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Activation and Induction of Cytosolic Phospholipase A_2 by IL-1β in Human Tracheal Smooth Muscle Cells: Role of MAPKs/p300 and NF-κB

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ABSTRACT

Cytosolic phospholipase A_2 ($cPLA_2$) plays a pivotal role in mediating agonist-induced arachidonic acid (AA) release for prostaglandin (PG) synthesis during inflammation triggered by IL-1β. However, the mechanisms underlying IL-1β-induced $cPLA_2$ expression and PGE_2 synthesis in human tracheal smooth muscle cells (HTSMCs) remain unknown. IL-1β-induced $cPLA_2$ protein and mRNA expression, PGE_2 production, or phosphorylation of p42/p44 MAPK, p38 MAPK, and JNK1/2, which was attenuated by pretreatment with the inhibitors of MEK1/2 (U0126), p38 MAPK (SB202190), and JNK1/2 (SP600125) or transfection with siRNAs of MEK1, p42, p38, and JNK2. IL-1β-induced $cPLA_2$ expression was also inhibited by pretreatment with a NF-κB inhibitor, helenalin or transfection with siRNA of NIK, IKKα, or IKKβ. IL-β-induced NF-κB translocation was blocked by pretreatment with helenalin, but not U0126, SB202190, and SP600125. In addition, transfection with p300 siRNA blocked $cPLA_2$ expression induced by IL-1β. Moreover, p300 was associated with the $cPLA_2$ promoter, which was dynamically linked to histone H4 acetylation stimulated by IL-1β. These results suggest that in HTSMCs, activation of MAPKs, NF-κB, and p300 are essential for IL-1β-induced $cPLA_2$ expression and PGE_2 secretion. J. Cell. Biochem. 109: 1045–1056, 2010.

KEY WORDS: IL-1β; CYTOSOLIC PHOSPHOLIPASE A_2; PGE_2; p300; NF-κB

A number of lipid mediators, such as ecosanoids generated from arachidonic acid (AA) have been identified in situ in airway secretion of asthmatics [Barnes, 1989]. These AA metabolites play an important role in the inflammatory pathogenesis, including asthma [Henderson et al., 2002]. The generation of ecosanoids is first initiated through the release of AA from membrane phospholipids hydrolyzed by the action of phospholipase A_2 (PLA_2) enzymes [Balsinde et al., 1999; Bonventre and Sapirstein, 2002]. AA is further converted to prostaglandins (PGs), such as PGE_2 by the constitutive enzyme cyclooxygenase (COX)-1 or the inducible COX-2 in various cell types [DeWitt, 1999; Yang et al., 2002]. The PLA_2 superfamily is composed of three main types of lipolytic enzymes, including secretory PLA_2, the 85 kDa cytosolic group IV PLA_2 ($cPLA_2$), and a calcium-independent group VI PLA_2 in mammalian cells [Six and Dennis, 2000]. Although several subtypes of PLA_2 have been described, $cPLA_2$ is the only one that exhibits specificity for AA, and its role in mediating agonist-induced AA release for eicosanoid production in various cell types is well studied [Leslie, 1997]. $cPLA_2$ has been shown to be implicated in acute lung injury induced by sepsis [Nagase et al., 2000] and bronchial reactivity associated with anaphylaxis [Uozumi et al., 1997]. Furthermore, increased synthesis of PGE_2 is dependent on an increase in $cPLA_2$ activity in various cell types [Dieter et al., 2002; Ghosh et al., 2004]. Tracheal smooth muscle cells also synthesize these lipid mediators that exert physiological or pathophysiological actions under various conditions. These results demonstrate that $cPLA_2$ plays an important role in mediating AA release for production of PGE_2 by inflammatory cells and airway resident cells.

Several lines of evidence have demonstrated that mesenchymal cells in several extrapulmonary sites synthesize PLA_2 upon stimulation by IL-1β, TNF-α, or IL-6 [Schalkwijk et al., 1991; Vadas et al., 1993]. Elevated levels of pro-inflammatory cytokines, including IL-1β in the bronchoalveolar lavage fluid have been detected in allergic asthmatic patients [Broide et al., 1992]. The

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expression of $cPLA_2$ induced by IL-1β may be integrated to the signaling networks that augment airway inflammation by enhancing eicosanoid synthesis. Increased levels of PLA_2 in circulation and affected tissues have been found to be associated with various pathological conditions, such as lung inflammation [Vadas et al., 1993]. However, the signaling pathways mediating IL-1β-induced $cPLA_2$ expression and PGE_2 synthesis in human tracheal smooth muscle cells (HTSMCs) have not been defined.

Several extracellular stimuli elicit a broad spectrum of biological responses through activation of MAPK cascades [Marshall, 1994]. Activation of MAPKs exerts distinct cellular responses mediated by phosphorylation of specific target proteins [Marshall, 1994]. Recently, we have demonstrated that IL-1β causes a rapid phosphorylation of p42/p44 MAPK and up-regulation of COX-2 expression in TSMCs [Yang et al., 2002; Lin et al., 2004]. These findings further imply the roles of these signaling pathways as important signaling pathways underlying the IL-1β-induced $cPLA_2$ expression and PGE_2 synthesis in HTSMCs. In addition, NF-κB has been shown to involve in $cPLA_2$ gene expression at the transcriptional level in various cell types [Goppelt-Struebe and Rehfeldt, 1992; Hulkower et al., 1994]. Therefore, whether activation of MAPKs and NF-κB pathways by IL-1β linked to $cPLA_2$ expression and PGE_2 synthesis is needed to be determined in HTSMCs.

Histone acetyltransferases (HATs), such as p300 and CREB-binding protein (CBP) functioning as transcriptional co-activators and signal integrators have been proved to play a vital role in expression of inflammatory genes. It has been demonstrated that pulmonary inflammation, exacerbate asthma, and chronic obstructive pulmonary disease induced by exposure to diesel exhaust particulate matter are related to the p300 activation and recruitment to the promoter region of COX-2 [Cao et al., 2007]. In airway smooth muscle cells, IL-1β has been proved to stimulate expression of COX-2 through modification of histone H4 acetylation state [Nie et al., 2003]. Although many evidences address the involvement of p300 in COX-2 expression, the role of p300 in $cPLA_2$ expression in response to IL-1β remained to be determined.

In addressing these questions, experiments were undertaken to investigate the mechanisms underlying IL-1β-induced $cPLA_2$ expression and PGE_2 synthesis in HTSMCs. These findings suggest that the increased expression of $cPLA_2$ correlates with the enhanced release of PGE_2 from IL-1β-challenged HTSMCs, at least in part, mediated through MAPKs and NF-κB signaling pathways. Activation of MAPKs and NF-κB regulated the activity of p300 and acetylation of histone H4 hence led to $cPLA_2$ expression. These results provide a new insight into the mechanisms that the MAPKs and NF-κB may be the critical components regulating $cPLA_2$ expression and PGE_2 synthesis in IL-1β-stimulated HTSMCs.

MATERIALS AND METHODS

MATERIALS

Recombinant human IL-1β was from R&D System (Minneapolis, MN). Anti-$cPLA_2$, anti-p300, anti-CBP, and anti-NF-κB (p65) antibodies were from Santa Cruz (Santa Cruz, CA). Anti-phospho p42/p44 MAPK, anti-phospho p38 MAPK, and anti-phospho JNK1/2 antibodies were from Cell Signaling (Danver, MA). U0126, SB202190, SP600125, helenalin, and garcinol were from Biomol (Plymouth Meeting, PA). Bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL).

CELL CULTURE

HTSMCs were purchased from ScienCell Research Lab (San Diego, CA) and grown as described previously [Lee et al., 2004]. Experiments were performed with cells from passages 4 to 7.

MEASUREMENT OF PGE_2 GENERATION

HTSMCs were cultured in six-well culture plates. After reaching confluence, cells were treated with IL-β for the indicated times at 37°C. After treatment, the medium were collected and stored at −80°C until being assayed. PGE_2 was assayed using the PGE_2 enzyme immunoassay kit (Cayman) according to the manufacturer's instructions.

TRANSIENT TRANSFECTION

SMARTpool RNA duplexes corresponding to human MEK1, p42, p38, JNK2, NIK, IKKα, IKKβ, and scrambled #2 siRNA were from Dharmacon Research, Inc. (Lafayette, CO). Transient transfection of siRNAs was carried out using Metafectene transfection reagent. siRNA (100 nM) was formulated with Metafectene transfection reagent according to the manufacturer's instruction. The transfection efficiency (~60%) was determined by transfection with EGFP.

WESTERN BLOT ANALYSIS

Growth-arrested HTSMCs were incubated with IL-1β at 37°C for the indicated times. The cells were washed, scraped, collected, and centrifuged at 45,000*g* at 4°C for 1 h to yield the whole cell extract, as previously described [Lee et al., 2004]. Samples were denatured, subjected to SDS–PAGE using a 12% running gel, and transferred to nitrocellulose membrane. Membranes were incubated with anti-$cPLA_2$ antibody for 24 h, and then membranes were incubated with anti-mouse horseradish peroxidase antibody for 1 h. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL.

QUANTITATIVE REAL-TIME RT-PCR

RNA was extracted using TRIzol and first-strand cDNA synthesis was done with 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocols. The primers and probes used for real-time PCR of human $cPLA_2\alpha$ and GAPDH were obtained from Applied Biosystems (Foster City, CA). Each PCR reaction (20 μl) contained 100 ng of cDNA, PCR master mix, and premade Taqman gene expression assay components containing a FAM reporter dye at the 5′-end of the Taqman probe and a nonfluorescent quencher (NFQ) at the 3′-end of the probe. Human GAPDH was used as a control to verify the quality of cDNA template. Real-time PCR was performed and analyzed by the ABI StepOnePlusR QPCR instrument, under standard thermal cycling conditions from Applied Biosystems.

LUCIFERASE ASSAY

For construction of the $cPLA_2$-luc plasmid, human $cPLA_2$ promoter, a region spanning −2,375 to +75 bp, was cloned into pGL3-basic

vector (Promega, Madison, WI). $cPLA_2$-luc activity was determined as previously described [Lee et al., 2008] using a luciferase assay system (Promega). Firefly luciferase activities were standardized for β-gal activity.

ISOLATION OF CELL FRACTION

Cells were harvested, sonicated for 5 s at output 1.5 with a sonicator (Ultrasonics, Inc., NY), and centrifuged at 8,000 rpm for 15 min at 4°C. The pellet was collected as the nuclear fraction. The supernatant was centrifuged at 14,000 rpm for 60 min at 4°C to yield the pellet (membrane fraction) and the supernatant (cytosolic fraction).

CHROMATIN IMMUNOPRECIPITATION ASSAY

To detect the association of nuclear proteins with human $cPLA_2$ promoter, chromatin immunoprecipitation (ChIP) analysis was conducted as previously described [Nie et al., 2003]. DNA immunoprecipitated by anti-p300 or anti-acetylated histone H4 antibody was purified. The DNA pellet was re-suspended in H_2O and subjected to PCR amplification with the forward primer 5′-GAATTCAACCTGATTTCATTTTCTTCC-3′ and the reverse primer 5′-CTTCAGGCTCCTCAATGCCTCTAGCTTTCAG-3′, which were specifically designed from the $cPLA_2$ promoter region. PCR products were analyzed on ethidium bromide-stained agarose gels (1%).

ANALYSIS OF DATA

Concentration–effect curves were fitted and EC_{50} values were estimated using the GraphPad Prism Program (GraphPad, San Diego, CA). Data were expressed as the mean ± SEM and analyzed by one-way ANOVA followed with Tukey's post hoc test. $P < 0.05$ was considered significant.

RESULTS

IL-1β INDUCES $cPLA_2$ EXPRESSION AND PGE_2 RELEASE

To determine the effect of IL-1β on $cPLA_2$ expression, HTSMCs were incubated with 30 ng/ml IL-1β for the indicated times. As shown in Figure 1A, IL-1β-induced $cPLA_2$ protein expression in a time-dependent manner with a maximal response within 16–24 h. Moreover, IL-1β also enhanced $cPLA_2$ mRNA accumulation in a time-dependent manner with a maximal response within 5 h (Fig. 1B). $cPLA_2$ is the major intracellular form of PLA_2, which selectively hydrolyzes membrane phospholipids at the sn-2 position and is the rate-limiting enzyme in the regulated release of AA [Dennis, 1997]. AA is further converted to PGs (i.e., PGE_2) by the constitutive enzyme COX-1 or by the inducible COX-2. We further tested the effect of IL-1β on PGE_2 synthesis as a parameter of $cPLA_2$ activity in HTSMCs. As shown in Figure 1C, IL-1β induced a time-dependent increase in PGE_2 synthesis in HTSMCs. These data suggested that IL-1β-induced PGE_2 generation was dependent on the up-regulation of $cPLA_2$ expression in HTSMCs.

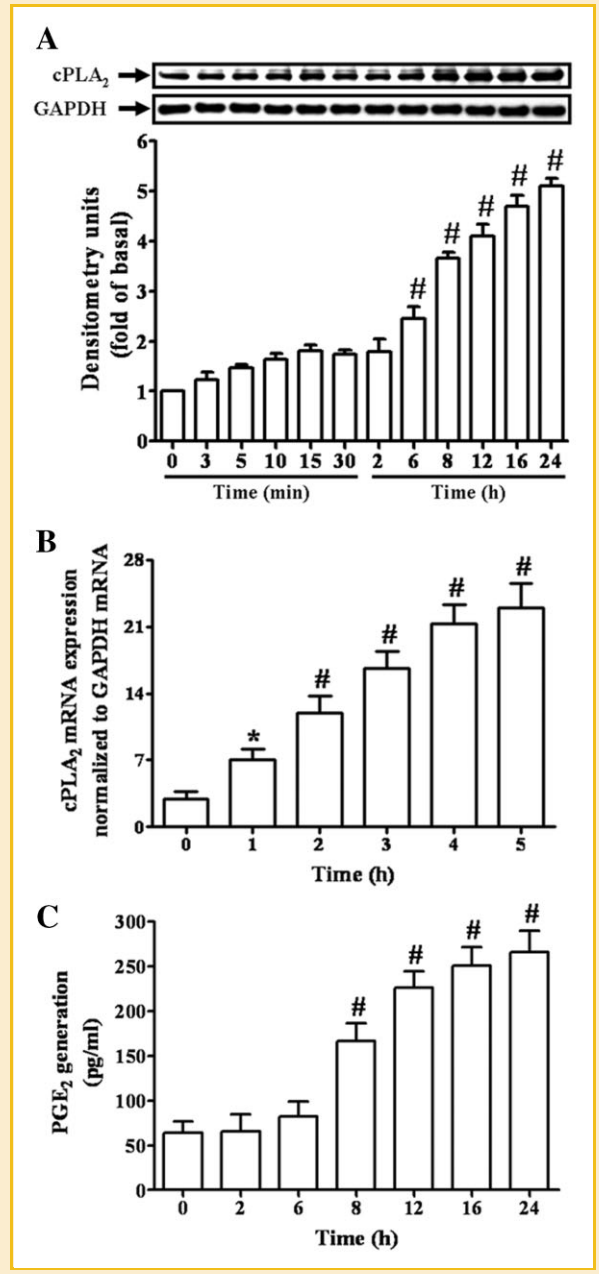


Fig. 1. IL-1β induces $cPLA_2$ protein levels and mRNA expression. A: HTSMCs were incubated with 30 ng/ml IL-1β for the indicated times. The expression of $cPLA_2$ was determined by Western blot analysis. B: Cells exposed to 30 ng/ml IL-1β for the indicated times were harvested and total RNA was extracted. The RNA samples were analyzed by real-time RT-PCR for the levels of $cPLA_2$ mRNA. C: Cells were incubated with 30 ng/ml IL-1β for the indicated times, and then the media were collected and analyzed for PGE2 release. Data are expressed as mean ± SEM of at least three independent experiments. $^*P < 0.05$; $^\#P < 0.01$ as compared with the cells exposed to vehicle.

IL-1β-INDUCED $cPLA_2$ EXPRESSION REQUIRES ONGOING TRANSCRIPTION AND TRANSLATION

To further examine if IL-1β-induced $cPLA_2$ expression required ongoing transcription or translation, HTSMCs were stimulated with IL-1β (30 ng/ml) in the absence or presence of a transcriptional level inhibitor, actinomycin D (Act. D) or a translational level inhibitor, cycloheximide (CHI) and $cPLA_2$ protein expression was determined by Western blot analysis. As shown in Figure 2A,B, IL-1β-mediated induction of $cPLA_2$ expression was abolished by either actinomycin D or CHI in a concentration-dependent manner.

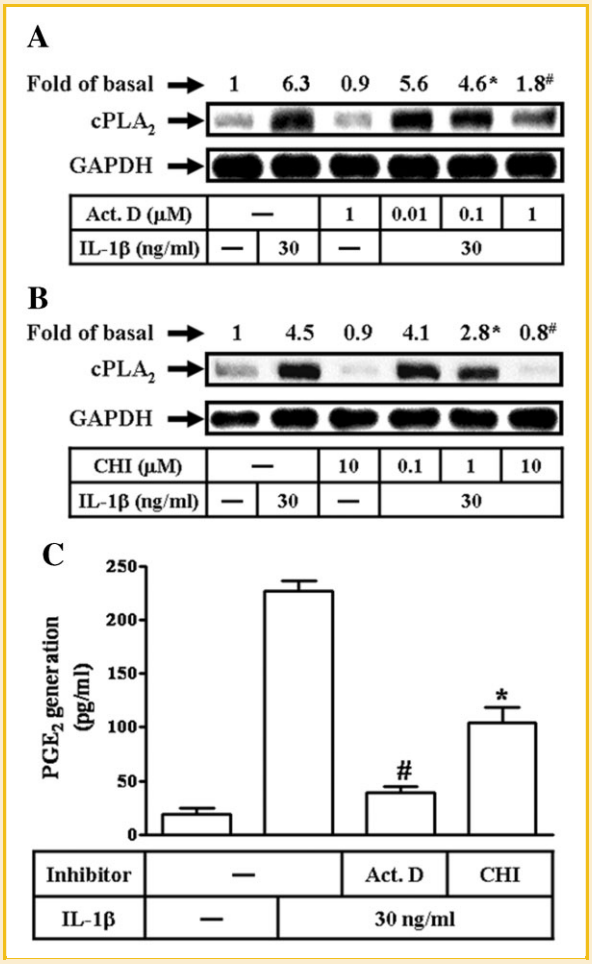


Fig. 2. The induction of $cPLA_2$ expression by IL-1β dependent on de novo protein synthesis in HTSMCs. Cells were pretreated with various concentrations of (A) actinomycin D (Act. D) or (B) cycloheximide (CHI) for 1 h, and then incubated with 30 ng/ml IL-1β for 24 h. The levels of $cPLA_2$ expression were determined by Western blot analysis. C: Cells were pretreated with Act. D (1 μM) or CHI (10 μM) for 1 h, and then incubated with 30 ng/ml IL-1β for 24 h. The generation of PGE_2 was determined. Data are expressed as mean ± SEM of at least three independent experiments. $^*P < 0.05$; $^\#P < 0.01$ as compared with the cells exposed to IL-1β alone.

In addition, IL-1β-induced PGE_2 synthesis was also inhibited by pretreatment with these two inhibitors (Fig. 2C). Taken together, these findings demonstrated that the induction of $cPLA_2$ by IL-1β depends on de novo protein synthesis in HTSMCs.

IL-1β INDUCES $cPLA_2$ EXPRESSION VIA p42/p44 MAPK

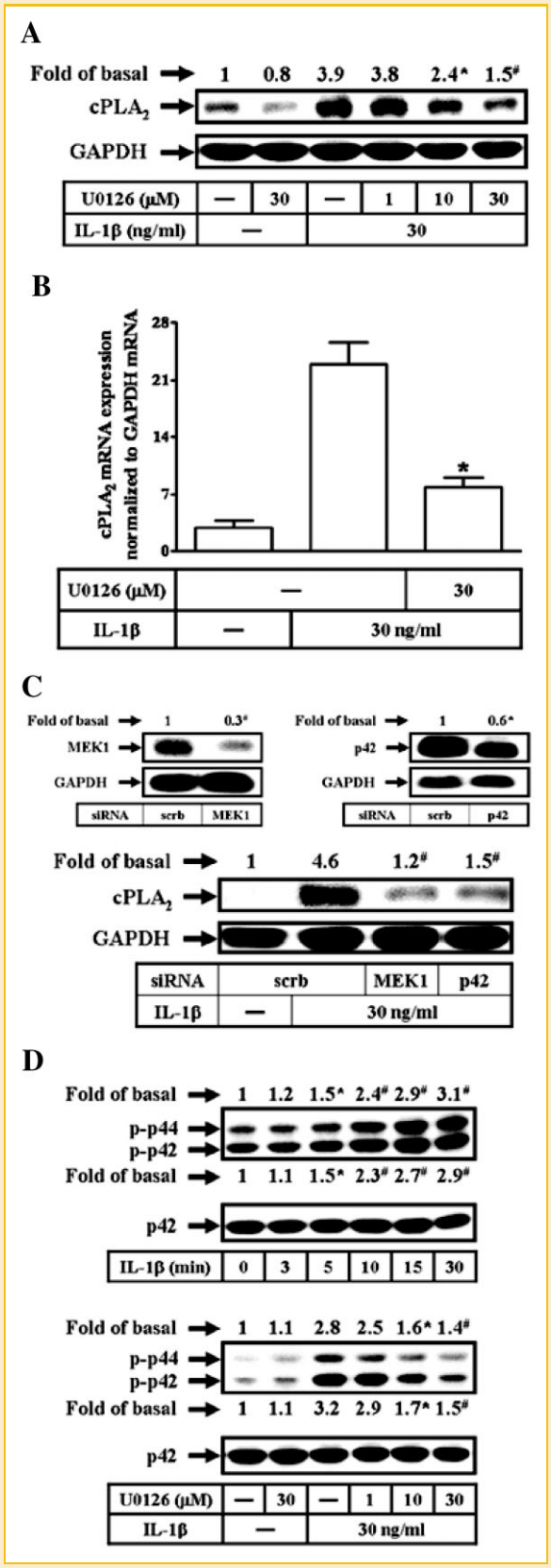
We have previously demonstrated that cytokines regulate expression of inflammatory genes dependent on activation of three MAPKs in HTSMCs [Lin et al., 2004; Wang et al., 2005]. Thus, we investigated whether IL-1β-induced $cPLA_2$ expression was also mediated via p42/p44 MAPK in these cells. As shown in Figure 3A,B, pretreatment with U0126 (an inhibitor of MEK1/2) attenuated IL-1β-induced $cPLA_2$ protein and mRNA expression. To further ensure that IL-1β-induced $cPLA_2$ expression was mediated via p42/p44 MAPK in HTSMCs, cells were transfected with MEK1 siRNA or p42 siRNA. As shown in Figure 3C, transfection with MEK1 siRNA or p42 siRNA significantly down-regulated MEK1 or p42 expression and subsequently led to a decrease of $cPLA_2$ protein expression in response to IL-1β. To determine whether p42/p44 MAPK was involved in IL-1β-induced $cPLA_2$ expression, activation of p42/p44 MAPK was assayed by Western blot analysis. As shown in Figure 3D, IL-1β stimulated a time-dependent phosphorylation of p42/p44 MAPK in HTSMCs. A maximal response was obtained within 30 min. Moreover, pretreatment with U0126 blocked IL-1β-stimulated p42/p44 MAPK phosphorylation (Fig. 3D). These data indicated that MEK1/2-p42/p44 MAPK cascade was involved in IL-1β-induced $cPLA_2$ expression in HTSMCs.

IL-1β ENHANCES $cPLA_2$ EXPRESSION VIA p38 MAPK

LPS has been shown to induce $cPLA_2$ expression via p38 MAPK in canine TSMCs [Luo et al., 2006]. To determine whether p38 MAPK was also involved in IL-1β-induced $cPLA_2$ expression in HTSMCs, a p38 MAPK inhibitor, SB202190 was used. As shown in Figure 4A,B, pretreatment with SB202190 inhibited $cPLA_2$ protein and mRNA expression induced by IL-1β. To further ensure that IL-1β-induced $cPLA_2$ expression was mediated via p38 MAPK, HTSMCs were transfected with p38 siRNA. As shown in Figure 4C, transfection with p38 siRNA significantly down-regulated p38 expression and subsequently led to a decrease of $cPLA_2$ protein expression in response to IL-1β. To determine whether p38 MAPK was involved in IL-1β-induced $cPLA_2$ expression, activation of p38 MAPK was assayed by Western blot analysis. As shown in Figure 4D, IL-1β stimulated a time-dependent phosphorylation of p38 MAPK in HTSMCs. A maximal response was obtained within 10 min, and then slightly declined within 30 min. Moreover, pretreatment with SB202190 blocked IL-1β-stimulated p38 MAPK phosphorylation (Fig. 4D). These results suggested that p38 MAPK activation was required for IL-1β-induced $cPLA_2$ expression in HTSMCs.

IL-1β INDUCES $cPLA_2$ EXPRESSION VIA JNK1/2

Expression of $cPLA_2$ in lung epithelial cells and nonsmall cell lung cancer is mediated by Sp1 and c-Jun through JNK1/2 activation [Blaine et al., 2001]. To characterize the role of JNK1/2 in IL-1β-induced $cPLA_2$ expression in HTSMCs, a selective inhibitor of JNK, SP600125 was used. As shown in Figure 5A,B, pretreatment of HTSMCs with SP600125 blocked $cPLA_2$ protein and mRNA expression induced by IL-1β. To further ensure that IL-1β-induced $cPLA_2$ expression was mediated via JNK1/2 in HTSMCs, cells were transfected with JNK2 siRNA. As shown in Figure 5C, transfection with JNK2 siRNA significantly down-regulated JNK2 expression and subsequently led to a decrease of $cPLA_2$ protein expression in response to IL-1β. To determine whether JNK1/2 was involved in IL-1β-induced $cPLA_2$ expression, activation of JNK1/2 was assayed by Western blot analysis. As shown in Figure 5D, IL-1β stimulated a time-dependent phosphorylation of JNK1/2 in HTSMCs. A maximal response was obtained within 15 min, and then slightly declined within 30 min. Moreover, pretreatment with SP600125 blocked IL-1β-stimulated JNK1/2 phosphorylation (Fig. 5D). These results suggested that JNK1/2 activation was required for IL-1β-induced $cPLA_2$ expression in HTSMCs.



INVOLVEMENT OF NF-κB IN IL-1β-INDUCED $cPLA_2$ EXPRESSION

Activation of NF-κB cascade is essential for $cPLA_2$ expression in various cell types [Hsieh et al., 2006; Luo et al., 2006]. Thus, we investigated whether NF-κB activation was involved in IL-1β-induced $cPLA_2$ expression in HTSMCs. We found that pretreatment of HTSMCs with a selective NF-κB inhibitor, helenalin attenuated IL-1β-induced $cPLA_2$ protein and mRNA expression (Fig. 6A,B). The pathways for how the cytokines, IL-1 and TNF-α, activated NF-κB through the IKK have been extensively investigated. To determine whether NIK, IKKα, or IKKβ was also involved in IL-1β-induced $cPLA_2$ expression in HTSMCs, siRNAs of NIK, IKKα, and IKKβ were used. As shown in Figure 6C, transfection of HTSMCs with siRNAs of NIK, IKKα, and IKKβ significantly attenuated IL-1β-induced $cPLA_2$ expression. In addition, we found that IL-1β-induced NF-κB (p65 and p50) translocation, which were inhibited by transfection with siRNAs of NIK, IKKα, and IKKβ (Fig. 6D). However, pretreatment of HTSMCs with U0126, SB202190, or SP600125 had no effect on NF-κB activation (Fig. 6E). These results indicated that activation of NF-κB by IL-1β was mediated through NIK/IKK pathway and subsequently led to $cPLA_2$ expression in HTSMCs.

IL-1β INDUCES $cPLA_2$ PROMOTER ACTIVITY

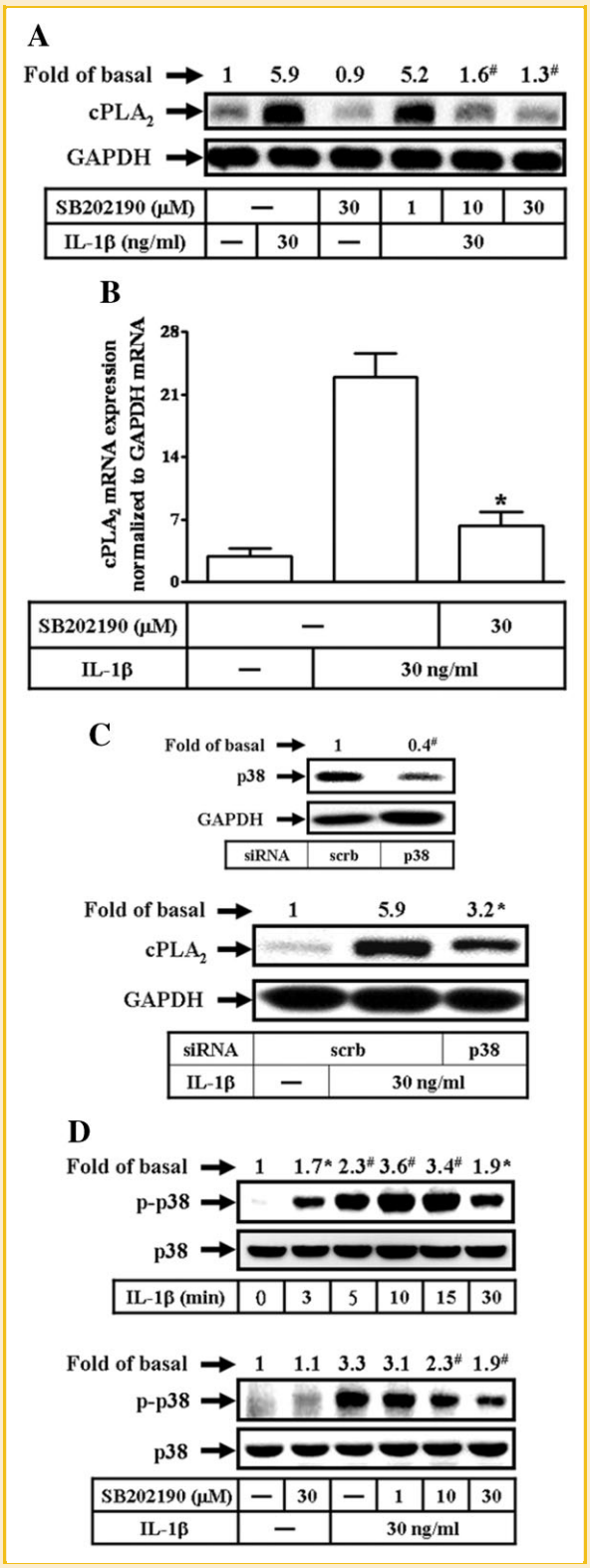
The regulation of $cPLA_2$ gene transcription through MAPKs and NF-κB pathways induced by IL-1β was further confirmed by a gene luciferase promoter activity assay. HTSMCs were transfected with $cPLA_2$ luciferase reporter gene, and then stimulated with IL-1β. As shown in Figure 7A, IL-1β markedly induced $cPLA_2$ promoter activity in HTSMCs. Moreover, we found that pretreatment with U0126, SB202190, SP600125, or helenalin reduced IL-1β-enhanced $cPLA_2$ promoter activity (Fig. 7B). These results suggested that IL-1β-stimulated $cPLA_2$ promoter activity at a transcriptional level was mediated through activation of MAPKs and NF-κB pathways.

INVOLVEMENT OF MAPKS AND NF-κB IN IL-1β-INDUCED PGE_2 PRODUCTION

We further determined whether MAPKs and NF-κB were involved in IL-1β-induced PGE_2 release in HTSMCs. As shown in Figure 8A, pretreatment of HTSMCs with the inhibitor of MEK1/2 (U0126), p38 MAPK (SB202190), JNK1/2 (SP600125), or NF-κB (helenalin) significantly attenuated IL-1β-induced PGE_2 synthesis.

Fig. 3. Involvement of MEK1/2 and p42/p44 MAPK in IL-1β-induced $cPLA_2$ expression. Cells were preincubated with U0126 for 1 h, and then incubated with IL-1β (30 ng/ml) for (A) 24 h or (B) 5 h. A: The levels of $cPLA_2$ expression were determined by Western blot analysis. B: The RNA samples were analyzed by real-time RT-PCR for the levels of $cPLA_2$ mRNA. C: HTSMCs were transfected with siRNA of MEK1 or p42, and then incubated with IL-1β (30 ng/ml) for 24 h. The levels of $cPLA_2$, MEK1, and p42 expression were determined by Western blot analysis. D: HTSMCs were treated with IL-1β for the indicated times or pretreated with U0126 for 1 h, and then incubated with 30 ng/ml IL-1β for 30 min. The cell lysates were subjected to Western blot analysis using an anti-phospho p42/p44 MAPK antibody. Data are expressed as mean ± SEM of at least three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the cells exposed to IL-1β alone (A,B,D, bottom). $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the cells transfected with scrambled siRNA (C, top) or exposed to IL-1β + scrambled siRNA (C, bottom). $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the basal level (D, top).

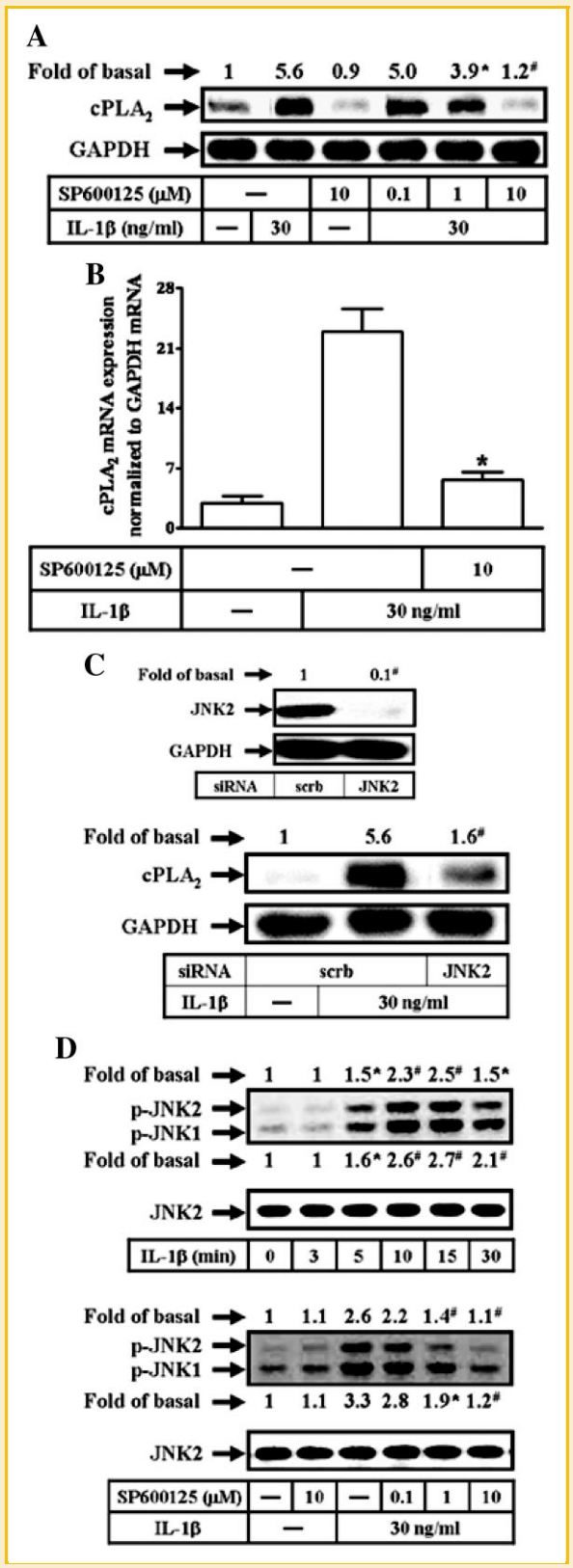
In addition, we also found that transfection with the siRNA of MEK1, p42, p38, or JNK2 markedly inhibited PGE_2 production induced by IL-1β in HTSMCs (Fig. 8B). These results further indicated that in HTSMCs, functional coupling of $cPLA_2$ expression to PGE_2 synthesis through activation of p42/p44 MAPK, p38 MAPK, JNK1/2, and NF-κB occurred at the transcriptional and translational levels. On the other hand, pretreatment with $AACOCF_3$ (an inhibitor of $cPLA_2$ activity) also significantly inhibited IL-1β-stimulated PGE_2 synthesis (data not shown), indicating that IL-1β-induced PGE_2 production in a $cPLA_2$-dependent manner. Taken together, these results suggested that IL-1β-induced $cPLA_2$ expression leading to PGE_2 synthesis was mediated through MAPKs and NF-κB in HTSMCs.



INVOLVEMENT OF p300 IN IL-1β-INDUCED $cPLA_2$ EXPRESSION

The transcriptional co-activator p300 displays an intrinsic HAT activity which participates in transcriptional activation through the destabilization of nucleosome structure. p300 is involved in the activity of several transcription factors that are nuclear endpoints of intracellular signal transduction pathways. To elucidate the mechanism underlying the expression of $cPLA_2$ by p300, a selective p300 inhibitor, garcinol was used. As shown in Figure 9A,B, pretreatment of HTSMCs with garcinol blocked IL-1β-induced $cPLA_2$ protein and mRNA expression in a concentration-dependent manner. To further ensure that IL-1β-induced $cPLA_2$ expression was mediated via p300 in HTSMCs, cells were transfected with p300 siRNA. As shown in Figure 9C, transfection with p300 siRNA significantly down-regulated p300 expression and subsequently led to a decrease of $cPLA_2$ protein expression in response to IL-1β. In addition, transfection with p300 siRNA also reduced IL-1β-enhanced PGE_2 production (Fig. 9D). p300 itself is a phosphoprotein, which is phosphorylated by MAPKs, has been demonstrated in various cell types [Poizat et al., 2005; Lee et al., 2007]. The in vivo recruitment of p300 and histone H4 to the $cPLA_2$ promoter was assessed by a ChIP assay. In vivo binding of p300 and histone H4 to the $cPLA_2$ promoter occurred as early as 30 min and was sustained for 120 min following IL-1β stimulation (Fig. 9E). The binding of p300 and histone H4 to the $cPLA_2$ promoter following IL-1β stimulation was attenuated by pretreatment with U0126, SB202190, or SP600125 (Fig. 9F). These data suggested that p300 and histone H4 involved in IL-1β-induced $cPLA_2$ transcription was regulated by p42/p44 MAPK, p38 MAPK, and JNK1/2 in HTSMCs.

Fig. 4. Involvement of p38 MAPK in IL-1β-induced $cPLA_2$ expression in HTSMCs. Cells were preincubated with SB202190 for 1 h, and then incubated with IL-1β (30 ng/ml) for (A) 24 h or (B) 5 h. A: The levels of $cPLA_2$ expression were determined by Western blot analysis. B: The RNA samples were analyzed by real-time RT-PCR for the levels of $cPLA_2$ mRNA. C: HTSMCs were transfected with siRNA of p38, and then incubated with IL-1β (30 ng/ml) for 24 h. The levels of $cPLA_2$ and p38 expression were determined by Western blot analysis. D: HTSMCs were treated with IL-1β for the indicated times or pretreated with SB202190 for 1 h, and then incubated with 30 ng/ml IL-1β for 10 min. The cell lysates were subjected to Western blot analysis using an anti-phospho p38 MAPK antibody. Data are expressed as mean ± SEM of at least three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the cells exposed to IL-1β alone (A,B,D, bottom). $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the cells transfected with scrambled siRNA (C, top) or exposed to IL-1β + scrambled siRNA (C, bottom). $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the basal level (D, top).



IL-1β STIMULATES TRANSIENT PHOSPHORYLATION OF $cPLA_2$

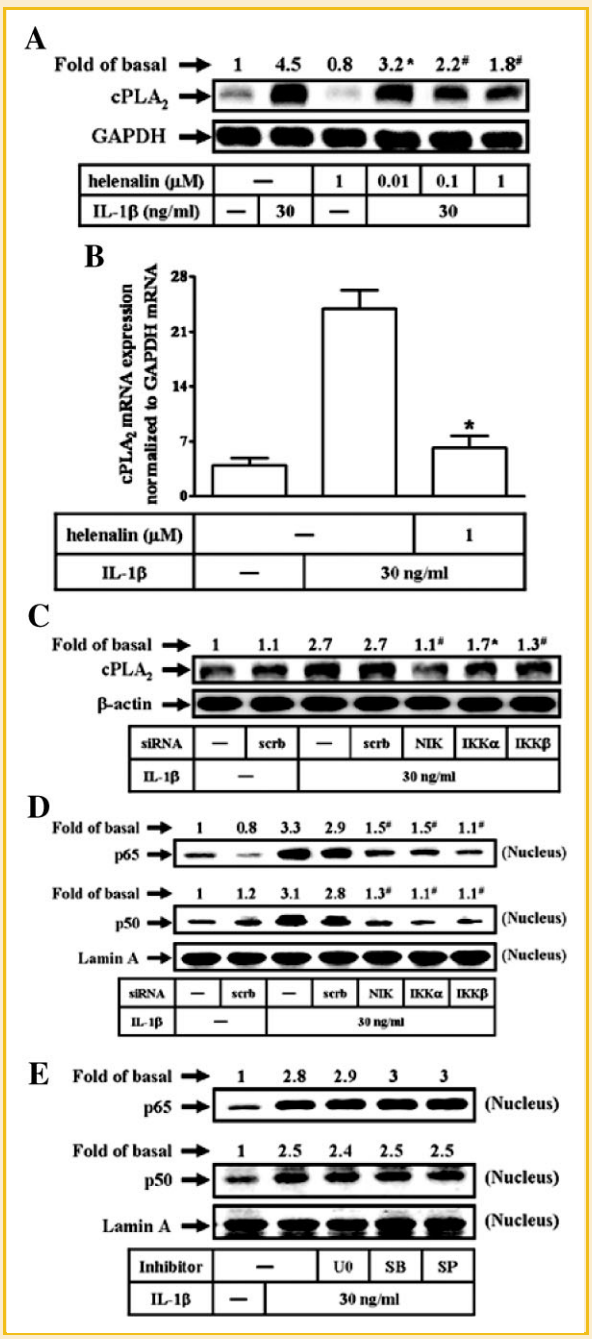
To determine whether the increase in $cPLA_2$ activity caused by IL-1β was associated with an increase in $cPLA_2$ phosphorylation, as shown in Figure 10, IL-1β-stimulated a time-dependent phosphorylation of $cPLA_2$ was attenuated by pretreatment with the inhibitors of MEK1/2 (U0126), p38 MAPK (SB202190), and JNK1/2 (SP600125). These results indicated that IL-1β-induced $cPLA_2$ phosphorylation via MAPKs in HTSMCs.

DISCUSSION

Up-regulation of $cPLA_2$ expression by mesenchymal cells in several extra-pulmonary sites may play a key role in generation of PGE_2, known as a biologically active lipid mediator implicated in inflammatory responses [Khanapure et al., 2007]. IL-1β has been confirmed to induce the late-phase airway hyperresponsiveness and inflammation mediated through activation of $cPLA_2$ [Yang et al., 2005], but little is known about the intracellular signaling pathways leading to its expression. IL-1β has also been shown to activate all of three MAPK pathways in several cell types [Bian et al., 2001; Wang et al., 2005]. However, in HTSMCs, whether IL-1β-induced $cPLA_2$ expression was mediated through the activation of these MAPKs and NF-κB was still unknown. In this study, IL-1β-induced $cPLA_2$ expression and PGE_2 production, which were attenuated by pretreatment with the inhibitors of MEK1/2 (U0126), p38 MAPK (SB202190), and JNK1/2 (SP600125) or transfection with siRNAs of MEK1, p42, p38, and JNK2. Moreover, abundant data support a key role for NF-κB signaling pathway in controlling the expression of inflammatory genes. Thus, the involvement of NF-κB in IL-1β-induced $cPLA_2$ expression was confirmed by using a selective NF-κB inhibitor, helenalin, indicating that pretreatment with helenalin significantly attenuated IL-1β-induced responses. Additionally, our results demonstrated that IL-1β-stimulated NF-κB translocation was inhibited by transfection with siRNAs of NIK, IKKα, and IKKβ. In this study, our results confirmed the notion that the mechanisms underlying activation of MAPKs, translocation of NF-κB, and association of p300 and histone H4 led to $cPLA_2$ gene transcription by IL-1β may be essential for up-regulation of $cPLA_2$ expression in HTSMCs. Several extracellular stimuli elicit a broad spectrum of biological responses mediated through activation of MAPK

Fig. 5. Involvement of JNK1/2 in IL-1β-enhanced $cPLA_2$ expression in HTSMCs. Cells were preincubated with SP600125 for 1 h, and then incubated with IL-1β (30 ng/ml) for (A) 24 h or (B) 5 h. A: The levels of $cPLA_2$ expression were determined by Western blot analysis. B: The RNA samples were analyzed by real-time RT-PCR for the levels of $cPLA_2$ mRNA. C: HTSMCs were transfected with siRNA of JNK2, and then incubated with IL-1β (30 ng/ml) for 24 h. The levels of $cPLA_2$ and JNK2 expression were determined by Western blot analysis. D: HTSMCs were treated with IL-1β for the indicated times or pretreated with SP600125 for 1 h, and then incubated with 30 ng/ml IL-1β for 15 min. The cell lysates were subjected to Western blot analysis using an anti-phospho JNK1/2 antibody. Data are expressed as mean ± SEM of at least three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the cells exposed to IL-1β alone (A,B,D, bottom). $^{\#}P < 0.01$ as compared with the cells transfected with scrambled siRNA (C, top) or exposed to IL-1β + scrambled siRNA (C, bottom). $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the basal level (D, top).

cascades, including p42/p44 MAPK, p38 MAPK, and JNK1/2. Since IL-1β plays an important role in different cellular responses, the phosphorylation of these MAPKs may not directly imply an involvement of these MAPKs in IL-1β-induced $cPLA_2$ expression. For example, activation of JNK1/2 and p42/p44 MAPK is required for up-regulation of $cPLA_2$ in response to oncogenic Ras in normal epithelial cells [Van Putten et al., 2001]. In canine TSMCs, up-regulation of $cPLA_2$ by LPS is mediated through these three MAPKs pathways [Luo et al., 2006]. In the present study, our results demonstrated that activation of p42/p44 MAPK was necessary for IL-1β-induced $cPLA_2$ expression in HTSMCs, since pretreatment



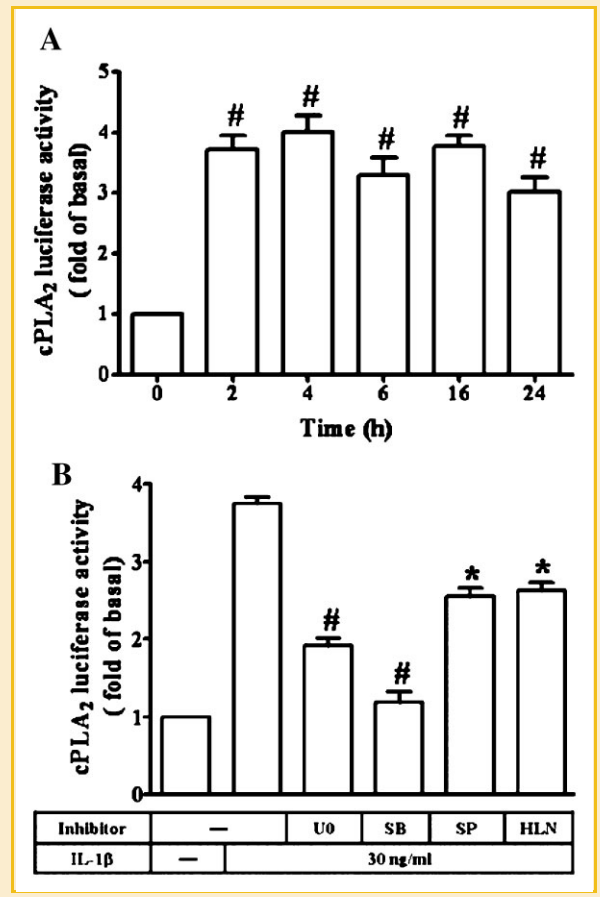


Fig. 7. Involvement of MAPKs and NF-κB in IL-1β-induced $cPLA_2$ promoter activity. Cells were transiently transfected with $cPLA_2$-luc reporter gene, and (A) challenged with IL-1β for the indicated times or (B) pretreated with U0126 (30 μM), SB202190 (30 μM), SP600125 (10 μM), or helenalin (HLN, 1 μM) for 1 h, and then incubated with IL-1β for 4 h. The $cPLA_2$ promoter activity was determined in the cell lysates. Data are expressed as mean ± SEM of at least three independent experiments. $^{\#}P < 0.01$ as compared with the basal level (A). $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the cells exposed to IL-1β alone (B).

Fig. 6. NF-κB is essential for IL-1β-induced $cPLA_2$ expression in HTSMCs. Cells were pretreated with helenalin for 1 h, and then incubated with IL-1β (30 ng/ml) for (A) 24 h or (B) 5 h. A: The levels of $cPLA_2$ expression were determined by Western blot analysis. B: The RNA samples were analyzed by real-time RT-PCR for the levels of $cPLA_2$ mRNA. C: HTSMCs were transfected with siRNAs of NIK, IKKα, and IKKβ, and then incubated with IL-1β (30 ng/ml) for 24 h. The expression of $cPLA_2$ was determined by Western blot analysis. D: HTSMCs were transfected with siRNAs of NIK, IKKα, and IKKβ, and then incubated with IL-1β (30 ng/ml) for 30 min. The nuclear fractions were prepared and subjected to Western blot analysis using an anti-p65 or anti-p50 antibody. E: HTSMCs were pretreated with U0126 (30 μM), SB202190 (30 μM), or SP600125 (10 μM) for 1 h, and then incubated with IL-1β for 30 min. The nuclear fractions were prepared and subjected to Western blot analysis using an anti-p65 or anti-p50 antibody. Data are expressed as mean ± SEM of at least three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the cells exposed to IL-1β alone (A,B). $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the cells exposed to IL-1β + scrambled scrambled siRNA (C,D).

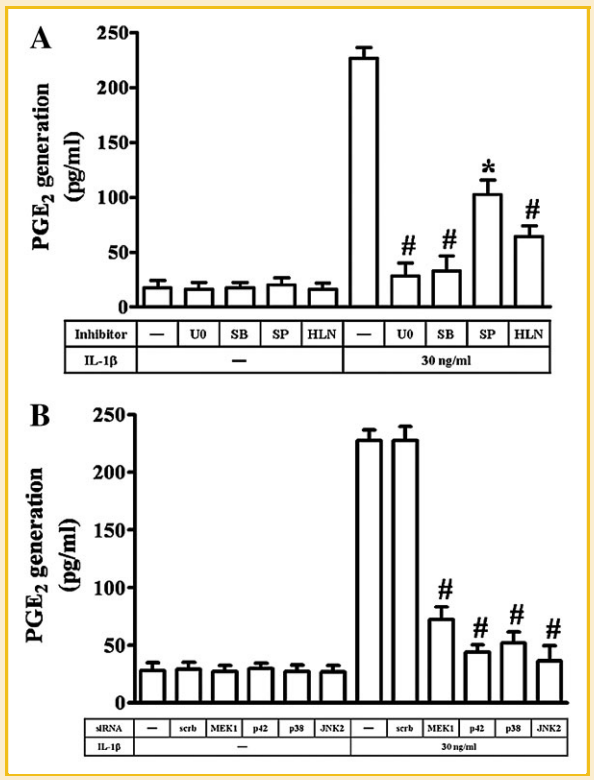


Fig. 8. Involvement of MAPKs and NF-κB in IL-1β-induced PGE_2 generation in HTSMCs. A: HTSMCs were pretreated with U0126 (30 μM), SB202190 (30 μM), SP600125 (10 μM), or HLN (1 μM) for 1 h, and then incubated with IL-1β for 24 h. The media were collected and analyzed for PGE_2 release. B: Cells were transfected with scrambled siRNA, MEK1 siRNA, p42 siRNA, p38 siRNA, or JNK2 siRNA, and then treated with IL-1β for 24 h. The media were collected and analyzed for PGE2 release. Data are expressed as mean ± SEM of at least three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the cells exposed to IL-1β alone (A). $^{\#}P < 0.01$ as compared with those of the scrambled siRNA-transfected cells exposed to IL-1β (B).

with U0126 attenuated IL-1β-induced $cPLA_2$ expression, PGE_2 synthesis, and p42/p44 MAPK phosphorylation. This notion was further supported by the results showing that transfection with siRNAs of MEK1 and p42 down-regulated IL-1β-induced $cPLA_2$ expression in HTSMCs. These results were consistent with the reports indicating that activation of p42/p44 MAPK plays a pivotal role in the expression of $cPLA_2$ in various cell types [Hsieh et al., 2006; Luo et al., 2006].

Further, we also found that IL-1β-induced p38 MAPK phosphorylation and $cPLA_2$ expression were attenuated by pretreatment with SB202190 and transfection with p38 MAPK siRNA, suggesting that IL-1β-induced responses were mediated through activation of p38 MAPK pathway, consistent with a study indicating that p38 MAPK is crucial for $cPLA_2$ expression in canine TSMCs [Luo et al., 2006]. We also investigated the involvement of JNK1/2 in IL-1β-induced $cPLA_2$ expression. It was found that IL-1β-induced $cPLA_2$ expression was mediated through activation of JNK1/2, since pretreatment with SP600125 or transfection with JNK2 siRNA attenuated IL-1β-mediated responses in HTSMCs. Our results were consistent with reports indicating that activation of JNK1/2 is linked to induction of $cPLA_2$ in several cell types [Van Putten et al., 2001; Luo et al., 2006]. In addition, the inhibitors of p42/p44 MAPK, p38 MAPK, and JNK1/2 displayed only a partial attenuation in the IL-1β-induced expression of $cPLA_2$, although U0126, SB201290, and SP600125 almost completely inhibited the phosphorylation of p42/p44 MAPK, p38 MAPK, and JNK1/2. The degree to which IL-1β-induced $cPLA_2$ expression was inhibited seemed to be dependent on the extents of different MAPKs phosphorylation. Since IL-1β is known to activate several signaling transduction pathways [Ghosh et al., 1998; Subbaramaiah et al., 2000], it is possible that treatment with one of these kinase inhibitors reduced only one of several components necessary for, or involved in, IL-1β-induced $cPLA_2$ expression. Apparently, combinatorial addition of U0126, SB202190, and SP600125 caused a more effective decrease of IL-1β-induced $cPLA_2$ expression by ~85–90% inhibition (data not shown). A balance or cross-talk may also exist among these pathways leading to induction of $cPLA_2$ expression by IL-1β. This explanation may also, in part, account for why MAPKs inhibitors do not completely abolish IL-1β-induced $cPLA_2$ expression. Thus, IL-1β-induced $cPLA_2$ expression was mediated through p42/p44 MAPK, p38 MAPK, and JNK1/2 pathways in HTSMCs.

MAPKs have been shown to phosphorylate transcription factors or intracellular enzymes, including NF-κB, c-Jun, ATF2, and Elk-1 [Van Putten et al., 2001]. It has also demonstrated that MEKK1 stimulates IKKα, and IKKβ leading to NF-κB activation [Barnes and Karin, 1997]. However, our previous studies have demonstrated that IL-1β-stimulated NF-κB activation is not inhibited by pretreatment with U0126, SB202190, and SP600125, indicating that MAPKs and NF-κB independently regulate gene expression [Luo et al., 2008]. This notion was further confirmed by the combinatorial addition of U0126, SB202190, SP600125, and helenalin caused a more effective decrease of IL-1β-induced $cPLA_2$ expression by >90% inhibition (data not shown). In the present study, we found that pretreatment with helenalin or transfection with siRNA of NIK, IKKα, and IKKβ attenuated IL-1β-induced NF-κB translocation and $cPLA_2$ expression, as well as PGE_2 production. These results suggested that IL-1β-stimulated NF-κB activation was mediated through NIK/IKK pathway leading to $cPLA_2$ expression, consistent with the reports indicating that activation of NF-κB promotes $cPLA_2$ gene expression in several cell types [Hsieh et al., 2006; Luo et al., 2006]. Moreover, our results showed that pretreatment with helenalin, U0126, SB202190, or SP600125 significantly attenuated $cPLA_2$ luciferase gene activity stimulated by IL-1β, suggesting that activation of MAPKs and NF-κB pathways independently regulated $cPLA_2$ expression at a transcription level.

In nonsmall cell lung cancer cells, $cPLA_2$ gene expression can be regulated by other transcription factors, including Sp1 and c-jun [Blaine et al., 2001]. Both Sp1 and c-jun have been reported to interact with co-activator, p300, one of HAT members [Hung et al., 2006; Wang et al., 2006]. HATs, such as p300 and CBP act as protein bridges, thereby connecting different transcriptional activators via protein-protein interactions to the basal transcriptional machinery, including transcription factor IIB (TFIIB), TATA-binding protein, and the RNA polymerase II complex [Goodman and Smolik, 2000]. They also function as a scaffolding protein which builds a multi-component transcriptional regulatory complex [Chan and La

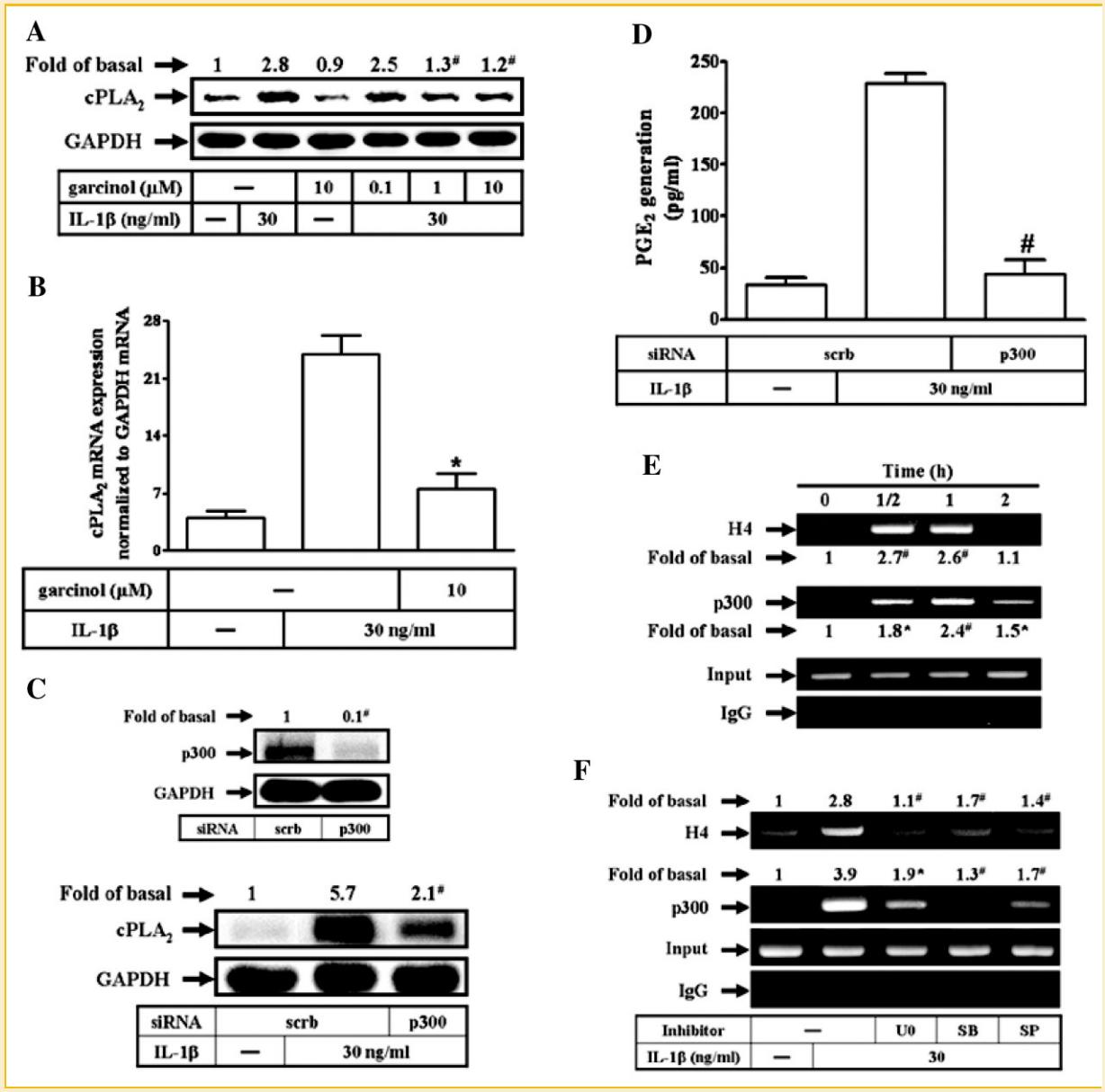


Fig. 9. Recruitment of p300 to $cPLA_2$ promoter in response to IL-1β in HTSMCs. Cells were preincubated with garcinol for 1 h, and then incubated with IL-1β (30 ng/ml) for (A) 24 h or (B) 5 h. A: The levels of $cPLA_2$ expression were determined by Western blot analysis. B: The RNA samples were analyzed by real-time RT-PCR for the levels of $cPLA_2$ mRNA. C: HTSMCs were transfected with siRNA of p300, and then incubated with IL-1β (30 ng/ml) for 24 h. The levels of $cPLA_2$ and p300 expression were determined by Western blot analysis. D: Cells were transfected with scrambled siRNA or p300 siRNA, and then treated with IL-1β for 24 h. The media were collected and analyzed for PGE_2 release. Cells were (E) treated with 30 ng/ml IL-1β for the indicated times or (F) pretreated with U0126 (10 μM), SB202190 (30 μM), or SP600125 (10 μM) for 1 h followed by stimulation with IL-1β for 1 h, and ChIP assay was then performed. Chromatin was immunoprecipitated with anti-histone H4 or anti-p300 antibody. One percent of the precipitated chromatin was assayed to verify equal loading (Input). Data are expressed as mean ± SEM of at least three independent experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the cells exposed to IL-1β alone (A,B,F). $^{\#}P < 0.01$ as compared with the cells transfected with scrambled siRNA (C, top) or exposed to IL-1β + scrambled siRNA (C, bottom and D). $^{*}P < 0.05$; $^{\#}P < 0.01$ as compared with the basal level (E).

Thangue, 2001]. Raised activity of intrinsic HAT may cause remodeling of chromatin structure by acetylation of the NH_2 terminus of core nucleosomal histones [Roth et al., 2001]. Chromatin remodeling after p300/CBP associated with histone acetylation is believed to participate in active transcription of pro-inflammatory genes upon stimulation by various mediators. On the basis of our results, IL-1β-induced $cPLA_2$ expression was significantly inhibited by pretreatment with a selective p300 inhibitor, garcinol or transfection with p300 siRNA. Moreover, an enrichment of p300 and histone H4-associated $cPLA_2$ promoter (−595 to +75) DNA complexes appeared in IL-1β-treated HTSMCs. Furthermore, activation of CBP/p300 mediated through MAPKs has been reported in various cell types [Poizat et al., 2005; Wang et al., 2006]. Here, we found that inhibition of p42/p44 MAPK, p38 MAPK, and JNK1/2 by their respective inhibitors attenuated the recruitment of 300 and histone H4 interacted with $cPLA_2$ promoter. These data suggested

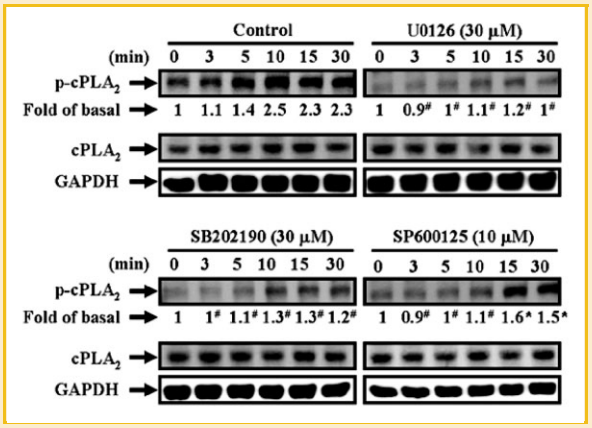


Fig. 10. IL-1β stimulates $cPLA_2$ phosphorylation via MAPKs in HTSMCs. Cells were pretreated with U0126 (10 μM), SB202190 (30 μM), or SP600125 (10 μM) for 1 h, and then treated with 30 ng/ml IL-1β for the indicated times. The cell lysates were subjected to Western blot analysis using an anti-phospho $cPLA_2$, anti-$cPLA_2$, or anti-GAPDH antibody.

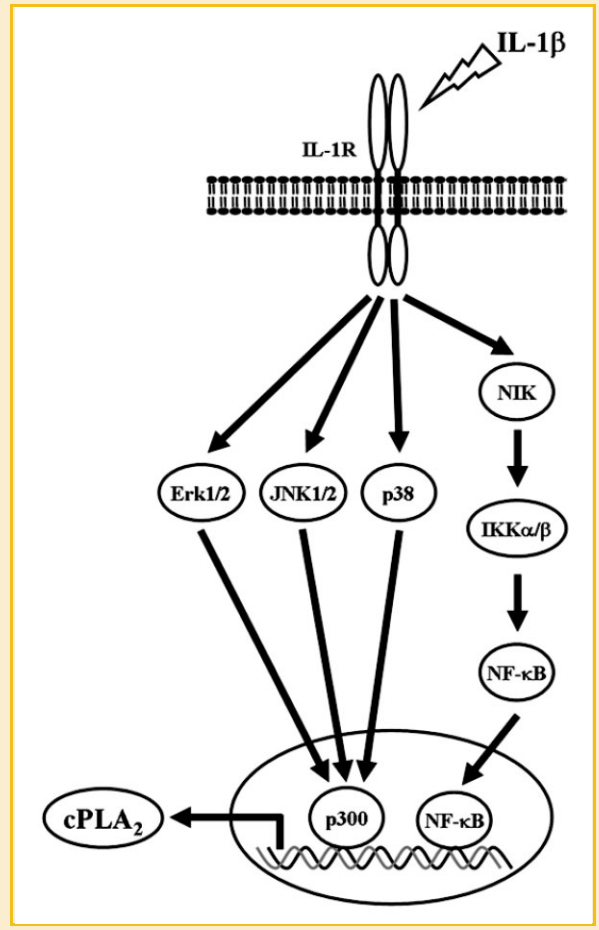


Fig. 11. Schematic representation of the signaling pathways involved in IL-1β-induced $cPLA_2$ expression in HTSMCs. IL-1β binds to its receptor and induces MAPKs, NF-κB, and p300 activation. Subsequently, $cPLA_2$ transcription is independently initiated by these signaling pathways. Moreover, these pathways might enforce each of the signaling pathways and contribute to sustained activation of transcription factors required for $cPLA_2$ expression.

that acetylation of histone H4 by p300 may depend on phosphorylation of p42/p44 MAPK, p38 MAPK, and JNK1/2, and then induce chromatin remodeling, and finally enhance NF-κB binding to the $cPLA_2$ promoter-binding site. In addition to $cPLA_2$ gene expression, phosphorylation of $cPLA_2$ is regulated by serine phosphorylation through the activation of kinases has been investigated in various cell types [Hirabayashi et al., 2004; Luo et al., 2006]. Depending on the cell types and stimuli, $cPLA_2$ is phosphorylated by MAPKs on Ser^{505}, by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) on Ser^{515}, and by MAPK-interacting kinase Mnk1 on Ser^{727}. Phosphorylation of these serine residues on $cPLA_2$ increases its intrinsic enzymatic activity and eventually leads to production of PGE_2 [Hirabayashi et al., 2004]. Our results showed that IL-1β-enhanced $cPLA_2$ phosphorylation on the Ser^{505} and PGE_2 production were reduced by pretreatment with these MAPKs inhibitors. These results indicated that activation of these MAPKs also stimulated $cPLA_2$ phosphorylation in HTSMCs.

On the basis of reported observations from literatures and our findings, a schematic pathway depicts a model for the roles of MAPKs, NF-κB, and p300 activation linked to $cPLA_2$ expression in HTSMCs exposed to IL-1β (Fig. 11). To our knowledge, this study is the first to demonstrate that in HTSMCs, the mechanisms underlying IL-1β-mediated activation of MAPKs, NF-κB, and p300 were required for the expression of $cPLA_2$. Finally, association of p300 and histone H4 led to $cPLA_2$ gene transcription. The mechanisms by which IL-1β-induced $cPLA_2$ expression may be an important link in the pathogenesis of airway inflammatory diseases. Therefore, understanding the mechanisms underlying IL-1β-induced $cPLA_2$ expression in HTSMCs is important to develop new therapeutic strategies.

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