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Thrombin induces cyclooxygenase-2 expression via the ERK and NF-κB pathways in human lung fibroblasts

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A B S T R A C T

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), NF-κB kinase α/β (IKKα/β), and nuclear factor-κB (NF-κB) 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 IκB phosphorylation inhibitor, and pyrrolidine dithiocarbamate (PDTC), an NF-κB inhibitor, inhibited thrombin-induced COX-2 expression. The thrombin-induced increase in COX-2-luciferase activity was also blocked by the dominant negative IκBα mutant (IκBαM). Treatment of WI-38 cells with thrombin induced IKKα/β and IκBα phosphorylation, IκBα degradation, and NF-κB-luciferase activity. The thrombin-mediated increases in IKKα/β phosphorylation and NF-κB-luciferase activity were inhibited by PD 98059. Taken together, these results suggest that the ERK-dependent IKKα/β/NF-κB 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-α 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)/NF-κB kinase α/β (IKKα/β)/nuclear factor-κB (NF-κB) 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
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-κB 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-κB is associated with IκBα to retain NF-κB in the cytosol. IκKs phosphorylates IκBα at Ser32 and Ser36 to produce ubiquitination and degradation of IκBα (Chen et al., 1995). This process releases active NF-κB, 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-κB, 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-κB in human lung fibroblasts. In this study, our results demonstrate that thrombin activates ERK to induce IKKα/β and NF-κB 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 α-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 IKKα/β, ERK phosphorylated at Tyr204, ERK2, IκBα phosphorylated at Ser32, and IκBα, 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-IKKα (Ser180)/IKKβ (Ser181) was purchased from New England Biolabs (Beverly, MA). A human COX-2 promoter-luciferase construct, pGS459 (−459/+9), which contains 2 IκBα-binding sites, was a kind gift from Prof. C.-C. Chen (National Taiwan University, Taipei, Taiwan). A dominant negative IκBα mutant (IκBαM) was purchased from BD Biosciences (Palo Alto, CA). pGL2-ELAM-Luc (which is under the control of a single NF-κB binding site) and pBK-CMV-LacZ 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 a MEM nutrient mixture, containing 10% fetal calf serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50U/ml penicillin G, and 100 μg/ml streptomycin, in a humidified 37 °C incubator with 5% CO2. 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, α-tubulin, phospho-ERK (Tyr204), ERK2, phospho-IKKα (Ser180)/IKKβ (Ser181), and IKKα/β 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 μg) 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, α-tubulin, phospho-ERK (Tyr204), ERK2, phospho-IKKα (Ser180)/IKKβ (Ser181), IKKα/β, phospho-IκBα (Ser32), and IκBα. 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 × 105 were seeded into 12-well plates. For the COX-2 promoter and IκB-luciferase assays, cells were transfected on the following day with the Lipofectamine plusTM reagent containing 1 μg of pGS459 (−459/+9) or 0.5 μg of pGL2-ELAM-Luc, respectively, and 1 μg of pBK-CMV-LacZ. After 24 h, 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 IκBαM, cells were cotransfected with pGS459 (−459/+9), Lac Z, and IκBαM. 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 ± 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 (0.1–10U/ml) for 24 h induced COX-2 protein expression in a concentration-dependent manner, with a maximum effect at 3–10U/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 ± 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 μM). Pretreating cells with 30 μM PD 98059 inhibited thrombin-induced
COX-2 expression by 94±4% (n = 3) (Fig. 2A). Moreover, the thrombin-induced increase in COX-2-luciferase activity was inhibited by PD 98059. PD 098059 at 30 μM inhibited thrombin-induced COX-2-luciferase activity by 74±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 (3U/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-κB in thrombin-induced COX-2 expression

Previous reports showed that NF-κB activation is necessary for COX-2 induction (Chen et al., 2004b, 2006; N’guessan et al., 2007). Pyrrolidine dithiocarbamate (PDTC), an NF-κB inhibitor, has been shown to inactivate NF-κB (Chen et al., 1998). PDTC was used to examine whether NF-κB activation is involved in the signal transduction pathway leading to COX-2 expression caused by thrombin. When cells were treated with 30 μM PDTC, thrombin-induced COX-2 expression was inhibited by 51±9% (n = 3) (Fig. 3A). Furthermore, pretreatment of cells with 10 μM Bay 117082, an IκB phosphorylation inhibitor (Pierce et al., 1997), inhibited thrombin response by 55±3% (n = 3) (Fig. 3B). In parallel with the inhibition by PDTC and Bay 117082, cells transfected with 0.5 μg IκBαM also exhibited inhibition of thrombin-induced COX-2 protein expression by 50±6% (n = 3) (Fig. 3B). These results suggest that NF-κB activation is necessary for thrombin-induced COX-2 expression in WI-38 fibroblasts.

3.4. Thrombin caused increases in IκKα/β phosphorylation, IκBα phosphorylation, IκBα degradation, and IκB-luciferase activity

We further determined the upstream molecules involved in thrombin-induced NF-κB activation. Stimulation of cells with 3U/ml
thrombin-induced increases in IKKα/β phosphorylation beginning at 3 min which was sustained to 30 min (Fig. 4A). We also found that thrombin-induced IκBα phosphorylation began at 5 min and was sustained to 20 min (Fig. 4B). In parallel with IKKα/β and IκBα phosphorylation, IκBα degradation was apparent after 5 min of treatment with 3 μM thrombin, and the IκBα protein was resynthesized after 30 min of treatment (Fig. 4C). To directly determine NF-κB activation after thrombin treatment, WI-38 cells were transiently transfected with pGL2-ELAM-Luc and 0.5 μg of pGS459 (−459/+9) and 0.5 μg of pBK-CMV-Lac Z or cotransfected with 0.5 μg IκBα 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 ± S.E.M. of three experiments performed in duplicate. *P<0.05, compared to thrombin treatment.

3.5. Involvement of ERK in thrombin-induced increases in IKKα/β phosphorylation and IκBα-luciferase activity

We further examined whether thrombin-induced IKKα/β phosphorylation and NF-κB activation occur through the ERK signaling pathway. As shown in Fig. 5A, pretreatment of cells for 30 min with PD 98059 (30 μM) markedly attenuated the thrombin-induced increases in IKKα/β phosphorylation by 80 ± 6% (Fig. 5A, upper panel). PD 98059 did not affect the basal level of IKKα/β protein expression (Fig. 5A, bottom panel). Similarly, the thrombin-induced increase in IκBα-luciferase activity was inhibited by 30 μM PD 98059 by 82 ± 12% (n = 3) (Fig. 5B). Taken together, these data suggest that activation of ERK pathway is required for thrombin-induced IKKα/β phosphorylation and NF-κB 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 IKKα/β phosphorylation and NF-κB activation in WI-38 cells. Previous studies demonstrated that the induction of COX-2 to 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-κB, 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
these transcription factors in modulating COX-2 expression. NF-κB, which comprises a family of Rel-related proteins that are normally bound to IκB inhibitors and retained in the cytoplasm, is the most important transcription factor regulating COX-2 expression. Activation of NF-κB plays a pivotal role in regulating lung inflammation (Lin et al., 2006; Martery et al., 2004). The results of this study showed that NF-κB activation is essential for COX-2 expression stimulated by thrombin in lung fibroblasts. This is based on the fact that the IκB phosphorylation inhibitor (Bay 117082) and NF-κB inhibitor (PDTC) inhibited thrombin-induced COX-2 expression. Moreover, the dominant negative IκBα mutant (IκBαM) inhibited thrombin-induced COX-2-luciferase activity. Furthermore, thrombin induced increases in IκBα/β and IκBα phosphorylation, IκBα degradation, and NF-κB-luciferase activity. Consistent with our study, NF-κB plays a critical role in regulating tobacco smoke-induced COX-2 expression in lung fibroblasts to mediate lung inflammation and cancer (Baglole et al., 2008; Martery et al., 2004). Therefore, these results suggest that NF-κB 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 IκBα-dependent NF-κB activation in human endothelial cells. In this study, we present data that confirms the role of the ERK pathway in thrombin-induced COX-2 protein 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-κB signaling pathway. ERK-mediated induction of NF-κB 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 IκBα/β and NF-κB activities in many cell types (Inoue et al., 1995; Kangaswami et al., 2006; Wei et al., 2008). In this study, we found that thrombin-induced increases in NF-κB activation was also inhibited by PD 98059. Therefore, ERK is involved in thrombin-induced NF-κB activation through phosphorylation of IκBα/β 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 (Carapagnano 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; Martery 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 IκBα/β phosphorylation and NF-κB 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.
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References


