 μg of *cox-2* promoter-luciferase reporter, 1 μg of pRL-TK, and 7 μg of dominant negative mutant of ERK. ***, ###: $p<0.001$ compared with Mock/vehicle and Mock/DNP-BSA group, respectively. (E) Effects of MAPK inhibitors on COX-2 expression. Cells were treated with increasing concentrations of SB 202190 or SP600125 for 20 min, followed by treatment with 1 μg/ml DNP-BSA for 8 hrs. Cell lysates were analyzed on the SDS-PAGE and blotted with antibodies against COX-2 and actin. Data are representative of three independent experiments.

regulation of *cox-2*, cells were treated with specific MAPK inhibitors or transfected with expression vectors encoding dominant negative (DN)-p38 MAPK and DN-ERK. The *cox-2* promoter activity was inhibited by SB 202190 (a p38 MAPK inhibitor)(Fig.5A) and by co-expression of DN-p38 MAPK (Fig.5B). On the other hand, SP600125, a JNK inhibitor, did not exert a significant inhibitory effect on the *cox-2* promoter activity (Fig.5C). Dominant negative mutants of ERK significantly inhibited the FcεRI-mediated increases in the transcriptional activity of the *cox-2* promoter (Fig.5D). As in the reporter gene assay, inhibition of p38 MAPK but not JNK resulted in the inhibition of COX-2 expression (Fig.5E). Overall, these results show that p38 MAPK and ERK but not JNK cascades are involved in the regulation of *cox-2* promoter activity in mast cells.

Contribution of ERK cascade on the transcriptional and translational regulation of COX-2 expression

The involvement of Ras/Raf-1/ERK pathways in the transcriptional regulation of the *cox-2* promoter has been reported in a murine mastocytoma cell line, MMC-34 [19]. In the case of RBL-2H3 mast cells, inhibition of MEK with PD98059, a specific inhibitor of MEK, did not affect COX-2 expression [23]. We were curious whether these discrepancies resulted from differences in cell type or at the level of regulation, *i.e.*, transcription or translation.

Co-expression of dominant negative mutants of Ras (data not shown), Raf-1, and MEK, inhibited the *cox-2* promoter activity (Fig.6A&B) but constitutively active MEK increased the *cox-2* promoter activity (Fig.6C). DN-ERK also inhibited COX-2 expression (data not

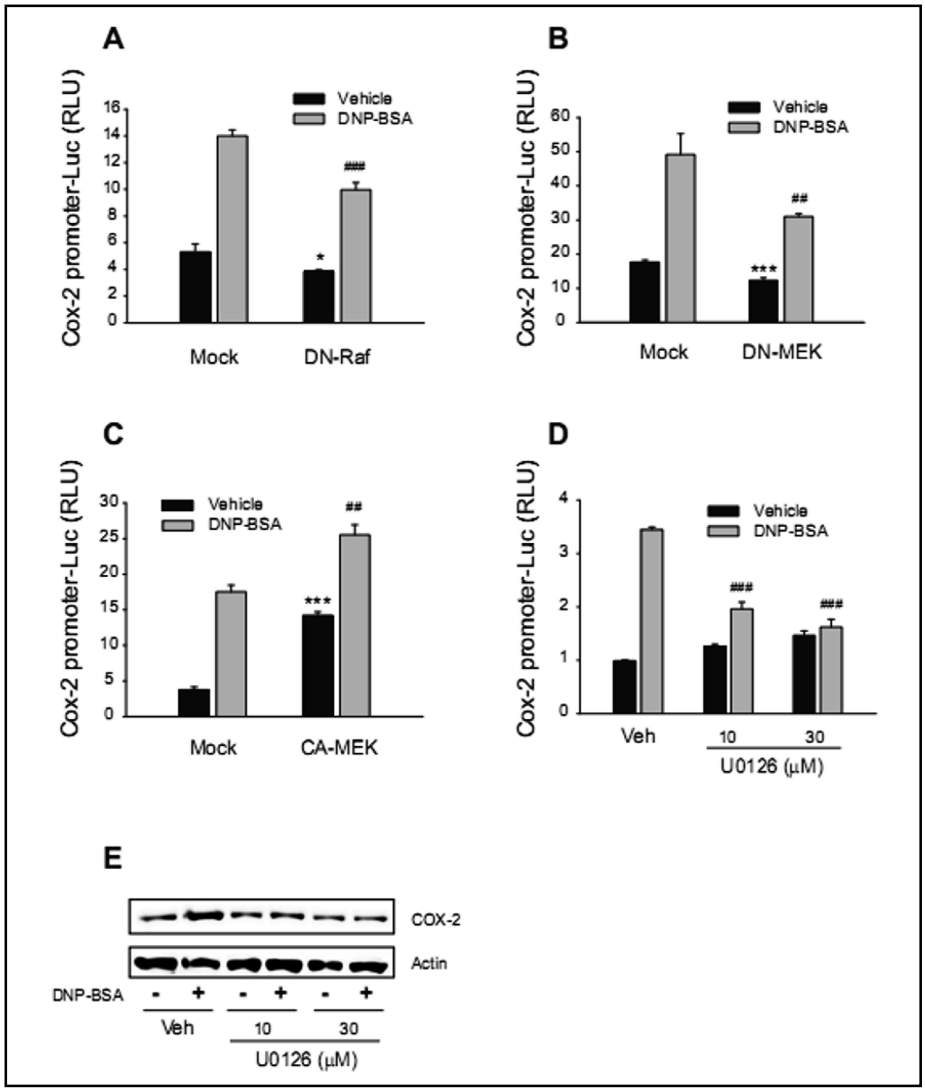

Fig. 6. Roles of Ras/Raf-1/MEK cascade in the COX-2 expression in RBL-2H3 cells. Effects of dominant negative mutant of Raf-1 (A), MEK (B), and constitutively active mutant of MEK (C) on the promoter activity of *cox-2*. *,***: $p<0.05$ and 0.001 compared with Mock/vehicle group. ##,###: $p<0.01$ and 0.001 compared with Mock/DNP-BSA group. (D) Effects of U0126 on the *cox-2* promoter activity. ###: $p<0.001$ compared with Veh/DNP-BSA group. (E) Effects of a MEK inhibitor on COX-2 expression. Cells were treated with increasing concentrations of U0126 for 20 min, followed by treatment with 1 μg/ml DNP-BSA for 8 hrs. Cell lysates were analyzed on the SDS-PAGE and blotted with antibodies against COX-2 and actin. Data are representative of three independent experiments.

shown). In agreement with this, treatment with U0126, a MEK inhibitor, inhibited *cox-2* reporter activity in a dose-dependent manner (Fig.6D) and the expression of COX-2 (Fig.6E).

Complex roles of PKC pathway on the regulation of COX-2

At least 6 different PKC subtypes are present in RBL-2H3 cells: conventional calcium-dependent PKC-α and PKC-β; novel calcium-independent PKC-δ, -ε, and -θ; and atypical PKC-ξ [24, 25]. PKC-β, -δ, and -θ mediate positive signals for secretion, while PKC-α and -ε subtypes mediate inhibitory signals for PLCγ activity [25, 26].

To determine the involvement of PKCs in the regulation of *cox-2* transcriptional activity, cells were treated with either PMA or PKC inhibitors. As shown in Fig.7A, Gö6976, which effectively inhibits PKC-α and PKC-β, showed inhibitory effects on *cox-2* promoter activity. On the other hand, Gö6983, which inhibits PKC-α, -β, -γ, -δ, and -ξ, potentiated it (Fig.7B). Considering that inhibition of PKC-α and -β results in the inhibition of COX-2 induction (from Gö6976 results), the results from Gö6983 treatments suggest that PKC-δ or -ξ might have negative feedback activities on COX-2 induction.

Inhibition of PLC, an upstream signaling component of PKCs, significantly inhibited the mast cell degranulation (data not shown). Inhibition of the *cox-2* promoter activity (Fig.7C) and protein expression (Fig.7D) was observed when cells were treated with U-73122, a PLC inhibitor. Therefore, the results obtained with the inhibitors of PKC and PLC, suggest that there are complex functional interactions between PKC subtypes and PLC.

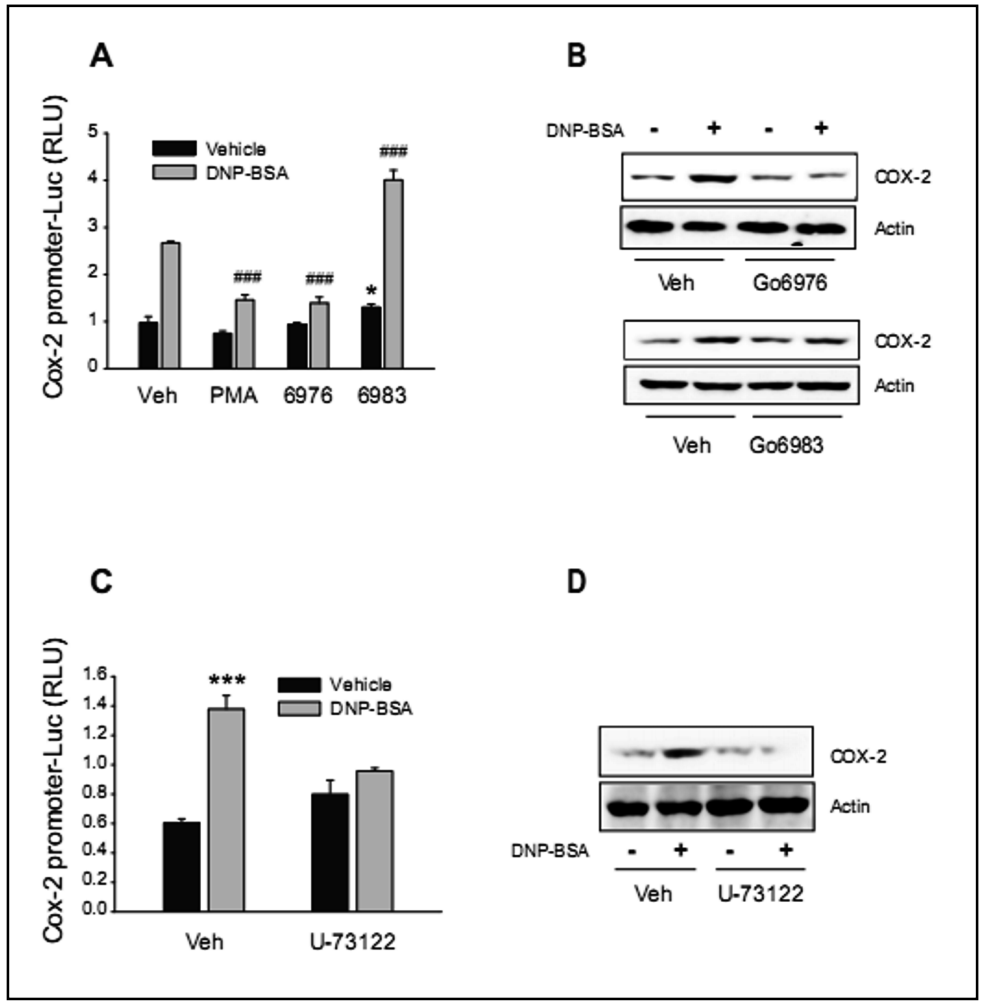

Fig. 7. Differential involvement of FcεRI signaling components in *cox-2* promoter activity in mast cells. (A) Effects of PKC activator or inhibitors on the transcriptional regulation of *cox-2* promoter in mast cells. RBL-2H3 cells transfected with 4 μg of *cox-2* promoter-luciferase reporter and 1 μg of pRL-TK were treated with 1 μM PMA, Gö6976, or Gö6983 for 20 min prior to antigenic stimulation. *:$p<0.05$ compared with Veh/vehicle group; ###:$p<0.001$ compared with Veh/DNP-BSA group. (B) Effects of PKC inhibitors on COX-2 expression. Cells were treated with 1 μM Gö6976 or Gö6983 for 20 min, followed by treatment with 1 μg/ml DNP-BSA for 8 hrs. Cell lysates were analyzed by SDS-PAGE and blotted with antibodies against COX-2 and actin. Data are representative of three independent experiments. (C) Effects of U-73122, a PLC inhibitor, on *cox-2* promoter activity. RBL-2H3 cells transfected with 4 μg of *cox-2* promoter-luciferase reporter and 1 μg of pRL-TK were treated with 10 μM U-73122 for 20 min prior to antigenic stimulation. ***:$p<0.001$ compared with Veh/vehicle group. (D) Effects of U-73122, a PLC inhibitor, on the COX-2 expression. Cells were treated with 10 μM U-73122 for 20 min, followed by treatment with 1 μg/ml DNP-BSA for 8 hrs. Cell lysates were analyzed by SDS-PAGE and blotted with antibodies against COX-2 and actin. Data are representative of three independent experiments.

Discussion

In this study, we wanted to identify (i) the *cis*-acting response element(s) responsible for the induction of COX-2 expression and (ii) the signal transduction pathways that interconnect FcεRI and COX-2 induction in resting and activated RBL-2H3 mast cells. For this, various deletion and point mutants of *cox-2* promoter, constitutively active as well as dominant-negative mutants, and selective inhibitors of the signaling components involved in the signaling of FcεRI and COX-2 induction, were utilized.

Previous studies in MMC-34 mast cells showed that CRE and NF-IL6 sites are involved in *cox-2* promoter activity [19]. In this study, we confirmed these previous results and, by creating additional mutants of the *cox-2* promoter in putative consensus sites, determined their authentic roles and the functional interactions needed for the regulation of *cox-2* promoter activity in RBL-2H3 cells. This set of constructs contained all the response elements that have been implicated in COX-2 regulation. Our study showed that the majority of binding sites in the *cox-2* promoter have active roles, that is, positive or negative activities, rather than neutral roles. In addition, functional interactions among these consensus binding sites seemed to be implied. The consensus binding sites in the proximal region of the *cox-2* promoter (AP-1 binding site and CREB binding site) were required for *cox-2* promoter activity. On the other hand, consensus binding sites in the distal region of the *cox-2* promoter (Ets-1 and NF-κB binding sites) had inhibitory activities. These results suggest that the transcriptional activity of the *cox-2*

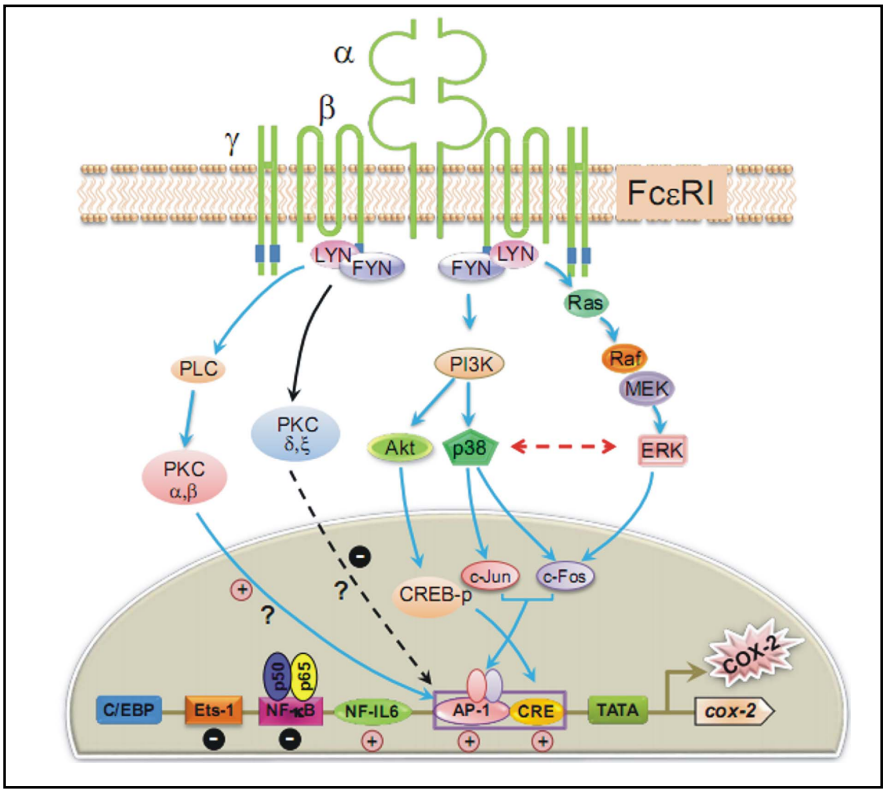

Fig. 8. Diagram showing the signal transduction pathways of FcεRI that regulate the transcriptional regulation of *cox-2*. (+) and (-) symbols shown in the *cox-2* promoter represent that stimulatory and inhibitory activities will be exerted on the promoter activity by the transcription factors that bind to each site, respectively. Different isozymes of PKCs have various regulatory activities on the *cox-2* promoter activity depending on the nature of their activities on the binding of c-Jun/c-Fos to AP-1 binding site. Akt phosphorylates CREB and increases binding to CRE site. p38 MAPK and ERK have functional interactions to regulate *cox-2* promoter.

promoter activity in mast cells is regulated in a complex manner and depends on the contribution of different transcription factors and activated signal transduction components.

Our results show that the proximal but not the distal NF-IL6 binding site in the murine *cox-2* promoter is selectively involved in regulating *cox-2* promoter activity (Fig.2B). Unlike the murine *cox-2* promoter used in Fig.2, the human and rat *cox-2* promoters each have one consensus NF-IL6 site. When the NF-IL6 binding site of the rat *cox-2* promoter was mutated, the *cox-2* promoter activity was inhibited (Fig.3A). Therefore, it is possible that the distal NF-IL6 site in the murine *cox-2* promoter might have different functional roles, other than transcriptional regulation of the *cox-2* promoter. Also structural differences in the *cox-2* promoter may contribute to species differences in the regulation of COX-2 expression in mast cells.

ERK, JNK, and p38 MAPK are activated in response to aggregation of FcεRIs [22], and ERK and p38 MAPK are functionally related [27]. A previous report suggests that *cox-2* transcription is regulated by ERK in the murine MMC-34 mast cell line [19]. On the other hand, another study showed that the induction of COX-2 in stimulated RBL-2H3 cells was abolished by inhibitor of p38 MAPK but not by an inhibitor of MEK, PD98059 [23]. Our study in RBL-2H3 cells shows that COX-2 expression is regulated by p38 MAPK and ERK which was confirmed both at promoter activity (Fig.5A&B; Fig.6D) and protein levels (Fig.5E; Fig.6E). These results agree with a previous study in the MMC-34 mast cell line [19] but somehow different from a previous study in RBL-2H3 cells, which reported that PD98059 another MEK inhibitor failed to inhibit COX-2 expression [23]. This discrepancy probably resulted from differences of MEK inhibitors employed in each study.

Allergic reactions start with the antigenic cross-linking of high affinity IgE receptors, leading to COX-2 induction. As expected, all of the signaling components of FcεRI tested showed inhibitory activities on COX-2 induction except that two different PKC inhibitors (Gö6976 and Gö6983) that inhibited the mast cell degranulation [28] showed opposite effects on *cox-2* promoter activity. Therefore, certain signaling components of FcεRI could have different roles in the mast cell degranulation and *cox-2* promoter activity depending on their relative contribution to the transcription factors that are responsible for *cox-2* transcription.

PKCs are suggested to regulate the binding of AP-1 to CRE site in the *cox-2* promoter [29]. PKC-δ, one of the downstream effectors of the PLCγ, is able to phosphorylate and activate Raf, thereby activating the MAPK pathway [30], suggesting a functional interaction

between PKC pathway and ERK.

FcεRI-mediated mast cell degranulation is regulated by two complementary signaling pathways [13]: Lyn→Syk→LAT→PLCγ and Fyn→PI3K→Akt. Several studies showed that Fyn and PI3K are the upstream of JNK and p38 MAPK. For example, Fyn deficiency caused defects in the activation of JNK, p38 MAPK, and NF-κB, however, ERK and PLA2 were normal [31]. Also it was reported that Fyn/PI3K, Rac, and p38 MAPK forms a pathway to production of cytokines such as IL4 [29, 32]. CREB and AP-1 have been suggested as the downstream components of Akt in the regulation of COX-2 expression [33, 34]. Overall, our results suggest that Fyn→PI3K pathway is the main pathway to stimulate COX-2 expression, and that Lyn→Syk pathway via PKC and MEK/ERK conducts an auxiliary roles (Fig.8). Further studies are needed to functionally analyze the relationship between the individual signaling components of FcεRI and the major transcription factors involved in the induction of COX-2.

In conclusion, the transcriptional regulation of the *cox-2* in RBL-2H3 mast cells is mediated (i) by the CRE element (60/56), the AP-1 binding site (65/60), and the proximal NF-IL6 binding site (90/86) in the *cox-2* promoter, (ii) by Fyn/PI3K/Akt/p38 MAPK, and (iii) by Lyn/Syk pathway which leads to the activation of PLCγ and ERK. There seem to be complex functional interactions among different PKCs for the regulation of COX-2 expression. Asthma and atopic diseases which involve allergic reactions are usually chronic and accompany inflammations in which COX-2 plays critical roles. In this sense, this work defined the signaling pathways and potential targets for intervention of inflammation-associated pathologies in allergic diseases.

Abbreviations

NF-IL6 (nuclear factor interleukin-6); CREB (cAMP response element-binding); C/EBP (CCAAT-enhancer-binding protein); PI 3-kinase (phosphatidylinositol 3-kinase); PMA (phorbol-12-myristate-13-acetate); JNK (c-Jun N-terminal kinase); MAPK (mitogen-activated protein kinase); ERK (Extracellular signal-regulated kinase); PLA2 (phospholipase A2); U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene); PLC (phospholipase C); PKC (protein kinase C).

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References

1 Blank U, Ra C, Miller L, White K, Metzger H, Kinet JP: Complete structure and expression in transfected cells of high affinity ige receptor. Nature 1989;337:187-189.
2 Turner H, Kinet JP: Signalling through the high-affinity ige receptor fc epsilonri. Nature 1999;402:B24-30.
3 Galli SJ: New concepts about the mast cell. N Engl J Med 1993;328:257-265.
4 Bischoff SC: Mucosal allergy: Role of mast cells and eosinophil granulocytes in the gut. Baillieres Clin Gastroenterol 1996;10:443-459.
5 Feng L, Sun W, Xia Y, Tang WW, Chanmugam P, Soyoola E, Wilson CB, Hwang D: Cloning two isoforms of rat cyclooxygenase: Differential regulation of their expression. Arch Biochem Biophys 1993;307:361-368.
6 Herschman HR: Prostaglandin synthase 2. Biochim Biophys Acta 1996;1299:125-140.
7 Kawata R, Reddy ST, Wolner B, Herschman HR: Prostaglandin synthase 1 and prostaglandin synthase 2 both participate in activation-induced prostaglandin d2 production in mast cells. J Immunol 1995;155:818-825.
8 Ashraf M, Murakami M, Kudo I: Cross-linking of the high-affinity ige receptor induces the expression of cyclo-oxygenase 2 and attendant prostaglandin generation requiring interleukin 10 and interleukin 1 beta in mouse cultured mast cells. Biochem J 1996;320:965-973.
9 Reddy ST, Herschman HR: Prostaglandin synthase-1 and prostaglandin synthase-2 are coupled to distinct phospholipases for the generation of prostaglandin d2 in activated mast cells. J Biol Chem 1997;272:3231-3237.
10 Kosaka T, Miyata A, Ihara H, Hara S, Sugimoto T, Takeda O, Takahashi E, Tanabe T: Characterization of the human gene (ptgs2) encoding prostaglandin-endoperoxide synthase 2. Eur J Biochem 1994;221:889-897.
11 Yeo SJ, Gravis D, Yoon JG, Yi AK: Myeloid differentiation factor 88-dependent transcriptional regulation of cyclooxygenase-2 expression by cpg DNA: Role of nf-kappab and p38. J Biol Chem 2003;278:22563-22573.
12 Nadler MJ, Kinet JP: Uncovering new complexities in mast cell signaling. Nature Immunol 2002;3:707-708.
13 Gilfillan AM, Tkaczyk C: Integrated signalling pathways for mast-cell activation. Nat Rev Immunol 2006;6:218-230.

14 Parravicini V, Gadina M, Kovarova M, Odom S, Gonzalez-Espinosa C, Furumoto Y, Saitoh S, Samelson LE, O'Shea JJ, Rivera J: Fyn kinase initiates complementary signals required for ige-dependent mast cell degranulation. Nature Immunol 2002;3:741-748.
15 Rivera J: Molecular adapters in fc(epsilon)ri signaling and the allergic response. Curr Opin Immunol 2002;14:688-693.
16 Gu H, Saito K, Klaman LD, Shen J, Fleming T, Wang Y, Pratt JC, Lin G, Lim B, Kinet JP, Neel BG: Essential role for gab2 in the allergic response. Nature 2001;412:186-190.
17 Saitoh S, Arudchandran R, Manetz TS, Zhang W, Sommers CL, Love PE, Rivera J, Samelson LE: Lat is essential for fc(epsilon)ri-mediated mast cell activation. Immunity 2000;12:525-535.
18 Oak MH, Cheong H, Kim KM: Activation of fc epsilon ri inhibits the pyruvate kinase through direct interaction with the gamma-chain. Int Arch Allergy Immunol 1999;119:95-100.
19 Reddy ST, Wadleigh DJ, Herschman HR: Transcriptional regulation of the cyclooxygenase-2 gene in activated mast cells. J Biol Chem 2000;275:3107-3113.
20 Blackwell TS, Christman JW: The role of nuclear factor-kappa b in cytokine gene regulation. Am J Respir Cell Mol Biol 1997;17:3-9.
21 Nishida K, Yamasaki S, Ito Y, Kabu K, Hattori K, Tezuka T, Nishizumi H, Kitamura D, Goitsuka R, Geha RS, Yamamoto T, Yagi T, Hirano T: Fc{epsilon}ri-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. J Cell Biol 2005;170:115-126.
22 Zhang C, Hirasawa N, Beaven MA: Antigen activation of mitogen-activated protein kinase in mast cells through protein kinase c-dependent and independent pathways. J Immunol 1997;158:4968-4975.
23 Hundley TR, Prasad AR, Beaven MA: Elevated levels of cyclooxygenase-2 in antigen-stimulated mast cells is associated with minimal activation of p38 mitogen-activated protein kinase. J Immunol 2001;167:1629-1636.
24 Ozawa K, Szallasi Z, Kazanietz MG, Blumberg PM, Mischak H, Mushinski JF, Beaven MA: Ca^{2+}-dependent and Ca^{2+}-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. Reconstitution of secretory responses with Ca^{2+} and purified isozymes in washed permeabilized cells. J Biol Chem 1993;268:1749-1756.
25 Ozawa K, Yamada K, Kazanietz MG, Blumberg PM, Beaven MA: Different isozymes of protein kinase c mediate feedback inhibition of phospholipase C and stimulatory signals for exocytosis in rat RBL-2H3 cells. J Biol Chem 1993;268:2280-2283.
26 Liu Y, Graham C, Parravicini V, Brown MJ, Rivera J, Shaw S: Protein kinase c theta is expressed in mast cells and is functionally involved in fcepsilon receptor i signaling. J Leukoc Biol 2001;69:831-840.
27 Zhang C, Baumgartner RA, Yamada K, Beaven MA: Mitogen-activated protein (map) kinase regulates production of tumor necrosis factor-alpha and release of arachidonic acid in mast cells. Indications of communication between p38 and p42 map kinases. J Biol Chem 1997;272:13397-13402.
28 Ryu H, Walker JK, Kim S, Koo N, Barak LS, Noguchi T, Kang BY, Kim KM: Regulation of m2-type pyruvate kinase mediated by the high-affinity ige receptors is required for mast cell degranulation. Br J Pharmacol 2008;154:1035-1046.
29 Subbaramaiah K, Cole PA, Dannenberg AJ: Retinoids and carnosol suppress cyclooxygenase-2 transcription by creb-binding protein/p300-dependent and -independent mechanisms. Cancer Res 2002;62:2522-2530.
30 Christofori G: Split personalities: The agonistic antagonist sprouty. Nat Cell Biol 2003;5:377-379.
31 Yoon E, Beom S, Cheong H, Kim S, Oak M, Cho D, Kim KM: Differential regulation of phospholipase cgamma subtypes through fcepsilonri, high affinity ige receptor. Biochem Biophys Res Commun 2004;325:117-123.
32 Hirasawa N, Sato Y, Fujita Y, Ohuchi K: Involvement of a phosphatidylinositol 3-kinase-p38 mitogen activated protein kinase pathway in antigen-induced il-4 production in mast cells. Biochim Biophys Acta 2000;1456:45-55.
33 Alique M, Calleros L, Luengo A, Griera M, Iniguez MA, Punzon C, Fresno M, Rodriguez-Puyol M, Rodriguez-Puyol D: Changes in extracellular matrix composition regulate cyclooxygenase-2 expression in human mesangial cells. Am J Physiol Cell Physiol 2011;300:C907-918.
34 Kim EH, Na HK, Kim DH, Park SA, Kim HN, Song NY, Surh YJ: 15-deoxy-delta12,14-prostaglandin j2 induces cox-2 expression through akt-driven ap-1 activation in human breast cancer cells: A potential role of ros. Carcinogenesis 2008;29:688-695.