



## Pulmonary, Gastrointestinal and Urogenital Pharmacology

Thrombin induces cyclooxygenase-2 expression via the ERK and NF- $\kappa$ B pathways in human lung fibroblastsChung-Huang Shih<sup>a,b</sup>, Mauo-Ying Bien<sup>a,b</sup>, Ling-Ling Chiang<sup>b</sup>, Chien-Ling Su<sup>b</sup>, Chien-Huang Lin<sup>c,d,e</sup>, Bing-Chang Chen<sup>b,\*</sup><sup>a</sup> Department of Respiratory Therapy, Taipei Medical University Hospital, Taipei, Taiwan<sup>b</sup> School of Respiratory Therapy, College of Medicine, Taipei Medical University, Taipei, Taiwan<sup>c</sup> Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan<sup>d</sup> Taipei Medical University-Wang-Fang Hospital, Taipei, Taiwan<sup>e</sup> Taipei Medical University-Shuang-Ho Hospital, Taipei County, Taiwan

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## ABSTRACT

There is growing evidence that increased expression of cyclooxygenase-2 (COX-2) in the lungs of patients is a key event in the pathogenesis of lung diseases. In this study, we investigated the involvement of the extracellular signal-regulated kinase (ERK), I $\kappa$ B kinase  $\alpha$ / $\beta$  (IKK $\alpha$ / $\beta$ ), and nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathways in thrombin-induced COX-2 expression in human lung fibroblasts (WI-38). Treatment of WI-38 cells with thrombin caused increased COX-2 expression in a concentration- and time-dependent manner. Treatment of WI-38 cells with PD 98059 (2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one, a MEK inhibitor) inhibited thrombin-induced COX-2 expression and COX-2-luciferase activity. Stimulation of cells with thrombin caused an increase in ERK phosphorylation in a time-dependent manner. In addition, treatment of WI-38 cells with Bay 117082, an I $\kappa$ B phosphorylation inhibitor, and pyrrolidine dithiocarbamate (PDTIC), an NF- $\kappa$ B inhibitor, inhibited thrombin-induced COX-2 expression. The thrombin-induced increase in COX-2-luciferase activity was also blocked by the dominant negative I $\kappa$ B $\alpha$  mutant (I $\kappa$ B $\alpha$ M). Treatment of WI-38 cells with thrombin induced IKK $\alpha$ / $\beta$  and I $\kappa$ B $\alpha$  phosphorylation, I $\kappa$ B $\alpha$  degradation, and  $\kappa$ B-luciferase activity. The thrombin-mediated increases in IKK $\alpha$ / $\beta$  phosphorylation and  $\kappa$ B-luciferase activity were inhibited by PD 98059. Taken together, these results suggest that the ERK-dependent IKK $\alpha$ / $\beta$ /NF- $\kappa$ B signaling pathway plays an important role in thrombin-induced COX-2 expression in human lung fibroblasts.

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## 1. Introduction

Thrombin plays a pivotal role in the coagulation cascade, and also promotes a wide range of cellular responses including modulation of lung inflammation (Wright and Dobbs, 1991). With vascular injury, which is an early event in most inflammatory lung diseases, thrombin may leave the circulation and become activated when it becomes part of the bronchoalveolar lavage fluid (Terada et al., 2004). Thrombin also plays important roles in acute lung inflammation such as inducing the accumulation of neutrophils in the airway and elevation of tumor necrosis factor- $\alpha$  levels in the bronchoalveolar lavage fluid from mice which had inhaled thrombin (Moffatt et al., 2004). Thus, the presence of thrombin in airway bronchoalveolar lavage fluid appears to be a common feature of a variety of lung diseases and plays critical roles in lung inflammation.

To date, four proteinase-activated receptors (PARs) have been identified and each receptor has been implicated in the development of lung diseases (Sokolova and Reiser, 2007). Thrombin activates PAR-1, PAR-3, and PAR-4 via cleavage of the extracellular N-terminal domain, which then enables the new N terminus to interact distally with the same molecule to activate G protein-coupled signal pathways and subsequently induce cellular responses (Coughlin, 2000). A previous report showed that thrombin can induce cyclooxygenase-2 (COX-2) expression in human lung fibroblasts (Sokolova et al., 2005). However, signal transduction events, especially the extracellular signal-regulated kinase (ERK)/I $\kappa$ B kinase  $\alpha$ / $\beta$  (IKK $\alpha$ / $\beta$ )/nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway, which lead to COX-2 expression by thrombin are unclear.

Arachidonic acid metabolites are important in the modulation of immunity in the lungs (Tilley et al., 2001). The major rate-limiting enzymes involved in their synthesis are COXs. COX-1 is constitutively expressed in most tissues and has general housekeeping functions, whereas COX-2 is responsible for high-level production of prostaglandins (PGs) in response to proinflammatory agents, which plays an important role in the regulation of inflammation (Vane et al., 1998). There is growing evidence that higher COX-2 expression in the lung of

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patients is key event in the pathogenesis of chronic obstructive pulmonary disease (COPD) (Xaubet et al., 2004). Therefore, COX-2 may be a major contributor to the pathogenesis of inflammation in COPD. Several consensus sequences, including NF- $\kappa$ B in the 5' region of the *cox-2* gene, have been identified as regulatory sequences which can induce COX-2 in response to various stimuli (Inoue et al., 1995). In a resting state, NF- $\kappa$ B is associated with I $\kappa$ Bs to retain NF- $\kappa$ B in the cytosol. IKKs phosphorylates I $\kappa$ B $\alpha$  at Ser32 and Ser36 to produce ubiquitination and degradation of I $\kappa$ B $\alpha$  (Chen et al., 1995). This process releases active NF- $\kappa$ B, which is then translocated from the cytosol to the nucleus, to bind specific DNA enhancer sequences and induce gene transcription (Chen et al., 2006). The regulation of COX-2 expression is subjected to a tight regulatory network involving NF- $\kappa$ B, which can be activated by complex kinase pathways including ERK (N'guessan et al., 2007). In an attempt to elucidate the molecular mechanisms underlying the regulation of COX-2 expression, we examined the activation of ERK and NF- $\kappa$ B in human lung fibroblasts. In this study, our results demonstrate that thrombin activates ERK to induce IKK $\alpha$ / $\beta$  and NF- $\kappa$ B activation, ultimately causing COX-2 expression in human lung fibroblasts.

## 2. Materials and methods

### 2.1. Materials

Thrombin (from bovine plasma) was purchased from Sigma (Saint Louis, MO). PD 98059 (2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one) was obtained from Calbiochem (San Diego, CA). Minimum essential medium (MEM), fetal calf serum, and penicillin/streptomycin were purchased from Life Technologies (Gaithersburg, MD). An antibody specific for  $\alpha$ -tubulin was purchased from Transduction Laboratories (Lexington, KY). An antibody specific for COX-2 was purchased from Cayman Chemical (Ann Arbor, MI). Antibodies specific for IKK $\alpha$ / $\beta$ , ERK phosphorylated at Tyr204, ERK2, I $\kappa$ B $\alpha$  phosphorylated at Ser32, and I $\kappa$ B $\alpha$ , as well as anti-mouse and anti-rabbit immunoglobulin G (IgG)-conjugated horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody specific for phospho-IKK $\alpha$  (Ser180)/IKK $\beta$  (Ser181) was purchased from New England Biolabs (Beverly, MA). A human COX-2 promoter-luciferase construct, pGS459 (-459/+9), which contains 2  $\kappa$ B-binding sites, was a kind gift from Prof. C.-C. Chen (National Taiwan University, Taipei, Taiwan). A dominant negative I $\kappa$ B $\alpha$  mutant (I $\kappa$ B $\alpha$ M) was purchased from BD Biosciences (Palo Alto, CA). pGL2-ELAM-Luc (which is under the control of a single NF- $\kappa$ B binding site) and pBK-CMV-Lac Z were kindly provided by Prof. W.-W. Lin (National Taiwan University, Taipei, Taiwan). All materials for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA). All other chemicals were obtained from Sigma.

### 2.2. Cell culture

WI-38 cells, a normal human embryonic lung fibroblast cell line, were obtained from American Type Culture Collection (Livingstone, MT). Cells were grown in an MEM nutrient mixture, containing 10% fetal calf serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 U/ml penicillin G, and 100  $\mu$ g/ml streptomycin, in a humidified 37 °C incubator with 5% CO<sub>2</sub>. Cells were used between passages 18 and 30 for all experiments. After reaching confluence, cells were seeded onto 6-cm dishes for cell immunoblotting and onto 12-well plates for the cell transfection and luciferase assays.

### 2.3. Protein preparation and Western blotting

To determine the levels of COX-2,  $\alpha$ -tubulin, phospho-ERK (Tyr204), ERK2, phospho-IKK $\alpha$  (Ser180)/IKK $\beta$  (Ser181), and IKK $\alpha$ / $\beta$  in WI-38

cells, proteins were extracted, and a Western blot analysis was performed as previously described (Chen et al., 2006). Briefly, WI-38 cells were cultured in 6-cm dishes. After reaching confluence, cells were treated with thrombin, or pretreated with specific inhibitors as indicated followed by thrombin. After incubation, cells were washed twice in ice-cold phosphate-buffered saline and solubilized in extraction buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM PMSF, 5 mM DTT, 0.5% NP-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin. Samples of equal amounts of protein (80  $\mu$ g) were subjected to SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes (2 h, 40 V), treated with 5% fat-free milk powder to block the nonspecific IgGs, and incubated for 2 h with specific antibodies for COX-2,  $\alpha$ -tubulin, phospho-ERK (Tyr204), ERK2, phospho-IKK $\alpha$  (Ser180)/IKK $\beta$  (Ser181), IKK $\alpha$ / $\beta$ , phospho-I $\kappa$ B $\alpha$  (Ser32), and I $\kappa$ B $\alpha$ . The blots were then incubated with anti-mouse or -rabbit IgG linked to HRP for 2 h. Subsequently, the immunoreactivity was detected by enhanced chemiluminescence. Quantitative data were obtained using a computing densitometer with Image-Pro Plus image analysis software systems (Kodak, Rochester, NY).

### 2.4. Transfection and luciferase reporter assays

WI-38 cells at  $1 \times 10^5$  were seeded into 12-well plates. For the COX-2 promoter and  $\kappa$ B-luciferase assays, cells were transfected on the following day with the Lipofectamine plus<sup>TM</sup> reagent containing 1  $\mu$ g of pGS459 (-459/+9) or 0.5  $\mu$ g of pGL2-ELAM-Luc, respectively, and 1  $\mu$ g of pBK-CMV-LacZ for 24 h. After incubation, the medium was aspirated and replaced with fresh MEM containing 10% fetal calf serum. Cells were then stimulated with thrombin for another 24 h before harvesting. To assay the effect of PD 98059, drug was added to the cells 30 min before thrombin stimulation. To assay the effect of I $\kappa$ B $\alpha$ M, cells were cotransfected with pGS459 (-459/+9), Lac Z, and I $\kappa$ B $\alpha$ M. Luciferase activity was determined with a luciferase assay system (Promega) and normalized on the basis of LacZ expression. The level of induction of luciferase activity was determined as a ratio compared to cells with no stimulation.

### 2.5. Statistical analysis

Results are presented as the mean  $\pm$  S.E.M. from at least three independent experiments. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple-range test, when appropriate, was used to determine the statistical significance of the difference between means. A *P* value of <0.05 was taken as statistically significant.

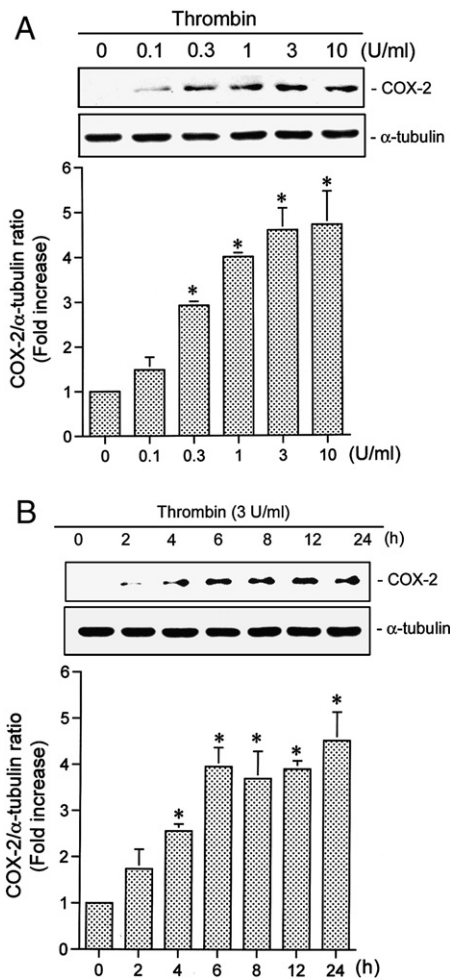
## 3. Results

### 3.1. Thrombin induces COX-2 expression in WI-38 cells

Human lung fibroblasts (WI-38) were chosen to investigate the signal pathways of thrombin in COX-2 expression. Treatment with thrombin (0.1–10 U/ml) for 24 h induced COX-2 protein expression in a concentration-dependent manner, with a maximum effect at 3–10 U/ml thrombin treatment (Fig. 1A). Similarly, this induction occurred in a time-dependent manner (Fig. 1B). After treatment, COX-2 protein bands began to appear at 4 h and reached a maximum at 6–24 h. After 12 h of treatment with 3 U/ml thrombin, the COX-2 protein had increased by  $291 \pm 18\%$  (Fig. 1B).

### 3.2. Involvement of ERK in thrombin-induced COX-2 expression

Next, we tested the role of ERK in mediating thrombin-induced COX-2 expression using the specific MAPK ERK kinase (MEK) inhibitor, PD 98059. As shown in Fig. 2A, thrombin-induced COX-2 expression was markedly attenuated by pretreating cells with PD 98059 (10 and 30  $\mu$ M). Pretreating cells with 30  $\mu$ M PD 98059 inhibited thrombin-induced

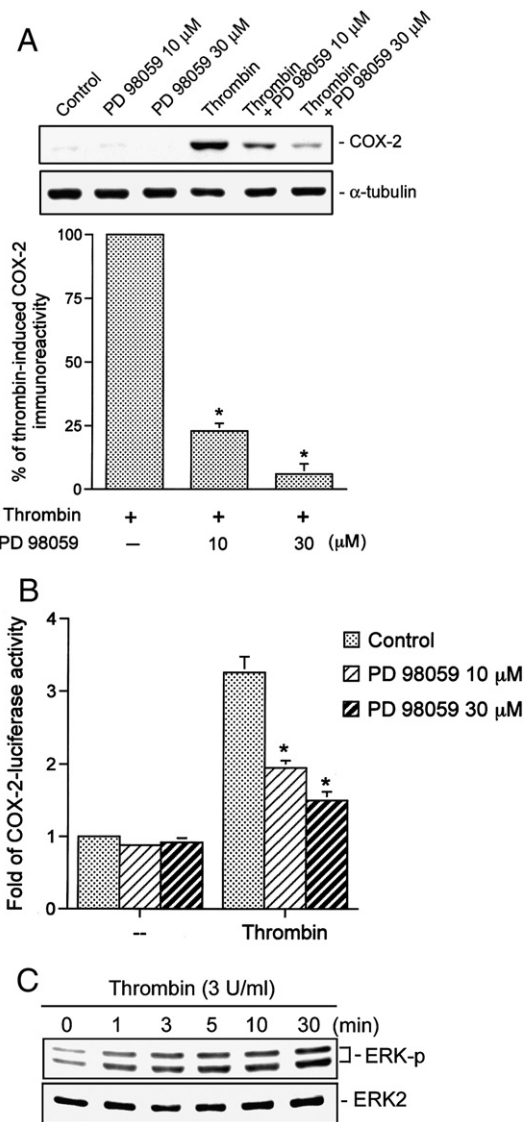


**Fig. 1.** Thrombin induces cyclooxygenase-2 (COX-2) expression in WI-38 cells. A, Cells were incubated with various concentrations of thrombin for 12 h, and then COX-2 and  $\alpha$ -tubulin protein levels were determined. Immunoblots are representative of three experiments, presented as the mean  $\pm$  S.E.M. \* $P < 0.05$ , compared to the control group. B, Cells were incubated for various time intervals with thrombin (3 U/ml), and then COX-2 and  $\alpha$ -tubulin protein levels were determined. Immunoblots are representative of three experiments, presented as the mean  $\pm$  S.E.M. \* $P < 0.05$ , compared to the control group.

COX-2 expression by  $94 \pm 4\%$  ( $n = 3$ ) (Fig. 2A). Moreover, the thrombin-induced increase in COX-2-luciferase activity was inhibited by PD 98059. PD 98059 at  $30 \mu\text{M}$  inhibited thrombin-induced COX-2-luciferase activity by  $74 \pm 5\%$  ( $n = 3$ ) (Fig. 2B). To directly confirm the crucial role of ERK in COX-2 expression, we determined ERK phosphorylation in response to thrombin. As shown in Fig. 2C, treating WI-38 cells with thrombin (3 U/ml) resulted in a time-dependent phosphorylation of ERK. ERK phosphorylation began at 1 min and was sustained to 30 min after thrombin treatment (Fig. 2C, upper panel). The protein level of ERK2 was not affected by thrombin treatment (Fig. 2C, bottom panel). Based on these results, we suggest that activation of ERK-mediated thrombin-induced COX-2 expression.

### 3.3. Involvement of NF- $\kappa$ B in thrombin-induced COX-2 expression

Previous reports showed that NF- $\kappa$ B activation is necessary for COX-2 induction (Chen et al., 2004b, 2006; N'guessan et al., 2007). Pyrrolidine dithiocarbamate (PDTC), an NF- $\kappa$ B inhibitor, has been shown to inactivate NF- $\kappa$ B (Chen et al., 1998). PDTC was used to examine whether NF- $\kappa$ B activation is involved in the signal transduction pathway leading to COX-2 expression caused by thrombin. When cells were treated with  $30 \mu\text{M}$  PDTC, thrombin-induced COX-2 expression was inhibited by  $51 \pm 9\%$  ( $n = 3$ ) (Fig. 3A). Furthermore,



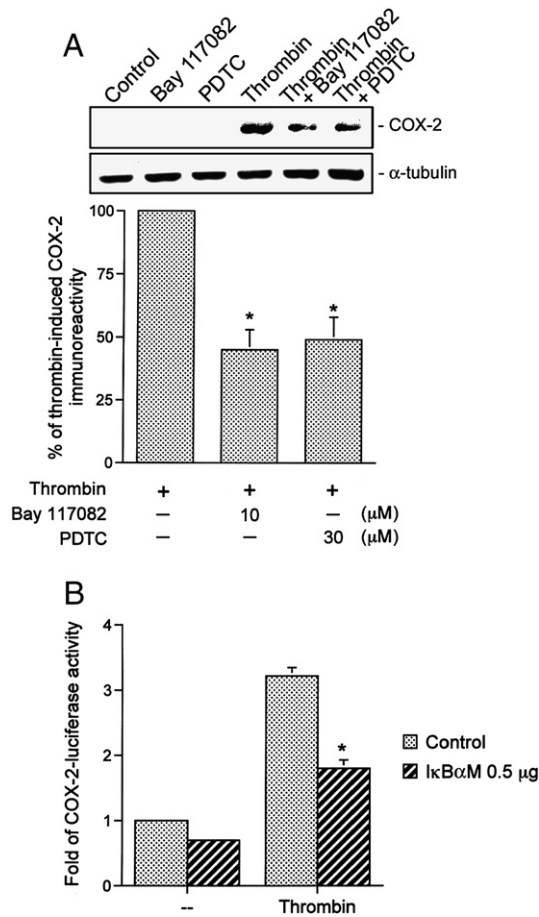
**Fig. 2.** Extracellular signal-regulated kinase (ERK) is involved in thrombin-induced cyclooxygenase-2 (COX-2) expression in WI-38 cells. A, Cells were pretreated for 30 min with PD 98059 (10 and  $30 \mu\text{M}$ ) and then stimulated with 3 U/ml thrombin. After 12 h incubation, COX-2 and  $\alpha$ -tubulin protein levels were determined. Immunoblots are representative of three experiments, presented as the mean  $\pm$  S.E.M. \* $P < 0.05$ , compared to thrombin treatment. B, Cells were transiently transfected with  $1 \mu\text{g}$  of pGS459 (-459/+9) and  $1 \mu\text{g}$  of pBK-CMV-Lac Z for 24 h, and then pretreated with PD 98059 (10 and  $30 \mu\text{M}$ ) for 30 min, before incubation with 3 U/ml thrombin for 24 h. Cells were then harvested for the COX-2 promoter-luciferase assay as described in "Materials and methods". Results are expressed as the mean  $\pm$  S.E.M. ( $n = 3$ ). \* $P < 0.05$ , compared to the thrombin-treated group. C, Cells were incubated with 3 U/ml thrombin for 0–30 min. Cell lysates were prepared, and ERK Tyr204 phosphorylation was determined by immunoblotting using the phospho-ERK Tyr204 antibody. Immunoblots are representative of three experiments with similar results.

pretreatment of cells with  $10 \mu\text{M}$  Bay 117082, an I $\kappa$ B $\alpha$  phosphorylation inhibitor (Pierce et al., 1997), inhibited thrombin response by  $55 \pm 3\%$  ( $n = 3$ ) (Fig. 3A). In parallel with the inhibition by PDTC and Bay 117082, cells transfected with  $0.5 \mu\text{g}$  I $\kappa$ B $\alpha$ M also exhibited inhibition of thrombin-induced COX-2 protein expression by  $50 \pm 6\%$  ( $n = 3$ ) (Fig. 3B). These results suggest that NF- $\kappa$ B activation is necessary for thrombin-induced COX-2 expression in WI-38 fibroblasts.

### 3.4. Thrombin caused increases in IKK $\alpha$ / $\beta$ phosphorylation, I $\kappa$ B $\alpha$ phosphorylation, I $\kappa$ B $\alpha$ degradation, and $\kappa$ B-luciferase activity

We further determined the upstream molecules involved in thrombin-induced NF- $\kappa$ B activation. Stimulation of cells with 3 U/ml



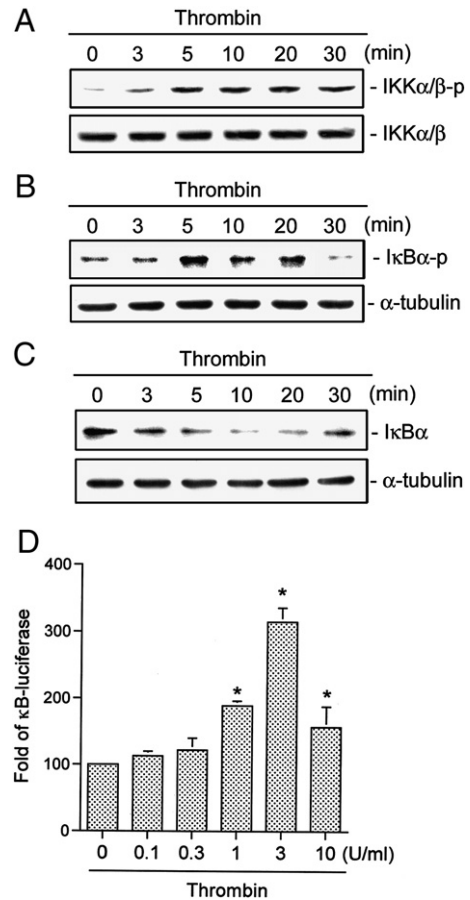


**Fig. 3.** Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is involved in thrombin-induced cyclooxygenase-2 (COX-2) expression in WI-38 cells. A, Cells were pretreated for 30 min with 10  $\mu$ M Bay 117082 and 30  $\mu$ M PDTC, and then stimulated with 3 U/ml thrombin. After 12 h of incubation, COX-2 and  $\alpha$ -tubulin protein levels were determined. Typical traces are representative of three experiments, presented as the mean  $\pm$  S.E.M. \* $P$  < 0.05, compared to thrombin treatment. B, WI-38 cells were transiently transfected with 1  $\mu$ g of pGS459 (–459/+9) and 0.5  $\mu$ g of pBK-CMV-Lac Z or cotransfected with 0.5  $\mu$ g IkB $\alpha$ M for 24 h, and then stimulated with 3 U/ml thrombin for another 24 h. Cells were then harvested for the COX-2-luciferase assay as described in “Materials and methods”. Data represent the mean  $\pm$  S.E.M. of three experiments performed in duplicate. \* $P$  < 0.05, compared to thrombin treatment.

thrombin induced increases in IKK $\alpha$ / $\beta$  phosphorylation beginning at 3 min which was sustained to 30 min (Fig. 4A). We also found that thrombin-induced I $\kappa$ B $\alpha$  phosphorylation began at 5 min and was sustained to 20 min (Fig. 4B). In parallel with IKK $\alpha$ / $\beta$  and I $\kappa$ B $\alpha$  phosphorylation, I $\kappa$ B $\alpha$  degradation was apparent after 5 min of treatment with 3 U/ml thrombin, and the I $\kappa$ B $\alpha$  protein was resynthesized after 30 min of treatment (Fig. 4C). To directly determine NF- $\kappa$ B activation after thrombin treatment, WI-38 cells were transiently transfected with pGL2-ELAM- $\kappa$ B-luciferase as an indicator of NF- $\kappa$ B activation. As shown in Fig. 4D, WI-38 cells treated with thrombin (0.1–10 U/ml) for 24 h exhibited a concentration-dependent increase in  $\kappa$ B-luciferase activity. Cells treated with 3 U/ml thrombin showed an increase in  $\kappa$ B-luciferase activity of  $3.2 \pm 0.2$ -fold (Fig. 4D).

### 3.5. Involvement of ERK in thrombin-induced increases in IKK $\alpha$ / $\beta$ phosphorylation and $\kappa$ B-luciferase activity

We further examined whether thrombin-induced IKK $\alpha$ / $\beta$  phosphorylation and NF- $\kappa$ B activation occur through the ERK signaling pathway. As shown in Fig. 5A, pretreatment of cells for 30 min with PD 98059 (30  $\mu$ M) markedly attenuated the thrombin-induced increases

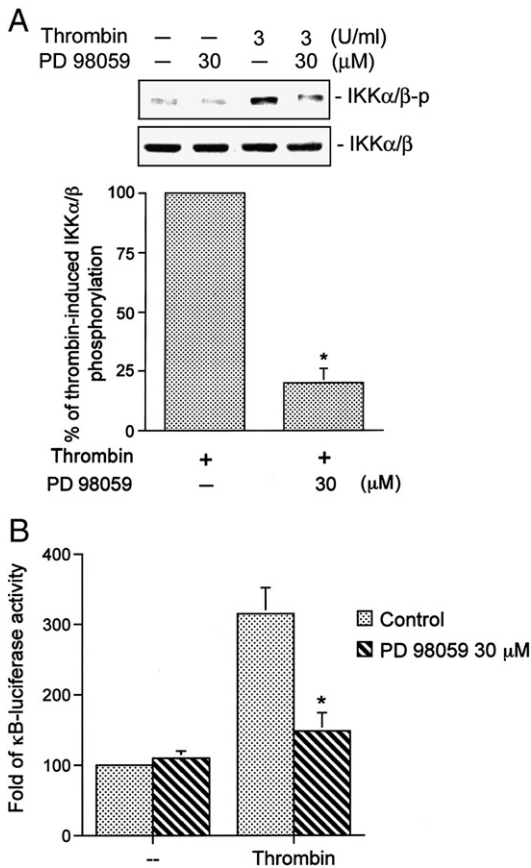


**Fig. 4.** Thrombin induced IKK $\alpha$ / $\beta$  phosphorylation, I $\kappa$ B $\alpha$  phosphorylation, I $\kappa$ B $\alpha$  degradation, and  $\kappa$ B-luciferase activity in WI-38 cells. Cells were incubated with 3 U/ml thrombin for different time intervals. Cell lysates were prepared, and then immunoblotted with antibodies for (A) phospho-IKK $\alpha$ / $\beta$  (top panel) or IKK $\alpha$ / $\beta$  (bottom panel), (B) phospho-I $\kappa$ B $\alpha$  (top panel) or  $\alpha$ -tubulin (bottom panel), and (C) I $\kappa$ B $\alpha$  (top panel) or  $\alpha$ -tubulin (bottom panel). Immunoblots are representative of three experiments with similar results. D, Cells were transiently transfected with 0.5  $\mu$ g of pGL2-ELAM-Luc and 0.5  $\mu$ g of pBK-CMV-Lac Z for 24 h. Cells were then stimulated with thrombin (0.1–10 U/ml) for another 24 h. Luciferase activities were determined as described in “Materials and methods”. The level of induction of luciferase activity was compared to that of cells without agonist treatment. Data are presented as the mean  $\pm$  S.E.M. of three experiments performed in duplicate. \* $P$  < 0.05, compared to the control group without thrombin treatment.

in IKK $\alpha$ / $\beta$  phosphorylation by  $80 \pm 6\%$  (Fig. 5A, upper panel). PD 98059 did not affect the basal level of IKK $\alpha$ / $\beta$  protein expression (Fig. 5A, bottom panel). Similarly, the thrombin-induced increase in  $\kappa$ B-luciferase activity was inhibited by 30  $\mu$ M PD 098059 by  $82 \pm 12\%$  ( $n = 3$ ) (Fig. 5B). Taken together, these data suggest that activation of ERK pathway is required for thrombin-induced IKK $\alpha$ / $\beta$  phosphorylation and NF- $\kappa$ B activation in WI-38 cells.

## 4. Discussion

In this study, we investigated the effects of thrombin-induced COX-2 expression in human lung fibroblasts (WI-38). Our data demonstrate that thrombin induces COX-2 protein expression via the activation of ERK-dependent IKK $\alpha$ / $\beta$  phosphorylation and NF- $\kappa$ B activation in WI-38 cells. Previous studies demonstrated that the induction of COX-2 in response to various stimulators is a consequence of *de novo* transcription (Chen et al., 2004a, 2004b). In humans, the *cox-2* gene promoter contains multiple potential regulatory transcription factor binding sites, including those for NF- $\kappa$ B, NF-IL6, and the activating transcription factor/cyclic AMP-responsive element (ATF/CRE) (Inoue et al., 1995; Kim and Fischer, 1998; Reddy et al., 2000), suggesting potential roles for



**Fig. 5.** Extracellular signal-regulated kinase (ERK) mediates thrombin-induced increases in I $\kappa$ B kinase  $\alpha/\beta$  (IKK $\alpha/\beta$ ) phosphorylation and  $\kappa$ B-luciferase activity in WI-38 cells. A, Cells were pretreated with 30  $\mu$ M PD 98059 for 30 min before treatment with 3 U/ml thrombin for another 20 min. Cell lysates were prepared, and then immunoblotted with antibodies for phospho-IKK $\alpha/\beta$  (top panel) or IKK $\alpha/\beta$  (bottom panel). Immunoblots are representative of three experiments with similar results. B, Cells were transiently transfected with 0.5  $\mu$ g of pGL2-ELAM-Luc and 0.5  $\mu$ g of pBK-CMV-Lac Z for 24 h, and then pretreated with 30  $\mu$ M PD 98059 for 30 min, before incubation with 3 U/ml thrombin for 24 h. Cells were then harvested for the  $\kappa$ B-luciferase assay as described in “Materials and methods”. Data represent the mean  $\pm$  S.E.M. of three experiments performed in duplicate. \* $P$  < 0.05, compared to thrombin treatment.

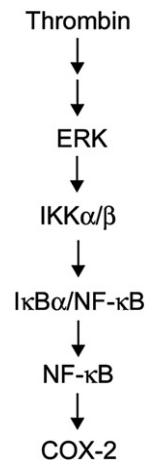
these transcription factors in modulating COX-2 expression. NF- $\kappa$ B, which comprises a family of Rel-related proteins that are normally bound to I $\kappa$ B inhibitors and retained in the cytoplasm, is the most important transcription factor regulating COX-2 expression. Activation of NF- $\kappa$ B plays a pivotal role in regulating lung inflammation (Lin et al., 2006; Martey et al., 2004). The results of this study showed that NF- $\kappa$ B activation is essential for COX-2 expression stimulated by thrombin in lung fibroblasts. This is based on the fact that the I $\kappa$ B $\alpha$  phosphorylation inhibitor (Bay 117082) and NF- $\kappa$ B inhibitor (PDTC) inhibited thrombin-induced COX-2 expression. Moreover, the dominant negative I $\kappa$ B $\alpha$  mutant (I $\kappa$ B $\alpha$ M) inhibited thrombin-induced COX-2-luciferase activity. Furthermore, thrombin induced increases in IKK $\alpha/\beta$  and I $\kappa$ B $\alpha$  phosphorylation, I $\kappa$ B $\alpha$  degradation, and  $\kappa$ B-luciferase activity. Consistent with our study, NF- $\kappa$ B plays a critical role in regulating tobacco smoke-induced COX-2 expression in lung fibroblasts to mediate lung inflammation and cancer (Bagloli et al., 2008; Martey et al., 2004). Therefore, these results suggest that NF- $\kappa$ B activation is required for COX-2 expression by thrombin stimulation of human lung fibroblasts.

The inflammatory activity of thrombin associated with COX-2 induction has been documented in different cell types (Sekiguchi et al., 2007; Seymour et al., 2003; Sokolova et al., 2005; Syeda et al., 2006). Moreover, Sekiguchi et al. (2007) reported that in rat gastric mucosal epithelial cells, MEK/ERK mediate thrombin-induced COX-2

expression. Syeda et al. (2006) showed that thrombin-induced COX-2 expression depends on ERK- and I $\kappa$ B $\alpha$ -dependent NF- $\kappa$ B activation in human endothelial cells. In this study, we present data that confirms the role of the ERK pathway in thrombin-induced COX-2 expression in human lung fibroblasts. We found that blocking ERK by PD 98059 significantly inhibited thrombin-induced COX-2 expression and COX-2-luciferase activity. Moreover, we also found that thrombin activated ERK phosphorylation. In addition, we focused our attention on the ERK pathway mediation of activation of the NF- $\kappa$ B signaling pathway. ERK-mediated induction of NF- $\kappa$ B transcriptional activity was shown to be necessary and sufficient for COX-2 expression (Chen et al., 2004a). Furthermore, several studies showed that ERK mediates increases in IKK $\alpha/\beta$  and NF- $\kappa$ B activities in many cell types (Inoue et al., 1995; Rangaswami et al., 2006; Wei et al., 2008). In this study, we found that thrombin-induced increases in  $\kappa$ B-luciferase activity were abolished by PD 98059, indicating that the ERK pathway is involved in the underlying mechanism of NF- $\kappa$ B activation. In addition, thrombin-induced IKK $\alpha/\beta$  phosphorylation was also inhibited by PD 98059. Therefore, ERK is involved in thrombin-induced NF- $\kappa$ B activation through phosphorylation of IKK $\alpha/\beta$  in WI-38 cells.

A large body of evidence has accumulated to suggest that COX-2 plays a pivotal role in respiratory diseases such as lung cancer, acute lung injury, pulmonary fibrous disorders, and COPD (Carpagnano et al., 2009; Chen et al., 2008; Fukunaga et al., 2005; Lappi-Blanco et al., 2006). For example, airway inflammation, especially small airway inflammation, is an important characteristic of COPD, whereas the chronic inflammation is typified by a heightened expression of COX-2. In COPD patients, COX-2 expression is increased in induced sputum, as well as in lung microvascular smooth muscle cells and fibroblasts (Chen et al., 2003; Martey et al., 2004). Furthermore, a recent study demonstrated that COX-2 is involved in severe airflow limitations during progression of COPD (Chen et al., 2008). In the present study, we found that thrombin induced COX-2 expression in human lung fibroblasts, suggesting that COX-2 expression is contributed to the pathogenesis of lung diseases.

In conclusion, our study supports the regulatory mechanisms of thrombin-induced COX-2 expression through the ERK signaling pathway in increasing IKK $\alpha/\beta$  phosphorylation and NF- $\kappa$ B activation in human lung fibroblasts (WI-38). Fig. 6 is a schematic representation of the signaling pathway involved in the enhancement of COX-2 expression in response to thrombin in human lung fibroblasts.



**Fig. 6.** Schematic summary indicating how signal transduction by thrombin induces cyclooxygenase-2 (COX-2) expression in human lung fibroblasts (WI-38). Thrombin activates the extracellular signal-regulated kinase (ERK) pathway, which in turn induces I $\kappa$ B kinase  $\alpha/\beta$  (IKK $\alpha/\beta$ ) phosphorylation and nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation, and ultimately leads to COX-2 expression in human lung fibroblasts.

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