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# Down-regulation of IL-1β-induced COX-2 Expression in A549 Lung Cancer Cells at Transcriptional Level by Leptomycin B Involves Inhibition of the IκB-α/NF-κB Pathway but Independent of CRM1

**Purpose:** Overexpression of COX-2, an enzyme responsible fro the synthesis of prostaglandins, is well linked to human chronic lung diseases. The mechanism by which COX-2 expression is increased or enhanced in cancer cells remains largely unknown. Any compound which can reduce COX-2 expression may be considered as an anti-cancer agent. **Materials and Methods:** Leptomycin B (LMB) is a metabolite of *Streptomyces* and a specific inhibitor of CRM1 nuclear export receptor. A549 is a human lung cancer cell line. To evaluate the effect of LMB on COX-2 expression induced by IL-1β, a pro-inflammatory cytokine, in A549 cells, Western blot and RT-PCR assays were applied to measure COX-2 protein and mRNA expressions in response to IL-1β, respectively. Luciferase experiments were done to measure promoter activity of COX-2, NF-κB or AP-1. CRM1 siRNA trasfection experiment was performed to knock-down endogenous CRM1. Biochemical protein fractionation method was also carried out to see intracellular localization of proteins. **Results:** LMB at 9 nM strongly suppressed IL-1β-induced expression of COX-2 protein that was attributable to decreased COX-2 transcript and promoter activity, but not mRNA stability. Distinctly, knock-down of CRM1 had no effect on COX-2 expression by IL-1β. Moreover, LMB did not affect IL-1β-induced phosphorylation of ERK-1/2, JNK-1/2, and p38 MAPK or AP-1 promoter activity. In contrast, LMB blocked IL-1β-mediated cytosolic IκB-α degradation, p65 NF-κB nuclear translocation, and NF-κB promoter activity. **Conclusion:** LMB potently down-regulates IL-1β-induced COX-2 at transcriptional level in A549 cells, in part, through modulation of the IκB-α/NF-κB pathway but independent of CRM1, MAPKs and AP-1. **(J Lung Cancer 2006;5(2):102－110)**

**Key Words:** Leptomycin B, COX-2, IL-1β, CRM1, IκB-α/p65 NF-κB, A549 cells

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## INTRODUCTION

Cyclooxygenase (COX)[1], also named as prostaglandin (PG) H synthase, is the rate-limiting enzyme in the biosynthesis of PGs and related eicosanoids from arachidonic acid(1). PGs involve in various physiological and pathophysiological events, including inflammatory and neoplastic diseases(2～4).

Two isoforms of COX have been identified and cloned in eukaryotic cells. COX-1 is the constitutively expressed isoform and involves in the maintenance of physiological functions. In contrast, COX-2 is inducible by extracellular stimuli such as tumor promoters, pro-inflammatory cytokines, mitogens, oncogenes, and growth factors in a variety of cells(1). Evidence that non-steroidal anti-inflammatory drugs (NSAIDs) to target COX-2 or COX-2 selective inhibitors reduce inflammatory symptoms such as pain and fever strongly suggests the role of COX-2 in inflammation. Expression of COX-2 is regulated at multiple steps, including transcription, post-transcription, and translation. COX-2 transcription is induced by various exogenous stimuli, which regulate intracellular signaling cascades that

in turn modulate the activity of various transcription factors (TFs) and hence stimulate transcriptional activation of the COX-2 promoter(5). Accordingly, CRE, NF-IL6, NF-κB, and AP-1 cis-acting elements were shown to be important for COX-2 transcriptional induction(6,7). COX-2 mRNA nuclear export and stabilization at post-transcriptional level are also critical for expression of COX-2(8～10). Moreover, recent studies have shown that COX-2 expression also requires activities of many intracellular signaling proteins, including ERKs, p38 MAPK, JNKs, and PKCs(11,12).

Leptomycin B (LMB) was originally discovered as a potent anti-fungal antibiotic from Streptomyces species(13). However, recent studies have demonstrated that LMB induces $G_1$ cell cycle arrest in mammalian cells(14) and apoptosis in cancer cells(15) and suppresses murine experimental tumors(16,17), suggesting its anti-cancer activity. Recently, the cellular target of LMB has been identified as chromosomal region maintenance 1 (CRM1), a nuclear export receptor(18). CRM1, also named as exportin 1, involves in nuclear trafficking of intracellular RNAs or proteins that contain nuclear export sequence (NES)(19). Supporting it, it has been shown that LMB blocks nuclear export of certain RNAs, including c-fos(20) or COX-2 (10), and the NES-containing α-catenin(21) or IκB-α(22). From these previous data, it is believed that LMB regulates expression of genes and/or movement of proteins primarily by controlling CRM1. However, we have recently shown that LMB inhibits LPS-induced iNOS expression by down-regulating iNOS transcript and promoter activity and the inhibition is the NF-κB-independent(23), suggesting that LMB may act on gene expression at transcriptional level. In a previous study (10), we have reported that LMB inhibits COX-2 expression at protein level in response to interleukin-1β (IL-1β), a pro-inflammatory cytokine, in human umbilical vein endothelial cells (HUVEC) or HT-29 human colonic epithelial cells. However, the regulatory mechanism by which LMB controls the cytokine-induced COX-2 expression is not fully known.

In this study, we investigated the effect of LMB on IL-1β-induced COX-2 expression in A549 lung cancer cells and the mechanisms associated. Here we demonstrate for the first time that LMB potently down-regulates IL-1β-induced COX-2 expression at transcriptional level in A549 cells and the down-regulation is in part mediated through blockage of cytosolic IκB-α degradation and the concomitant inactivation of NF-κB but not through CRM1, MAPKs, or AP-1.

## MATERIALS AND METHODS

### 1) Materials

Anti-actin antibody, aprotinin, leupeptin, phenanthroline, and benzamidine-HCl were from Sigma (St. Louis, MO). Anti-rabbit or mouse secondary horseradish peroxidase antibodies and ECL Western detection reagents were from Amersham Biosciences (Amersham, UK). Bradford reagent was from Bio-rad (Hercules, CA). An anti-COX-2 polyclonal antibody was from Cayman Chemicals (Ann Arbor, MI). Antibodies of ERK-1/2, phospho-ERK-1/2 (p-ERK-1/2), JNK-1/2, phospho-JNKs (p-JNK-1/2), p38 MAPK, and phospho-p38 MAPK were from Cell Signaling Tech. (Beverly, MA). Control or CRM1 small interference RNA (siRNA) and antibodies against IκB-α, p65 NF-κB, or HuR were from Santa Cruz Company (Santa Cruz, CA). RPMI 1640 and mixtures of penicillin/streptomycin were from GIBCO-BRL (Gaithersberg, MD).

### 2) Cell culture and preparation of whole cell lysates

A549 lung cancer cells were cultured in RPMI supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. To get whole cell lysates, A549 cells were washed with ice-cold phosphate-buffered saline (PBS) containing 1 mM $Na_3VO_4$ and 1 mM NaF, and lysed in a buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40 (NP40), 1 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine-HCl]. After centrifugation at 12,000 rpm for 20 min at 4°C, the supernatant was collected, and the protein concentration was determined by Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard.

### 3) Subcellular protein fractionation

To get cytosolic and nuclear proteins, A549 cells were initially exposed to a hypotonic buffer (10 mM Hepes, 10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40, 2 mM PMSF, 1 mM DTT, and 200 nM aprotinin) for 20 min and centrifuged 12,000 rpm for 10 min, saving the supernatant as the cytosolic fraction. The pellets were washed with the hypotonic buffer and exposed to a nuclear extract buffer [10 mM Tris-Cl (pH 7.5), 0.5 M NaCl,

2.5% glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 2 mM PMSF, and 200 nM aprotinin] for 20 min and centrifuged 12,000 rpm for 10 min, saving the supernatant as the nuclear fraction.

**4) Western blot analysis**

Forty micrograms of protein were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto a nitrocellulose membrane (Millipore, Bedford, MA). The membrane was washed with Tris-buffered saline (TBS, 10 mM Tris, 150 mM NaCl) containing 0.05% Tween 20 [TBST] and blocked in TBST containing 5% non-fat dried milk. The membrane was incubated with respective specific antibodies, COX-2, ERK-1/2, p-ERK-1/2, JNK-1/2, p-JNK-1/2, p38 MAPK, p-p38 MAPK, I$\kappa$B-$\alpha$, p65 NF-$\kappa$B, CRM1, HuR in 1 : 1,000 dilution, except actin in 1 : 5,000. The membrane was incubated with appropriate secondary antibodies coupled to horseradish peroxidase, and developed in the ECL western detection reagents.

**5) Luciferase assay**

Briefly, 1 $\mu$g of human COX-2, NF-$\kappa$B, or AP-1 promoter/luciferase DNA along with 20 ng of control pRL-TK DNA (Promega, Madison, WI) was transfected into $1.5\times10^6$ A549 cells/well in 6-well plates using LipoFectamine/Plus reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's manual instruction. After 24 h, cells were treated with IL-1$\beta$ in absence or presence of LMB for additional 4 h. Cells were then washed, lysed, followed measurement of luciferase activity using a luciferase assay kit (Promega, Madison, WI). The luciferase activity was normalized with expression of control pRL-TK.

**6) Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated with the RNAzol-B (Tel-Test). Three micrograms of total RNA were reverse- transcribed using murine-molony leukemia virus (M-MLV) reverse transcriptase (Promega). Single stranded cDNA was amplified by PCR with the following primers: COX-2 sense, 5'-TTGAAGACCAGG-AGTACAGC-3'; COX-2 anti-sense, 5'-GGTACAGTTCCAT-GACATCG-3'; GAPDH sense, 5'-GGTGAAGGTCGGTGTG-AACG-3'; GAPDH anti-sense, 5'-GGTAGGAACACGGAA-GGCCA-3'. The PCR conditions applied were: COX-2, 25 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s; GAPDH, 18 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, andextension at 72°C for 30 s. GAPDH was used as an internal control to evaluate the relative expression of COX-2.

**7) Determination of COX-2 mRNA stability**

A549 cells were firstly grown in absence or presence of IL-1$\beta$ for 4 h to highly induce COX-2 mRNA. Cells were then treated with IL-$\beta$ alone or IL-1$\beta$ plus LMB in the presence of actinomycin D, a transcription inhibitor, for 0, 3, 6, or 9 h. At each time, total RNA was prepared and subjected to COX-2 or GAPDH RT-PCR to determine the amounts of COX-2 or GAPDH mRNA remained in the cells.

**8) Transfection of CRM1 siRNA**

Briefly, 80 nM of control or CRM1 siRNA was transfected into $0.4\times10^6$ A549 cells/well in 6-well plates using Lipo-Fectamine/Plus reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's manual instruction. After 60 h, cells were treated without or with IL-1$\beta$ in absence or presence of LMB for 4 h. Cells were then washed, lysed, followed extraction of whole cell lysates that were used for immunoblot analyses to measure the expression level of CRM1 or COX-2 protein in control or CRM1 siRNA-transfected cells treated with IL-1$\beta$ in absence or presence of LMB.

## RESULTS

**1) LMB potently inhibits IL-1$\beta$-induced COX-2 protein and mRNA expressions and COX-2 promoter activity in A549 lung cancer cells**

Initially, the effects of different concentrations of LMB on COX-2 protein and mRNA expressions by IL-1$\beta$ in A549 cells were investigated. As shown in Fig. 1A, LMB suppressed IL-1$\beta$-induced COX-2 protein in A549 cells in a concentration-dependent manner. Results of RT-PCR analyses also showed that LMB dose-dependently down-regulated IL-1$\beta$-induced COX-2 mRNA in A549 cells (Fig. 1B). These results suggest that suppression of IL-1$\beta$-induced COX-2 protein by LMB is due to decreased COX-2 transcript. Notably, the concentration of LMB at 5 ng/ml (9 nM) was sufficient to

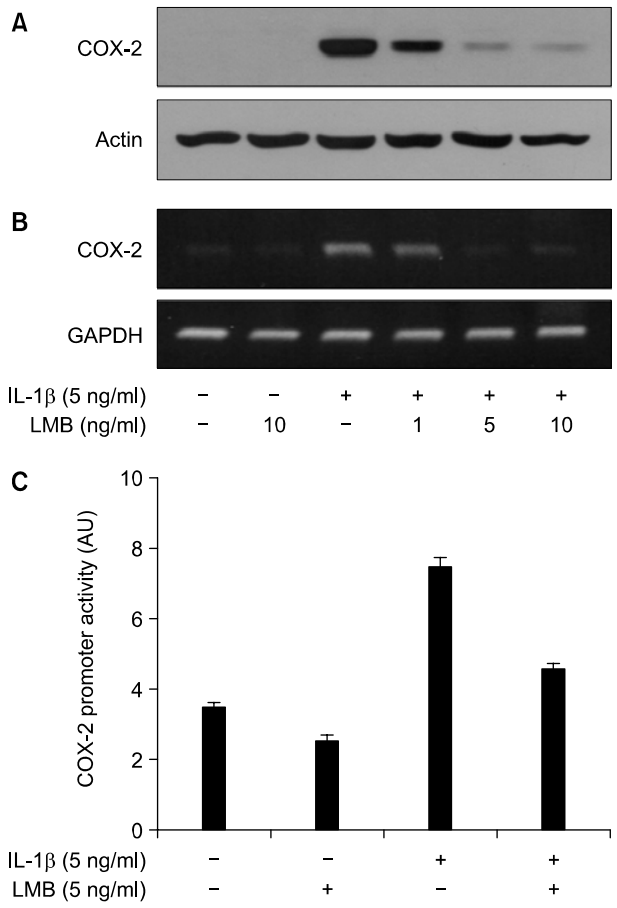


Fig. 1. Effect of LMB on IL-1$\beta$-induced COX-2 protein and mRNA and promoter activity in A549 lung cancer cells. (A, B) A549 cells were pre-treated with the indicated concentrations of LMB for 1 h. Cells were then exposed to IL-1$\beta$ for 4 h. Total cell lysates and RNA were prepared, and analyzed for COX-2 immunoblot (A) and RT-PCR (B), respectively. Actin or GAPDH was used to evaluate the relative expression of COX-2 protein or mRNA. (C) A549 cells were transfected with COX-2 promoter/luciferase DNA along with control pRL-TK DNA for 24 h and then exposed to IL-1$\beta$ for 4 h in absence or presence of LMB. Cell lysates were prepared and used for reporter gene activity. Data are mean±S.E. of three independent experiments.

largely inhibit IL-1$\beta$-induced COX-2 protein and mRNA expressions, suggesting the potency of LMB. Considering that regulation of COX-2 expression occurs primarily at transcriptional level(5～7), the effect of LMB on IL-1$\beta$-mediated COX-2 promoter-driven luciferase expression was next determined. As shown in Fig. 1C, in A549 cells, IL-1$\beta$ increased COX-2 promoter-driven luciferase expression (lane 3) that was largely inhibited by LMB (lane 4). Collectively, these results suggest that LMB potently inhibits IL-1$\beta$-induced COX-2 expression at transcriptional level in A549 cells.

**2) LMB inhibits degradation of cytosolic I$\kappa$B-$\alpha$ and the concomitant inactivation of NF-$\kappa$B in response to IL-1$\beta$**

It has been reported that IL-1$\beta$ signals to degrade I$\kappa$B-$\alpha$, a cytosolic inhibitory protein of NF-$\kappa$B(24) and the resultantly free NF-$\kappa$B enters into the nucleus where this transcription factor involves in COX-2 transcriptional activation(25). This led us to examine the effect of LMB on the I$\kappa$B-$\alpha$/NF-$\kappa$B activation pathway in response to IL-1$\beta$ signal in A549 cells. In this study, the degree of NF-$\kappa$B activation was assessed by the degree of I$\kappa$B-$\alpha$ degradation and of p65 NF-$\kappa$B nuclear localization as well as of NF-$\kappa$B promoter-driven luciferase expression in response to IL-1$\beta$. As shown in Fig. 2A, as anticipated, IL-1$\beta$ caused almost complete degradation of I$\kappa$B-$\alpha$ in A549 cells (lane 3) compared with control (lane 1). Remarkably, LMB strongly blocked IL-1$\beta$-mediated degradation of I$\kappa$B-$\alpha$ (lane 4). Total protein level of actin was constant after treatment with IL-1$\beta$ and/or LMB. Remarkably, subcellular protein fractionation experiments in Fig. 2B demonstrated that IL-1$\beta$ signaled to degrade I$\kappa$B-$\alpha$ in the cytosol (lane 3) and LMB suppressed it (lane 4). Moreover, IL-1$\beta$ increased nuclear localization of p65 NF-$\kappa$B (lane 3) that was also in part inhibited by LMB (lane 4). Total protein level of p65 NF-$\kappa$B was constant after treatment with IL-1$\beta$ or LMB and both (Fig. 2A). Importantly, as shown in Fig. 2C, IL-1$\beta$ increased NF-$\kappa$B promoter-driven luciferase expression (lane 3) that was also largely suppressed by LMB (lane 4). Total protein level of HuR, a nuclear protein, was constant by treatment with IL-1$\beta$ or LMB and both (Fig. 2A). Distribution of HuR was much higher in the nuclear fraction than in the cytosolic one (Fig. 2D), suggesting the effectiveness of our subcellular protein fractionation. Collectively, these results strongly suggest that inhibition of IL-1$\beta$-induced COX-2 expression in A549 cells by LMB involves blockage of cytosolic I$\kappa$B-$\alpha$ degradation and the concomitant NF-$\kappa$B inactivation in response to IL-1$\beta$.

**3) No effect of LMB on MAPKs phosphorylation and AP-1 promoter activity in response to IL-1$\beta$**

We next wanted to see the specificity of LMB down-regulation of the I$\kappa$B-$\alpha$/NF-$\kappa$B pathway in response to IL-1$\beta$ signal.

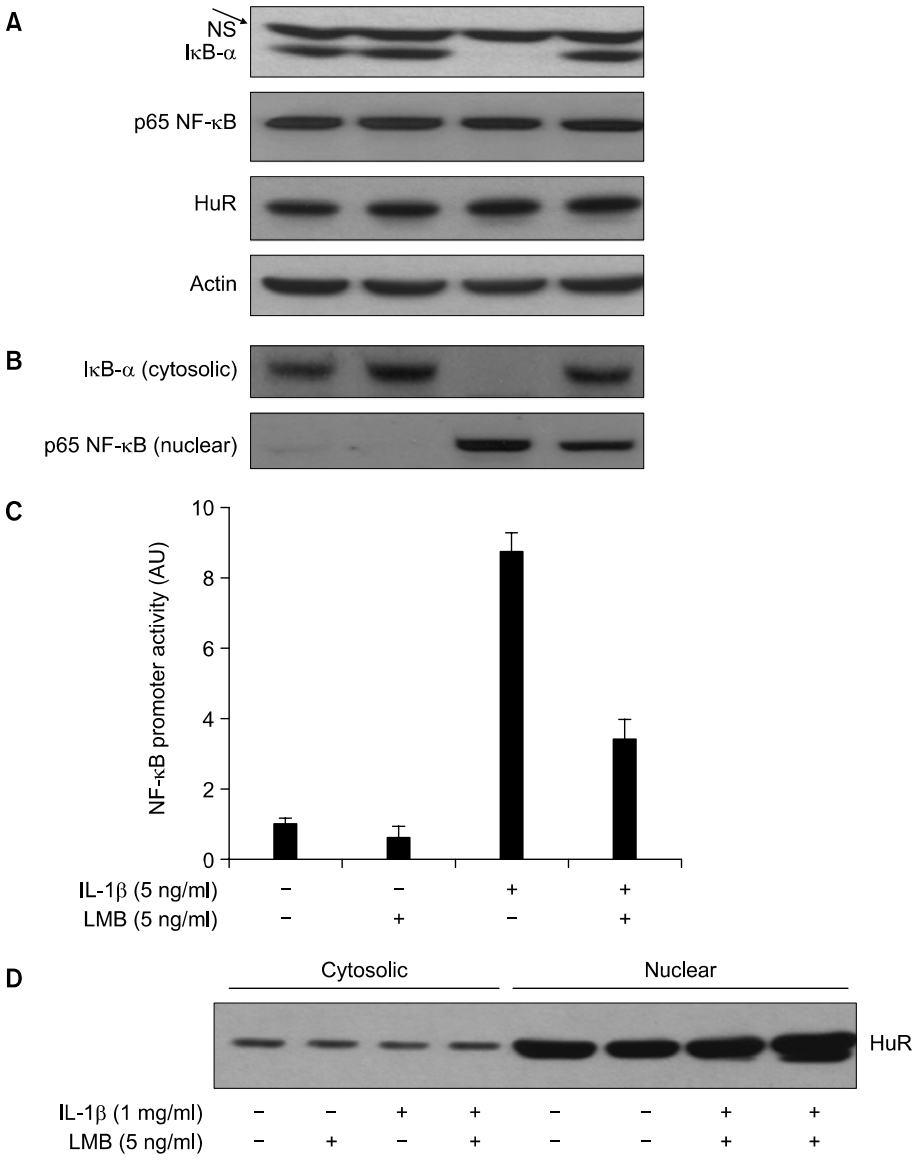


**Fig. 2.** Effect of LMB on IL-1$\beta$-induced proteolysis of I$\kappa$B-$\alpha$ and NF-$\kappa$B nuclear localization and its promoter activity in A549 cells. (A) A549 cells were pre-treated with LMB for 1 h. Cells were then exposed to IL-1$\beta$ for 0.5 h in absence or presence of LMB. Total cell lysates were prepared, and used to measure total protein of I$\kappa$B-$\alpha$, p65 NF-$\kappa$B, HuR, or actin by immunoblot with respective antibody. NS indicates nonspecific protein band. (B) Cytosolic and nuclear proteins were prepared and used to measure the level of cytosolic I$\kappa$B-$\alpha$ and nuclear p65 NF-$\kappa$B by immunoblot with respective antibody. (C) A549 cells were transfected with NF-$\kappa$B promoter/luciferase DNA along with control pRL-TK DNA for 24 h and then exposed to IL-1$\beta$ for 4 h in absence or presence of LMB. Cell lysates were prepared and used for reporter gene activity. Data are mean±S.E. of three independent experiments. (D) The same as in (C) except HuR immunoblot.

Studies have shown that activities of various signaling proteins, including ERKs, p38 MAPK and JNKs, contribute to IL-1$\beta$-induced COX-2 expression(11,12,24) and IL-1$\beta$-mediated activation of AP-1 transcription factor involves in COX-2 transcription(26). Therefore we investigated the effect of LMB on IL-1$\beta$ signaling to activate ERKs, p38 MAPK or JNKs, and AP-1 in A549 cells. As shown in Fig. 3A, IL-1$\beta$ increased phosphorylation of ERK-1/2, JNK-1/2, and p38 MAPK (lane 3) compared with control (lane 1). Total protein level of each protein was constant after IL-1$\beta$ treatment (lane 1 and 3). However, IL-1$\beta$-mediated phosphorylation of these MAPKs (lane 3) was not greatly influenced by LMB (lane 4). As shown in Fig. 3B, though low, IL-1$\beta$ induced AP-1 promoter-driven luciferase expression (lane 3) that was also not inhibited by LMB (lane 4). These results suggest that inhibition of IL-1$\beta$-induced COX-2 expression in A549 cells by LMB is not through modulation of MAPKs or AP-1.

#### 4) Inhibition of IL-1$\beta$-induced COX-2 expression by LMB appears to be independent of CRM1 and COX-2 mRNA stability

Given that LMB is a specific inhibitor of CRM1 nuclear export receptor(18), we speculated that IL-1$\beta$ might induce COX-2 expression in A549 cells via CRM1-dependent COX-2

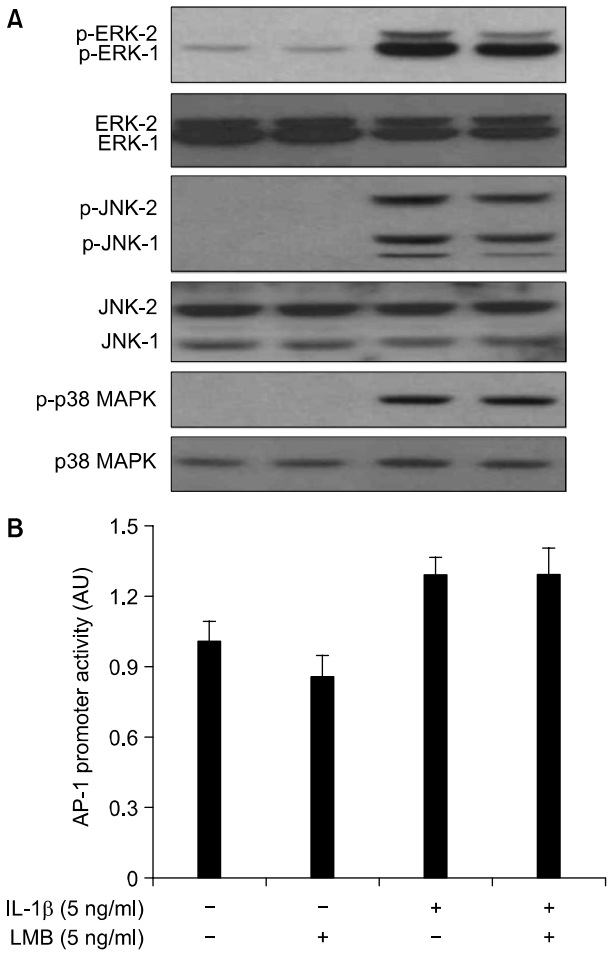


Fig. 3. Effect of LMB on IL-1$\beta$-mediated activation of MAPKs and AP-1 promoter activity in A549 cells. (A) A549 cells were pre-treated with LMB for 1 h. Cells were then exposed to IL-1$\beta$ for 0.5 h in absence or presence of LMB. Total cell lysates were prepared, and used to measure the extent of phosphorylation of ERK-1/2, JNK-1/2, or p38 MAPK. Total protein level of each protein was confirmed with striping and reprobing the membrane by immunoblot using respective antibody. (B) A549 cells were transfected with AP-1 promoter/luciferase DNA along with control pRL-TK DNA for 24 h and then exposed to IL-1$\beta$ for 4 h in absence or presence of LMB. Cell lysates were prepared and used for reporter gene activity. Data are mean±S.E. of three independent experiments.

mRNA nuclear export and LMB might inhibit it. To directly test it, CRM1 siRNA transfection experiment was next carried out to knock-down expression of endogenous CRM1 and to measure COX-2 expression by IL-1$\beta$. As shown in Fig. 4A, CRM1 siRNA effectively knock-downed expression of endogenous CRM1 protein (lanes 5～8) compared with control siRNA (lanes 1～4). Actin protein expression was not affected in control or CRM1 siRNA-transfected A549 cells (lanes 1～8), suggesting the specificity and effectiveness of CRM1 siRNA to down-regulate CRM1. IL-1$\beta$ induced high COX-2 protein in control siRNA-trnasfected A549 cells (lane 3) that was largely inhibited by LMB (lane 4), as expected. Remarkably, in CRM1 siRNA-transcfected A549 cells, IL-1$\beta$ was still able to induce high COX-2 protein (lane 7) and LMB also inhibited IL-1$\beta$-induced COX-2 protein expression (lane 8). The less efficacy of LMB to down-regulate IL-1$\beta$-induced COX-2 protein in CRM1 siRNA-transcfected A549 cells (lane 8) than that in control siRNA-transcfected A549 cells (lane 4) seems to be due to overall slightly increased COX-2 protein level after transfection of CRM1 siRNA itself (lanes 5～8), given that this phenomena was not seen by that of control siRNA (lanes 1～4). Together, these results strongly suggest that inhibition of IL-1$\beta$-induced COX-2 expression in A549 cells by LMB is the CRM1-independent. Post-transcriptional COX-2 mRNA stabilization is important for maximal COX-2 expression. This led us to determine the effect of LMB on COX-2 mRNA stability in IL-1$\beta$-treated A549 cells. As shown in Fig. 4B, actinomycin D chase experiments showed that IL-1$\beta$-induced COX-2 mRNA was stable (by 9 hrs) in the absence of LMB (lanes 3～6), which was not significantly changed by LMB (lanes 7～10), suggesting that LMB does not target COX-2 at its mRNA stability in response to IL-1$\beta$. The slightly reduced COX-2 mRNA in presence of LMB (lane 10) compared with no LMB (lane 6) is likely the relatively low level of control GAPDH mRNA.

## DISCUSSION

COX-2 is suggested to play a carcinogenic role in a variety of tumors, including lung. Thus, any compound which can inhibit COX-2 has the potential to be clinically useful against tumors in which COX-2 plays a causative role. LMB is a *Streptomyces* metabolite and a specific inhibitor of CRM1 nuclear export receptor. In this study, we have evaluated the effect of on COX-2 expression induced by IL-1$\beta$ in A549 human airway epithelial cells. Here we demonstrate for the first time that LMB potently suppresses IL-1$\beta$-induced COX-2 expression in A549 cells at transcriptional level and the suppression is associated with blockage of cytosolic I$\kappa$B-$\alpha$ degradation and the concomitant inactivation of NF-$\kappa$B transcription factor but not with CRM1.

LMB is a specific inhibitor of CRM1(18), which delivers

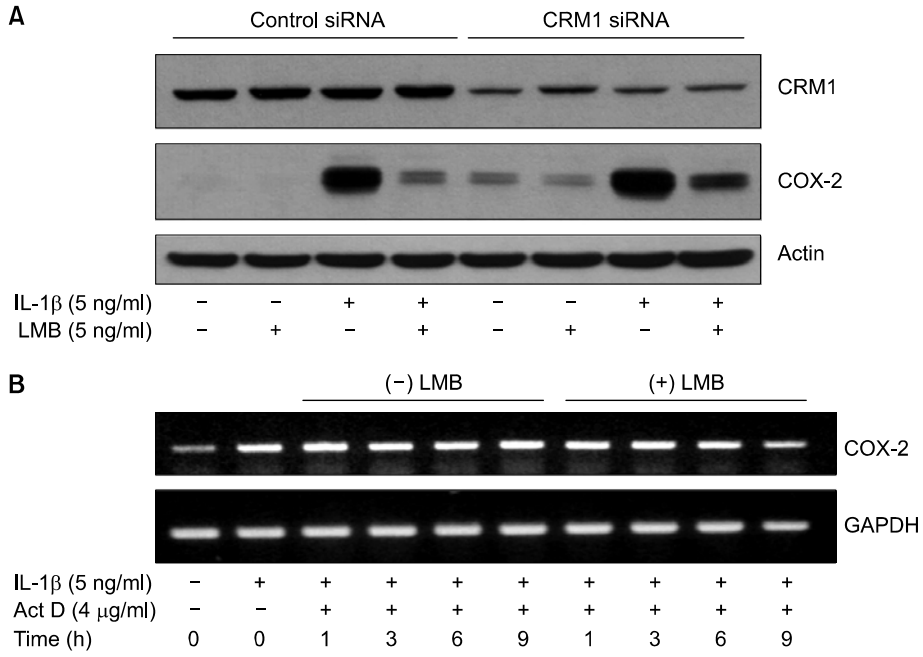


**Fig. 4.** No dependence of CRM1 and COX-2 mRNA stability in LMB inhibition of IL-1$\beta$-induced COX-2 expression in A549 cells. (A) A549 cells were transfected with 80 nM of control or CRM1 siRNA. Cells were then exposed to IL-1$\beta$ in absence or presence of LMB for 4 h. Cell lysates were prepared and used to measure the expression level of CRM1, COX-2, or actin by immunoblot with respective antibody. (B) A549 cells were primarily treated without (lane 1) or with IL-1$\beta$ (lanes 2~10) for 4 h to highly induce COX-2 mRNA. Cells were then exposed to IL-1$\beta$ in the presence of actinomycin D (Act D), a transcription inhibitor, (lanes 3~10) in absence (lane 3~6) or presence (lanes 7~10) of LMB for the indicated times. At each time, total RNA was prepared, and used for COX-2 or GAPDH RT-PCR to measure the amount of respective RNA remained in the cells.

intracellular RNAs or proteins containing NES form the nucleus to the cytoplasm. Timely nuclear export of RNA upon transcription into the cytoplasm where translation occurs is critical for gene expression(27). In view of this, LMB may be a useful experimental tool to study regulation of gene expression in the context of RNA nuclear export. Truly, evidence demonstrates that LMB inhibits nuclear export of certain RNA such as c-fos (20). We have previously also shown that LMB inhibits COX-2 expression induced by serum withdrawal in MDA-MB-231 cells in part via blockage of COX-2 mRNA nuclear export and decreased COX-2 mRNA stability at post-transcriptional but not transcriptional level(10). However, the present findings that LMB inhibits IL-1$\beta$-induced COX-2 protein and mRNA expressions as well as COX-2 promoter activity, but not mRNA stability, in A549 cells strongly suggest that the inhibition is at COX-2 transcriptional level, which seems to, be to our knowledge, the first reporting LMB down-regulation of COX-2 transcription particularly in response to IL-1$\beta$ signal.

COX-2 transcription is greatly affected by transcription factors and/or signaling proteins. IL-1$\beta$ signals to induce degradation of I$\kappa$B-$\alpha$, a cytosolic inhibitory protein of NF-$\kappa$B(24) and the resultantly free NF-$\kappa$B then enters into the nucleus where this transcription factor by binding its cognate cis-acting element within the COX-2 promoter stimulates COX-2 transcription (25). I$\kappa$B-$\alpha$ has nuclear localization sequence along with NES within its structure and thus shuttles between the cytoplasm and the nucleus(22). Importantly, a recent study has shown that LMB induces nuclear retention of I$\kappa$B-$\alpha$ by blocking its nuclear exporting, which protects IL-1$\beta$- or TNF-$\alpha$-induced degradation of I$\kappa$B-$\alpha$ and eventually inhibits IL-1$\beta$- or TNF-$\alpha$-induced NF-$\kappa$B activation in HeLa cells(28). In agreement with it, we have shown that LMB effectively inhibits IL-1$\beta$-mediated degradation of I$\kappa$B-$\alpha$ (particularly the cytosolic one), nuclear localization of p65 NF-$\kappa$B, and NF-$\kappa$B promoter-driven luciferase expression in A549 cells. However, in this study, though not clear, we have not seen any nuclear retention of I$\kappa$B-$\alpha$ following the exposure of LMB in IL-1$\beta$-treated A549 cells (data not shown). Nevertheless, these data strongly suggest that one mechanism by which LMB inhibits IL-1$\beta$-induced COX-2 transcription in A549 cells includes inhibition of the cytosolic I$\kappa$B-$\alpha$ degradation and the concomitant NF-$\kappa$B inactivation in response to IL-1$\beta$. However, at this moment, it is uncertain how LMB blocks IL-1$\beta$-induced proteolysis of the cytosolic I$\kappa$B-$\alpha$ in A549 cells. Presently, the ubiquitin-proteasome system is regarded as a major pathway mediating agonist-driven proteolysis of I$\kappa$B-$\alpha$. Interestingly, a previous study has demonstrated that calpain, an intracellular cytosolic calcium-dependent cysteine protease, involves in tumor necrosis factor-alpha-inducible I$\kappa$B-$\alpha$ proteolysis(29). In line of this, it will be interesting to see whether LMB affects

these protein degradative factors. However, it should be noted that LMB does affect LPS-induced degradation of I$\kappa$B-$\alpha$ in BV2 cells(23), suggesting that LMB regulation of I$\kappa$B-$\alpha$ may be agonist- and/or cell type-specific. Multiple signaling pathways, including ERKs, p38 MAPK, JNKs, and AP-1, have been shown to be linked to COX-2 up-regulation in response to IL-1$\beta$(25). However, the present findings showing that LMB does not affect IL-1$\beta$-mediated phosphorylation of ERKs, p38 MAPK or JNKs and AP-1 promoter-driven luciferase expression in A549 cells rule out the possibility that LMB down-regulation of IL-1$\beta$-induced COX-2 is through modulation of these signaling proteins.

Considering that LMB is a CRM1 specific inhibitor(18) and it inhibits COX-2 expression by IL-1$\beta$ in A549 cells as shown herein, we hypothesized that IL-1$\beta$ might induce COX-2 expression in A549 cells via a CRM1-dependent mRNA nuclear exporting pathway. However, the present data of CRM1 siRNA transfection experiment showing that IL-1$\beta$ is still able to induce high COX-2 protein and LMB effectively inhibits IL-1$\beta$-induced COX-2 protein expression in CRM1 siRNA-transcfected A549 cells where 50% of endogenous CRM1 protein is knock-downed by CRM1 siRNA clearly suggest that LMB inhibition of IL-1$\beta$-induced COX-2 expression and probably IL-1$\beta$-mediated COX-2 mRNA nuclear exporting in A549 cells is the CRM1-independent.

In summary, we report for the first time the action mechanism of LMB to down-regulate the cytokine IL-1$\beta$-induced COX-2 expression in A549 lung cancer cells, which involves COX-2 transcriptional suppression and blockage of the cytosolic I$\kappa$B-$\alpha$ proteolysis and the concomitant inactivation of NF-$\kappa$B but independent of CRM1, MAPKs, and AP-1.

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