Thrombin-Induced CCAAT/Enhancer-Binding Protein β Activation and IL-8/CXCL8 Expression via MEKK1, ERK, and p90 Ribosomal S6 Kinase 1 in Lung Epithelial Cells

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Thrombin-Induced CCAAT/Enhancer-Binding Protein β Activation and IL-8/CXCL8 Expression via MEKK1, ERK, and p90 Ribosomal S6 Kinase 1 in Lung Epithelial Cells

Chien-Huang Lin,* Po-Ling Nai,* Maou-Ying Bien,†‡ Chung-Chi Yu,* and Bing-Chang Chen‡

Thrombin, a serine protease, is a well-known coagulation factor generated during vascular injury and plays an important role in lung inflammation. We previously showed that the c-Src– and Rac/Pi3K/Akt-dependent NF-κB pathways are involved in thrombin-induced IL-8/CXCL8 expression in human lung epithelial cells (A549). In this study, we investigated the role of the MEK kinase (MEKK)1/ERK/p90 ribosomal S6 kinase (RSK)1–dependent C/EBPβ signaling pathway in thrombin-induced IL-8/CXCL8 expression. Thrombin-induced IL-8/CXCL8 release and IL-8/CXCL8-luciferase activity were attenuated by small interfering RNA (siRNA) of C/EBPβ and by cells transfected with the C/EBPβ site mutation of the IL-8/CXCL8 construct. Moreover, thrombin-induced c-Jun-luciferase activity was also inhibited by C/EBPβ siRNA. The thrombin-induced increases in IL-8/CXCL8 release and IL-8/CXCL8-luciferase were also inhibited by MEKK1 siRNA, PD98059 (an MEK inhibitor), U0126 (an ERK inhibitor), and RSK1 siRNA. Stimulation of cells with thrombin caused an increase in C/EBPβ phosphorylation at Thr235, C/EBPβ-luciferase activity, recruitment of C/EBPβ to the IL-8/CXCL8 promoter, and C/EBPβ-specific DNA complex formation. Furthermore, thrombin-mediated C/EBPβ phosphorylation and C/EBPβ-luciferase activity were inhibited by MEKK1 siRNA, PD98059, and RSK1 siRNA. Stimulation of cells with thrombin resulted in an increase in RSK1 phosphorylation at Thr359/Ser363, and this effect was inhibited by MEKK1 siRNA and PD98059. The thrombin-induced increase in ERK activation was inhibited by MEKK1 siRNA. These results imply that thrombin activates the MEKK1/ERK/RSK1 signaling pathway, which in turn initiates C/EBPβ activation, recruitment of C/EBPβ to the IL-8/CXCL8 promoter, and C/EBPβ-specific DNA complex formation, and ultimately induces IL-8/CXCL8 expression and release in lung epithelial cells. The Journal of Immunology, 2014, 192: 000–000.

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Abbreviations used in this article: BEGM, bronchial epithelial growth medium; ChIP, chromatin immunoprecipitation; Lac, luciferase; MEKK, MEK kinase; MMP, matrix metalloproteinase; mt, mutant; PAR, protease-activated receptor; RSK, p90 ribosomal S6 kinase; siRNA, small interfering RNA; wt, wild-type.
(also known as NF-IL-6) is known to play critical roles in the immune response and inflammation (16). C/EBPβ can be activated by phosphorylation through several protein kinases, including ERK (17). Human C/EBPβ has several known phosphorylation sites, including Thr235, Thr266, and Thr273, which are important for intracellular localization and transcriptional activity (17, 18). It was shown that Thr235 in the DNA binding site of C/EBPβ was phosphorylated by ERK, and this phosphorylation conferred major transcriptional activity on C/EBPβ, which mediated IL-1β–induced MMP-1 expression in human lung epithelial cells (16, 19).

Mammalian MAPKs are a family of serine/threonine kinases that play essential roles in connecting cell surface receptors to changes in transcriptional programs (20). Upon stimulation, MAP3Ks such as MEK kinase (MEKK1) phosphorylate MEK, which then phosphorylates and activates ERK (21). Signaling initiated with the typically membrane-associated MEKK1 ends with activation of the C/EBP transcription factor (22). In airway epithelial cells, MEKK1 is involved in TNFα–mediated IL-8/CXCL8 expression (23). Moreover, several studies showed that the MEKK1/ERK signaling pathway plays a crucial role in C/EBPβ–dependent gene expression in murine macrophages (22, 24). Taken together, those reports suggested that MEKK1/ERK may function as an upstream activator of C/EBPβ–dependent pathways that mediate inflammatory protein expressions.

Members of the p90 ribosomal S6 kinase (RSK)1 family play critical roles in cell proliferation, survival, and gene expression (25). Among the RSK isoforms, RSK1 is the most represented critical roles in cell proliferation, survival, and gene expression (25). Among the RSK isoforms, RSK1 is the most represented.

**Materials and Methods**

**Materials**

Thrombin (from bovine plasma), U0126, MEKK1 small interfering RNA (siRNA), RSK1 siRNA, C/EBPβ siRNA, and control siRNA (scrambled) were purchased from Sigma-Aldrich (St. Louis, MO). PD98059 was purchased from Calbiochem (San Diego, CA). A chromatin immunoprecipitation (ChIP) assay kit was purchased from Upstate Biotech Millipore (Lake Placid, NY). The human lung epithelial cell line (A549) was obtained from the American Type Culture Collection (Manassas, VA). DMEM/Ham’s F-12 nutrient mixture containing 5% BSA. Proteins were visualized by specific primary Abs specific for ERK, ERK phosphorylation at Tyr204 (E-4, SC-7383), and C/EBPβ, as well as anti-mouse and anti-rabbit IgG-conjugated HRP, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs specific for ERK, ERK phosphorylation at Tyr204, and ERK in A549 cells, proteins were extracted and a Western blot analysis was performed as described previously (12). Briefly, cells were cultured in 6-cm dishes. After reaching confluence, cells were treated with the vehicle and thrombin, pretreated with specific inhibitors, or transfected with various siRNAs as indicated followed by thrombin addition. To assay the effects of MEKK1 siRNA, RSK1 siRNA, and C/EBPβ siRNA, cells were cotransfected with IL-8/CXCL8-Luc, C/EBPβ-Luc, pBK-CMV-luc-Z, control siRNA, MEKK1 siRNA, RSK1 siRNA, or C/EBPβ siRNA. Luciferase activity was determined with a luciferase assay system (Promega, Madison, WI) and was normalized on the basis of lacZ expression. The level of induction of luciferase activity was compared as a ratio of cells with and without stimulation.

**Western blot analysis**

To determine the expressions of C/EBPβ phosphorylation at Thr235, C/EBPβ, RSK1 phosphorylation at Thr858/Ser863, RSK1, ERK phosphorylation at Tyr204, and ERK in A549 cells, proteins were extracted and a Western blot analysis was performed as described previously (12). Briefly, cells were cultured in 6-cm dishes. After reaching confluence, cells were treated with the vehicle and thrombin, pretreated with specific inhibitors, or transfected with various siRNAs as indicated followed by thrombin. After incubation, cells were washed twice in ice-cold PBS and solubilized in extraction buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM PMSF, 5 mM DTT, 0.5% Nonidet P-40, 0.05 mM mephitin A, and 0.2 mM leupeptin. Samples of equal amounts of protein (80 μg) were subjected to SDS-PAGE and then transferred onto a polyvinyliden difluoride membrane, which was then incubated in TBST buffer (150 mM NaCl, 20 mM Tris-HCl, and 0.02% Tween 20; pH 7.4) containing 5% BSA. Proteins were visualized by specific primary proteins using specific primary antibodies and detection was performed using the ECL system (Amersham Biosciences).
Abs and then incubated with HRP-conjugated secondary Abs. The immunoreactivity was detected using ECL following the manufacturer’s instructions. Quantitative data were obtained using a densitometer with Image-Pro Plus image analysis software systems (Eastman Kodak, Rochester, NY).

**ChIP assay**

ChIP assays were performed using a ChIP assay kit according to the manufacturer’s instructions. Briefly, A549 cells (4 × 10^7 cells) were incubated with thrombin (10 U/ml) for 2 h and then cross-linked with formaldehyde at 37˚C for another 10 min. Cell lysates were sonicated and then centrifuged for 10 min at 15,000 × g at 4˚C to spin down the cell debris. Soluble cross-linked chromatin was immunoprecipitated with anti-C/EBP\(\beta\) and anti-rabbit IgG Abs. DNA was purified and eluted with 50 μl elution buffer using a spinning filter. PCR amplifications of the C/EBP\(\beta\) response elements on the IL-8/CXCL8 promoter region were performed using the following primers: 5’-GGT ACT ATG ATA AAG TTA TC-3’ (sense) and 5’-CAT CAC CCT ACT AGA GAA C-3’ (antisense). Extracted DNA (2 μl) was used for 40 cycles of amplification in 50 μl reaction mixture under the following conditions: 95˚C for 30 s, 54˚C for 60 s, and 72˚C for 30 s. The PCR products were analyzed by 2% agarose gel electrophoresis.

**EMSA**

A549 cells were cultured in 10-cm dishes. After reaching confluence, cells were treated with vehicle or 10 U/ml thrombin for 90 min, and then cells were scraped and collected. The preparation of nuclear extracts and the EMSA were performed essentially described previously (12). Briefly, the double-stranded oligonucleotide probes containing C/EBP\(\beta\) consensus and C/EBP\(\beta\) mutant sequences—C/EBP\(\beta\) consensus, 5’-TGC AGA TTG CGC AAT CTG CA-3’ and 3’-ACG TCT AAC GCG TTA GAC GT-5’; and C/EBP\(\beta\) mutant, 5’-TGC AGA GAC TAG TCT CTG CA-3’ and 3’-ACG TCT CTG ATC AGA GAC GT-5’ (Santa Cruz Biotechnology)—were purchased.

**FIGURE 1.** Involvement of C/EBP\(\beta\) in thrombin-induced IL-8/CXCL8 expression and release in human lung epithelial cells. (A) A549 cells and BEAS-2B cells were transiently transfected with control siRNA (con siRNA, 50 nM) or C/EBP\(\beta\) siRNA (50 nM) for 24 h. Cells were stimulated with 10 U/ml thrombin for another 24 h and then IL-8/CXCL8 levels were determined. Results are expressed as the mean ± SEM of three experiments performed in duplicate. *p < 0.05, compared with the thrombin-treated group. (B) A549 cells and BEAS-2B cells were either transiently transfected with 0.2 μg IL-8/CXCL8 wt-Luc and 0.1 μg pBK-CMV-lacZ or were cotransfected with control siRNA (con siRNA, 50 nM) or C/EBP\(\beta\) siRNA (50 nM) for 24 h and then stimulated with 10 U/ml thrombin for another 24 h. Cells were harvested for the luciferase assay as described in Materials and Methods. Results are expressed as the mean ± SEM of three experiments performed in duplicate. *p < 0.05, compared with the thrombin-treated group. (C) A549 cells and BEAS-2B cells were transiently transfected with 50 nM control siRNA (con siRNA) or 50 nM C/EBP\(\beta\) siRNA for 48 h. Cell lysates were prepared and then immunoblotted with Abs for C/EBP\(\beta\) (top panel) or α-tubulin (bottom panel). Traces represent results from three independent experiments. (D) A549 cells were either transiently transfected with 0.2 μg IL-8/CXCL8 wt-Luc (IL-8 wt-Luc), IL-8/CXCL8 C/EBP\(\beta\) mt-Luc (IL-8 C/EBP\(\beta\) mt-Luc), or 0.1 μg pBK-CMV-lucZ for 24 h, and then cells were incubated with 10 U/ml thrombin for another 24 h. Luciferase activities were determined as described in Fig. 1B. Results are expressed as the mean ± SEM of three experiments performed in duplicate. *p < 0.05, compared with the thrombin-treated group. (E) A549 cells were either transiently transfected with 0.5 μg pGL2-ELAM-xb-Luc and 0.5 μg pBK-CMV-lucZ or were cotransfected with control siRNA (con siRNA, 50 nM) or C/EBP\(\beta\) siRNA (50 nM) for 24 h and then stimulated with 10 U/ml thrombin for another 24 h. Cells were harvested for the luciferase assay as described in Materials and Methods. Results are expressed as the mean ± SEM of three experiments performed in duplicate. *p < 0.05, compared with the thrombin-treated group.
and end-labeled with \([\gamma^{-32}\text{P}]\text{ATP}\) using T4 polynucleotide kinase. The nuclear extract (5 μg) was incubated with 1 ng \([32\text{P}]\)-labeled C/EBPβ probe (50,000–75,000 cpm) in 10 μl binding buffer containing 1 μg poly(dI-dC), 15 mM HEPES (pH 7.6), 80 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol at 30˚C for 25 min. DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on 5% polyacrylamide gels. Gels were vacuum-dried and subjected to autoradiography with an intensifying screen at −80˚C. For supershift experiments, 4 μg Ab for C/EBPβ was mixed with the nuclear extract proteins.

Statistical analysis

Results are presented as the mean ± SEM of at least three independent experiments. One-way ANOVA followed by, when appropriate, a Dunnett multiple comparisons test was used to determine the statistical significance of the difference between means. The \(p\) values, \(0.05\) were considered statistically significant.

Results

Involvement of C/EBPβ in thrombin-induced IL-8/CXCL8 expression and release

Many studies revealed that IL-8/CXCL8 expression is regulated by activation of several transcription factors, including NF-κB and C/EBPβ (14, 29). Our previous studies showed that NF-κB activation plays a role in thrombin-induced IL-8/CXCL8 expression (12, 13). Our previous study found that thrombin (0.3–10 U/ml) induced IL-8/CXCL8 release in a concentration-dependent manner, with a maximal effect at 10 U/ml in A549 cells (12). To explore whether C/EBPβ might mediate thrombin-induced IL-8/CXCL8 expression in human lung epithelial cells, C/EBPβ siRNA was used. The siRNA experiments revealed that C/EBPβ siRNA (50 nM)

**FIGURE 2.** Thrombin induced C/EBPβ activation in human lung epithelial cells. A549 cells (A) and BEAS-2B cells (B) were incubated with the vehicle or 10 U/ml thrombin for the indicated intervals. Cell lysates were prepared and then immunoblotted with Abs for phospho-C/EBPβ Thr235 and C/EBPβ. Results are expressed as the mean ± SEM of three experiments. (C) A549 cells were transiently transfected with 0.5 μg C/EBPβ-Luc and 0.1 μg pBK-CMV-lacZ for 24 h and then stimulated with 10 U/ml thrombin for another 24 h. Cells were harvested for the luciferase assay as described in Materials and Methods. Results are expressed as the mean ± SEM of three experiments performed in duplicate. (D) Schematic diagram of transcription factor–binding elements and the ChIP primer location on the IL-8/CXCL8 I promoter. ChIP primer pairs with 272-bp PCR products were designed to amplify DNA corresponding to the putative C/EBPβ binding site. A549 cells were incubated with the vehicle or thrombin (10 U/ml) for 2 h and then cross-linked with formaldehyde at 37˚C for another 10 min. Cell lysates were sonicated and prepared for the ChIP assay using an Ab specific for C/EBPβ. PCR amplification using primers designed against C/EBPβ binding sites was performed. Equal amounts of the soluble cross-linked chromatin present in each PCR were confirmed by the product for input. Rabbit polyclonal IgG was used as a negative control. Input represents 1% of sonicated cross-linked chromatin. Typical traces are representative of three experiments with similar results. (E) A549 cells were incubated with 10 U/ml thrombin for 90 min. Following incubation, a nuclear extract was prepared and EMSA was performed as described in Materials and Methods. The top band represents C/EBPβ. To determine the specificity of C/EBPβ binding, we incubated the nuclear extracts with mutated C/EBPβ oligonucleotides. A supershift experiment with 4 μg anti-C/EBPβ performed on the nuclear extract from thrombin-stimulated A549 cells for 90 min is also shown. Typical traces are representative of three experiments with similar results. *\(p < 0.05\), compared with the control group.
attenuated thrombin-induced IL-8/CXCL8 release by 74 ± 7% (n = 5) in A549 cells (Fig. 1A). Similarly, transfection of BEAS-2B cells with C/EBPβ siRNA (50 nM) also inhibited thrombin-induced IL-8/CXCL8 release by 70 ± 8% (n = 4) (Fig. 1A). Furthermore, transfection of A549 cells with C/EBPβ siRNA (50 nM) almost completely inhibited thrombin-induced IL-8/CXCL8-Luc activity (n = 5) (Fig. 1B). Moreover, in BEAS-2B cells, C/EBPβ siRNA (50 nM) attenuated thrombin-induced IL-8/CXCL8-Luc activity by 60 ± 10% (n = 3) (Fig. 1B). To confirm the results of the C/EBPβ siRNA experiment, we also used C/EBPβ siRNA to suppress C/EBPβ protein expression. We found that C/EBPβ siRNA markedly inhibited C/EBPβ protein expression in A549 cells and BEAS-2B cells (Fig. 1C). Because the regulation of thrombin-induced IL-8/CXCL8-Luc activity and IL-8/CXCL8 release by C/EBPβ in A549 cells is similar to that of the BEAS-2B response, this supported the use of A549 cells for further studies. To further confirm that C/EBPβ is involved in thrombin-induced IL-8/CXCL8 expression, the IL-8/CXCL8-Luc plasmid or C/EBPβ site mutant of the IL-8/CXCL8-Luc (C/EBPβ-mt-IL-8/CXCL8-Luc) plasmid was transfected into A549 cells. As shown in Fig. 1D, thrombin (10 U/ml)-induced IL-8/CXCL8-Luc activity was reduced by 77 ± 5% (n = 3) in cells transfected with the C/EBPβ-mt-IL-8/CXCL8-Luc construct. These results suggest that C/EBPβ is involved in thrombin-induced IL-8/CXCL8 expression in A549 cells. Our previous studies showed that NF-kB activation plays a role in thrombin-induced IL-8/CXCL8 expression (12, 13). Moreover, a previous study revealed that C/EBPβ exerts a positive effect on TNF-α- or LPS-induced NF-kB activation in RAW 264.7 macrophages (30). Therefore, we further evaluated whether C/EBPβ could modulate thrombin-induced NF-kB activation. As shown in Fig. 1E, transfection of cells with C/EBPβ siRNA (50 nM) inhibited thrombin-induced NF-kB-Luc activity by 58 ± 8% (n = 3) (Fig. 1E), suggesting that C/EBPβ may exert positive regulation of NF-kB activation by thrombin stimulation in A549 cells. These results together with our previous data (12, 13) suggest that NF-kB and C/EBPβ both contribute to thrombin-induced IL-8/CXCL8 expression and release.

**Thrombin-induced C/EBPβ activation in human lung epithelial cells**

As mentioned above, C/EBPβ is involved in thrombin-mediated IL-8/CXCL8 expression and release in A549 cells and BEAS-2B cells. We further elucidated whether C/EBPβ activation is involved in the signaling cascade of thrombin-induced IL-8/CXCL8 expression. Thr phosphorylation of residue 235 in C/EBPβ causes functional activation (17), and the Ab against phosphorylated Thr235 was used to examine C/EBPβ activation. Fig. 2A shows that for treatment of A549 cells with thrombin (10 U/ml) for 0–180 min, C/EBPβ Thr235 phosphorylation significantly increased at 30 min and reached a maximum at 120 min (Fig. 2A). Moreover, treatment of BEAS-2B cells with thrombin (10 U/ml) induced an increase in C/EBPβ phosphorylation in a time-dependent manner. The C/EBPβ Thr235 phosphorylation significantly increased at 20 min and reached a maximum at 30–60 min (Fig. 2B). To directly determine C/EBPβ activation after thrombin treatment, cells were transiently transfected with C/EBPβ-Luc as an indicator of C/EBPβ activation. Stimulation of A549 cells with thrombin (0.3–10 U/ml) induced an increase in C/EBPβ-Luc activity in a concentration-dependent manner, with an increase of 3.0 ± 0.4-fold with 10 U/ml thrombin treatment (Fig. 2C). To determine whether C/EBPβ is recruited to the endogenous IL-8/CXCL8 promoter region in response to thrombin, ChIP experiments were performed on A549 cells stimulated with thrombin. Treatment of cells with 10 U/ml thrombin induced an increase in recruitment of C/EBPβ to the C/EBPβ response element on the promoter region of IL-8/CXCL8 (Fig. 2D; IgG, control IgG; input, positive control). To further determine whether thrombin can induce C/EBPβ-specific DNA protein complex formation, an EMSA experiment was performed on A549 cells stimulated with thrombin. Fig. 2E shows that stimulation of thrombin (10 U/ml) resulted in an increase in C/EBPβ-specific DNA protein complex formation (Fig. 2E, lane 2). To determine the specificity of C/EBPβ binding, the C/EBPβ mutated site probe was used. Incubation with C/EBPβ mutant probe did not increase in C/EBPβ-specific DNA complex formation by thrombin stimulation (Fig. 2E, lane 3). To confirm the specificity of C/EBPβ-specific DNA complex formation, a supershift assay was performed using Ab specific for anti-C/EBPβ. Incubation of nuclear extracts with anti-C/EBPβ induced a supershift band and attenuated the C/EBPβ-specific DNA complex formation (Fig. 2E, lane 4). Taken together, these results suggest that activation of C/EBPβ plays a role in...
thrombin-induced IL-8/CXCL8 expression in human lung epithelial cells.

Involvement of RSK1 in thrombin-induced IL-8/CXCL8 expression and release

A recent study suggested that RSK1 participates in signaling pathways leading to the induction of IL-8/CXCL8 expression by *Pseudomonas aeruginosa* infection (31). To examine whether RSK1 might mediate thrombin-induced IL-8/CXCL8 expression and release, RSK1 siRNA was used. As shown in Fig. 3A, transfection of cells with RSK1 siRNA (100 nM) markedly inhibited thrombin-induced IL-8/CXCL8 release. When cells were treated with 100 nM RSK1 siRNA, basal levels of IL-8/CXCL8 and thrombin-induced IL-8/CXCL8 release were attenuated by 75 ± 3 and 49 ± 11%, respectively (n = 3) (Fig. 3A). Furthermore, we found that RSK1 siRNA (100 nM) almost completely attenuated thrombin-induced IL-8/CXCL8-Luc activity (n = 3) (Fig. 3B). Moreover, RSK1 siRNA markedly attenuated RSK1 protein expression (Fig. 3C). These results suggest that RSK1 activation is involved in thrombin-induced IL-8/CXCL8 expression in human lung epithelial cells.

**RSK1 mediates thrombin-induced C/EBPβ phosphorylation and C/EBPβ-Luc activity**

Next, we further examined whether thrombin can induce RSK1 activation. Phosphorylation of RSK1 Thr359/Ser363 residues causes enzymatic activation (25), and an Ab specific against phosphorylated RSK1 Thr359/Thr363 was used to determine RSK1 phosphorylation. When cells were treated with 10 U/ml thrombin for various intervals, RSK1 Thr359/Ser363 phosphorylation increased at 5 min, peaked at 10 min, and declined after 20 min of treatment (Fig. 4A, upper panel). The protein level of RSK1 was not affected by thrombin treatment (Fig. 4A, lower panel).

Next, we investigated the role of RSK1 in thrombin-induced C/EBPβ Thr235 phosphorylation. As shown in Fig. 4B, transfection of cells with RSK1 siRNA (100 nM) markedly inhibited thrombin-induced C/EBPβ Thr235 phosphorylation (Fig. 4B).

**FIGURE 4.** RSK1 is involved in thrombin-induced C/EBPβ activation in A549 cells. (A) Cells were incubated with the vehicle or 10 U/ml thrombin for 0–60 min. Cell lysates were prepared and then immunoblotted with Abs for phospho-RSK1 Thr359/Ser363 and RSK1. Results are expressed as the mean ± SEM of three experiments. *p < 0.05, compared with the control group. (B) Cells were transiently transfected with control siRNA (con siRNA, 100 nM) or RSK1 siRNA (100 nM) for 24 h and then stimulated with 10 U/ml thrombin for another 10 min. Cell lysates were prepared and then immunoblotted with Abs for phospho-C/EBPβ Thr235 and C/EBPβ. Results are expressed as the mean ± SEM of three experiments. *p < 0.05, compared with the control group. (C) Cells were either transiently transfected with control siRNA (con siRNA, 100 nM) or RSK1 siRNA (100 nM) for 24 h and then stimulated with 10 U/ml thrombin for another 24 h. Cells were harvested for the luciferase assay as described in Materials and Methods. Results are expressed as the mean ± SEM of three experiments performed in duplicate. *p < 0.05, compared with the control group.

**FIGURE 5.** ERK is involved in thrombin-mediated IL-8/CXCL8-Luc activity in A549 cells. (A) Cells were transfected with 0.5 μg C/EBPβ-Luc and 0.1 μg pBK-CMV-lacZ for 24 h or were pretreated with an equivalent vehicle control (DMSO) or PD98059 (30 μM) for 30 min and then incubated with 10 U/ml thrombin for another 24 h. Cells were harvested for the luciferase assay as described in Materials and Methods. Results are expressed as the mean ± SEM of three experiments performed in duplicate. *p < 0.05, compared with the thrombin-treated group. (B) Cells were pretreated with an equivalent vehicle control (DMSO) or U0126 (10 μM) for 30 min and then incubated with 10 U/ml thrombin for another 24 h. IL-8/CXCL8 levels were determined. Results are expressed as the mean ± SEM of three experiments. *p < 0.05, compared with the thrombin-treated group.
parallel with C/EBPβ Thr235 phosphorylation, thrombin-induced C/EBPβ-Luc activity was also completely inhibited by RSK1 siRNA (100 nM) (Fig. 4C). These results suggest that RSK1 is an upstream molecule of thrombin-induced C/EBPβ activation in human lung epithelial cells.

Involvement of ERK in thrombin-induced IL-8/CXCL8-Luc activity

ERK might activate a number of signal pathways, including RSK1 (25). Next, to determine whether ERK is involved in the signal transduction pathway leading to IL-8/CXCL8 expression caused by thrombin, a MEK inhibitor (PD98059) and an ERK inhibitor (U0126) were used. Our previous study showed that PD98059 attenuated thrombin-induced IL-8/CXCL8 release in A549 cells (13). In this study, we found that thrombin-induced IL-8/CXCL8-Luc activity was also inhibited by PD98059 (30 μM) (Fig. 5A). Moreover, we found that U0126 (10 μM) inhibited thrombin-induced IL-8/CXCL8 release by 78 ± 3% (n = 3) (Fig. 5B). These results, together with our previous data, suggest that ERK is involved in thrombin-induced IL-8/CXCL8 expression and release in A549 cells.

**FIGURE 6.** Involvement of ERK in thrombin-induced RSK1 and C/EBPβ activation in A549 cells. (A) Cells were pretreated with 30 μM PD98059 for 30 min and then stimulated with thrombin for another 120 min. Cell lysates were prepared and then immunoblotted with Abs for phospho-C/EBPβ Thr235 and C/EBPβ. Results are expressed as the mean ± SEM of three experiments. *p < 0.05, compared with thrombin stimulation. (B) Cells were transiently transfected with 0.5 μg C/EBPβ-Luc and 0.1 μg pBK-CMV-lacZ for 24 h or pretreated with 30 μM PD98059 for 30 min and then stimulated with 10 U/ml thrombin for another 24 h. Cells were harvested for the luciferase assay as described in Materials and Methods. Results are expressed as the mean ± SEM of three experiments performed in duplicate. *p < 0.05, compared with the thrombin-treated group. (C) Cells were transiently transfected with 30 μM PD98059 for 30 min and then stimulated with thrombin for another 10 min. Cell lysates were prepared and then immunoblotted with Abs for phospho-RSK1 Thr359/Ser363 and RSK1. Results are expressed as the mean ± SEM of three experiments. *p < 0.05, compared with thrombin stimulation.

**FIGURE 7.** Involvement of MEKK1 in thrombin-induced IL-8/CXCL8 release and IL-8/CXCL8-Luc activity in A549 cells. (A) Cells were transiently transfected with control siRNA (con siRNA, 100 nM) or MEKK1 siRNA (100 nM) for 24 h. Cells were stimulated with 10 U/ml thrombin for another 24 h and then IL-8/CXCL8 levels were determined. Results are expressed as the mean ± SEM of three experiments performed in duplicate. (B) Cells were either transiently transfected with 0.2 μg IL-8/CXCL8 wt-Luc and 0.1 μg pBK-CMV-lacZ or were cotransfected with control siRNA (con siRNA, 100 nM) or MEKK1 siRNA (100 nM) for 24 h and then stimulated with 10 U/ml thrombin for another 24 h. Cells were harvested for the luciferase assay as described in Materials and Methods. Results are expressed as the mean ± SEM of three experiments performed in duplicate. (C) Cells were transiently transfected with 100 nM control siRNA (con siRNA) or 100 nM MEKK1 siRNA for 48 h. Cell lysates were prepared, and then immunoblotted with Abs for MEKK1 (top panel) or α-tubulin (bottom panel). Traces represent results from three independent experiments. *p < 0.05, compared with thrombin-treated group.
ERK mediated thrombin-induced RSK1 phosphorylation and C/EBPβ activation

As mentioned above, ERK is involved in thrombin-mediated IL-8/CXCL8 expression in A549 cells. To further examine whether RSK1 and C/EBPβ activation occurs through the ERK signaling pathway, treatment of A549 cells with 30 μM PD98059 markedly inhibited thrombin-induced C/EBPβ Thr235 phosphorylation (n = 3) (Fig. 6A). Similarly, thrombin-induced C/EBPβ-Luc activity was almost completely attenuated by PD98059 (30 μM) (Fig. 6B). Moreover, thrombin-induced RSK1 Thr359/Ser363 phosphorylation was inhibited by treatment of cells with 30 μM PD98059 (Fig. 6C). These results suggest that ERK activation occurs upstream of RSK1 and C/EBPβ in thrombin-induced IL-8/CXCL8 expression and release in A549 cells.

MEKK1 mediated thrombin-induced IL-8/CXCL8 release and IL-8/CXCL8-Luc activity

To examine whether MEKK1, an upstream protein of ERK, might play a crucial role in thrombin-induced IL-8/CXCL8 release and IL-8/CXCL8-Luc activity, transfection of cells with MEKK1 siRNA (100 nM) significantly inhibited thrombin-induced IL-8/CXCL8 release (Fig. 7A). When cells were treated with 100 nM MEKK1 siRNA, thrombin-induced IL-8/CXCL8 release was inhibited by 58 ± 22% (n = 3) (Fig. 7A). Moreover, thrombin-induced IL-8/CXCL8-Luc activity was attenuated in cells transfected with 100 nM MEKK1 siRNA (Fig. 7B). Moreover, MEKK1 siRNA markedly inhibited MEKK1 protein expression (Fig. 7C). These results suggest that activation of MEKK1 is involved in thrombin-induced IL-8/CXCL8 expression in human lung epithelial cells.

MEKK1 mediates thrombin-induced ERK, RSK1, and C/EBPβ activation

Next, we investigated the role of MEKK1 in thrombin-induced C/EBPβ Thr235 phosphorylation and C/EBPβ-Luc activity. As shown in Fig. 8A and 8B, transfection of A549 cells with MEKK1 siRNA (100 nM) attenuated thrombin-induced C/EBPβ Thr235 phosphorylation and C/EBPβ-Luc activity by 90 ± 23 and 70 ± 44%, respectively. We further examined whether MEKK1 mediates ERK Tyr249 phosphorylation and RSK1 Thr363/Ser363 phosphorylation. We found that thrombin-induced ERK Tyr249 phosphorylation was inhibited by 100 nM MEKK1 siRNA. Moreover, transfection of cells with 100 nM MEKK1 siRNA attenuated thrombin-induced RSK1 Thr363/Ser363 phosphorylation (Fig. 8C, 8D). These results suggest that activation of MEKK1 occurs upstream of ERK, RSK1, and C/EBPβ in thrombin-induced IL-8/CXCL8 expression and release.

Discussion

A growing body of evidence demonstrates that increased thrombin levels occur in lungs of asthmatic patients and play a crucial role in...
the pathological process. Thrombin mediates a variety of inflammatory and tissue repair responses associated with vascular injury (32). Additionally, thrombin stimulates secretion of mediators involved in the pathogenesis of lung inflammation, such as IL-6, IL-8/CXCL8, and PGE\(_2\) in lung epithelial cells and fibroblasts (11, 13). Our previous studies showed that thrombin induced NF-\(\kappa\)B activation and IL-8/CXCL8 expression and release through two separate pathways, including the protein kinase Ca/c-Src/IkB kinase \(\alpha/\beta\) and Rac1/PI3K/Akt/IkB kinase \(\alpha/\beta\) pathways (12, 13). In the present study, we provide a description of the signaling pathway through which thrombin stimulated activation of MEKK1, ERK, and RSK1 to induce C/EBP\(\beta\) activation and recruitment to the IL-8/CXCL8 promoter, which in turn induced IL-8/CXCL8 expression and release in human lung epithelial cells.

C/EBPs comprise a family of six structurally related transcription factors that play vital roles in tissue development, cellular proliferation, and differentiation (16). C/EBPs were reported to play important roles in pulmonary inflammation through induction of the expressions of several proinflammatory mediators (16, 33). The IL-8/CXCL8 gene possesses several binding sites, including NF-\(\kappa\)B, AP-1, and C/EBP\(\beta\) (14). Our previous studies demonstrated that NF-\(\kappa\)B activation is involved in thrombin-induced IL-8/CXCL8 expression in human lung epithelial cells (12, 13). Recent studies suggested that C/EBP\(\beta\) also plays a critical role in regulating IL-8/CXCL8 expression by various stimuli in lung epithelial cells (15, 34). In human airway epithelial cells, ozone-induced IL-8/CXCL8 expression requires C/EBP\(\beta\) activation (34). In A549 cells, C/EBP\(\beta\) also participates in bacterial flagellin-induced IL-8/CXCL8 expression (15). The results of this study showed that C/EBP\(\beta\) activation is involved in IL-8/CXCL8 expression and release stimulated by thrombin. This is based on the thrombin-induced increases in C/EBP\(\beta\) phosphorylation, C/EBP\(\beta\)-Luc activity, recruitment of C/EBP\(\beta\) to the IL-8/CXCL8 promoter, and C/EBP\(\beta\)-specific DNA complex formation. Furthermore, transfection of cells with C/EBP\(\beta\) siRNA reduced thrombin-induced IL-8/CXCL8 expression and IL-8/CXCL8 release. Moreover, a mutational analysis demonstrated that thrombin-induced IL-8/CXCL8-Luc activity was inhibited by transfection with the C/EBP\(\beta\)-mt-IL-8/CXCL8-Luc construct. Therefore, these results suggest that C/EBP\(\beta\) activation also participates in thrombin-induced IL-8/CXCL8 expression and release.

Previous studies have shown that C/EBP\(\beta\) plays a complex role in NF-\(\kappa\)B–dependent gene regulation (16, 35). Several promoters have been identified, such as IL-8/CXCL8, which are positively regulated via interaction of C/EBP\(\beta\) and NF-\(\kappa\)B (34, 35). A previous study showed that \(P.\ aeruginosa\) induces IL-8/CXCL8 gene expression through the recruitment of both RelA and C/EBP\(\beta\) to the IL-8/CXCL8 promoter in human conjunctival epithelial cells (35). Moreover, Cappello et al. (30) indicated that C/EBP\(\beta\) enhances TNF-\(\alpha\)– or LPS-induced NF-\(\kappa\)B activation in RAW 264.7 macrophages. In agreement with previous studies, our previous study found that stimulation of cells with thrombin resulted in an increase in NF-\(\kappa\)B–specific DNA protein complex formation (12). In this study, we found that C/EBP\(\beta\) markedly bound on the IL-8/CXCL8 promoter upon thrombin treatment. Furthermore, transfection of cells with C/EBP\(\beta\) siRNA significantly inhibited thrombin-induced NF-\(\kappa\)B activation, indicating that C/EBP\(\beta\) exerts a positive modulation on thrombin-induced NF-\(\kappa\)B activation. Taken together, our results suggest that thrombin induces IL-8/CXCL8 expression in human lung epithelial cells by activating NF-\(\kappa\)B and C/EBP\(\beta\) and by inducing their cooperative binding to the IL-8/CXCL8 promoter that, in turn, activates transcription.

RSK1 was shown to activate transcription factors and mediate protein expression by various stimuli (25, 26). Bezzerrri et al. (31) reported that activation of RSK1 is required for induction of IL-8/CXCL8 caused by \(P.\ aeruginosa\) stimulation. Moreover, RSK was shown to be activated by a PAR1 agonist and to modulate IL-8/CXCL8 expression in intestinal epithelial cells (36). In this study, we present data that confirm the role of RSK1 in thrombin-induced IL-8/CXCL8 expression and release. We found that treatment of A549 cells with thrombin caused RSK1 phosphorylation at Thr\(^{359}\)/Thr\(^{363}\), and RSK1 siRNA inhibited thrombin-induced IL-8/CXCL8-Luc activity and IL-8/CXCL8 release. These results suggest that RSK1 is required for thrombin-induced IL-8/CXCL8 expression and release in human lung epithelial cells.

MEKK1, which belongs to the MAP3K family, was shown to activate MAPK signaling cascades (21). A large body of evidence has implicated MEKK1 involvement in IL-8/CXCL8 expression by various stimuli in different cell types (23, 37). Zhou et al. (23) reported that MEKK1 mediates TNF-\(\alpha\)–induced IL-8/CXCL8 expression in airway epithelial cells. Another study demonstrated that streptococcal pyrogenic exotoxin B–induced IL-8/CXCL8 expression depends on MEKK1 activation in human lung epithelial cells (A549) (38). Additionally, several reports demonstrated that the ERK signaling pathway is involved in thrombin-induced IL-8/CXCL8 release (37, 39). In this study, we further demonstrated roles of MEKK1 and RSK in thrombin-induced IL-8/CXCL8 expression in human lung epithelial cells. We found that MEKK1 siRNA and a MEK inhibitor (PD98059) both inhibited thrombin-induced IL-8/CXCL8-Luc activity. Our previous study demonstrated that PD98059 attenuated thrombin-induced IL-8/CXCL8 release in human lung epithelial cells (13). Similarly, we found that MEKK1 siRNA and an ERK inhibitor (U0126) both also inhibited thrombin-induced IL-8/CXCL8 release. Taken together, we suggest that MEKK1 and ERK are involved in thrombin-induced IL-8/CXCL8 expression and release in human lung epithelial cells.
We also focused our attention on the MEKK1-mediated activation of the ERK signaling pathway, which is involved in thrombin-induced RSK1 phosphorylation. Mounting evidence showed that phosphorylation of RSK1 linked a growth factor to activation of transcription factor cascades and mediation of inflammatory responses (25). Several reports demonstrated that ERK or 3-phosphoinositide–dependent protein kinase-1 mediated phosphorylation of RSK1 in several cell types (25, 26). However, the molecular mechanisms through which RSK1 becomes activated by thrombin in lung epithelial cells are still unclear. A previous study showed that ERK mediated the IL-6–induced RSK1 phosphorylation pathway in human cholangiocarcinomas (33). Moreover, Zhou et al. (23) demonstrated that MEKK1 mediates TNF-α–induced ERK activation and IL-8/CXCL8 expression in human bronchial epithelial cells. In this study, we found that thrombin induced phosphorylation of RSK1 at Thr235 and Ser363, which were abolished by cell transfeciton with MEKK1 siRNA and a MEK inhibitor (PD98059). Moreover, we also found that MEKK1 siRNA inhibited thrombin-induced ERK Tyr205 phosphorylation. In support of our findings, Malik and Storey (40) demonstrated that the dehydration stress-induced MEKK1/ERK signaling pathway is required for RSK1 phosphorylation in Xenopus laevis tissues. Taken together, these results suggest that MEKK1/ERK is an upstream kinase of RSK1 with thrombin treatment in lung epithelial cells. Transcriptional activity of C/EBPβ may be regulated by its expression and also by multiple phosphorylations. In addition to recruiting C/EBPβ to the IL-8/CXCL8 promoter, thrombin appears to regulate the activity of C/EBPβ through phosphorylation. It is known that activation of both ERK and RSK are required for ligand-induced phosphorylation of C/EBPβ. It was demonstrated that ERK is involved in events leading to the enhanced phosphorylation of C/EBPβ at Thr235, and this phosphorylation confers major transcriptional activity on C/EBPβ (16). Given that ERK/RSK1 plays an important role in C/EBPβ transactivation, we speculated that MEKK1, ERK, and RSK1 might be upstream of C/EBPβ and investigated this possibility. In this study, we found that MEKK1 siRNA, PD98059, and RSK1 siRNA all blocked thrombin-induced C/EBPβ reporter activity. Moreover, we found that thrombin-mediated Thr235 phosphorylation of C/EBPβ was attenuated by MEKK1, ERK, and RSK1. Taken together, these results suggest that thrombin-induced phosphorylation of C/EBPβ at Thr235 and C/EBPβ activation are regulated by the MEKK1/ERK/RSK1 signaling pathway in A549 cells. In conclusion, the present study shows that thrombin activates the MEKK1/ERK/RSK1 signaling pathway, which in turn initiates C/EBPβ phosphorylation at Thr235, C/EBPβ activation, and recruitment to the IL-8/CXCL8 promoter, and finally induces IL-8/CXCL8 expression and release in human lung epithelial cells. This study shows that RSK1 mediates C/EBPβ phosphorylation of Thr235 and C/EBPβ activation caused by thrombin in human lung epithelial cells. Fig. 9 is a schematic representation of the signaling pathway that participates in thrombin-induced IL-8/CXCL8 expression and release in lung epithelial cells. Our results present a mechanism linking thrombin and IL-8/CXCL8, and they provide support for the development of therapeutic strategies to reduce inflammation caused by thrombin.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


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