Thalidomide suppresses IL-1β-induced NFκB activation and destabilizes cyclooxygenase-2 mRNA

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Directed by Professor Won Ho Kim

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ABSTRACT

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Thalidomide which used as a sedative and anti-nausea drug during late 1950's has been shown to have both anti-inflammatory and anti-oncogenic properties. The anti-inflammatory effect of thalidomide is associated with suppression of cytokine expression and the anti-oncogenic effect is related to inhibition of angiogenesis. Its cellular target and action mechanism are poorly understood. The NFκB and cyclooxygenase (COX)-2 have been known to be a critical regulator of immune and inflammatory response. Therefore, this research investigated the molecular mechanism of the anti-inflammatory effect of thalidomide, especially in NFκB pathway, NFκB-mediated COX-2 expression and post-transcriptional level of COX-2 expression.

This research shows here that thalidomide can block NFκB activation through a mechanism that involves the inhibition of activity of the IκB kinase

and NIK dependent pathway. These data indicate that anti-inflammatory property for thalidomide may be based on its ability to inhibit NFκB activation through suppression of NFκB activation through NIK dependent pathway.

COX-2, an inducible prostaglandin synthase, is overexpressed in cancer and chronic inflammatory disease. Regulation of COX-2 expression is essential in the suppression of tumor and inflammation. Therefore, the effect of thalidomide was examined on COX-2 expression in colon cancer cell line, Caco-2. This research identified that thalidomide did not affect COX-2 transcription through reporter assay with COX-2 promoter deletion series. But, on post-transcriptional level, thalidomide and p38 MAPK inhibitor, SB203580 increase COX-2 mRNA degradation after actinomycin D treatment. Thalidomide also suppressed p38 MAPK activation. This research also shows that thalidomide changes the subcellular localization of HuR which is a mRNA stabilizing protein and nuclear shuttling protein. These data indicate that thalidomide downregulates COX-2 expression through p38 MAPK suppression and alteration of HuR localization. Therefore, this research suggests that thalidomide acts dependently as a transcriptional or post-transcriptional regulator on NFκB and COX-2 and also, the effects of thalidomide on NFκB and COX-2 are important to understanding its anti-inflammatory and anti-oncogenic properties.

Key words :Thalidomide, Cyclooxygenase-2(COX-2), Nuclear factor-κB(NFκB), mRNA stabilization, p38 MAPK, HuR

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I. INTRODUCTION

Thalidomide, a synthetic derivative of glutamic acid, was used as a sedative and anti-nausea medication in 1950's, but its use was discontinued because it was linked to serious congenital birth defects such as limb deformity.[1,2] Nevertheless, thalidomide has been used to treat some diseases such as rheumatoid arthritis,[3,4] inflammatory bowel disease[5] and some cancers[6,7] because of its anti-inflammatory and anti-angiogenic properties.[8-10] The advantage of thalidomide compared with other anti-inflammatory drugs is its selective inhibition of TNF-α production and its wide therapeutic range,[11] but the action mechanisms of that remain poorly understood. In terms of anti-inflammatory effects, thalidomide has been shown to suppress important inflammatory cytokine expression such as TNF-α [12,13]and IL-12.[14,15] And its anti-angiogenic effect has been demonstrated by its inhibitory effect on growth factor -induced neovascularization.[16] Neovascularization

occurs through a process that requires the induction of a number of cellular genes including IL-8.[17,18] Transcriptional upregulation of IL-8, as well as TNF-α and IL-12, can occur through activation of the transcription factor NFκB.[19]

The transcriptional factor NFκB has been identified as a critical component of several signal transduction pathways and has been known as a key regulator of inflammation, tumorigenensis and angiogenesis.[20,21] Its widespread biological significance was demonstrated in part by its activation in response to several different agents in variety of cell types and by its regulation of a variety of genes involved in immune and inflammation responses such as those encoding IL-2, major histocompatibility complex class I, IL-6, and cellular adhesion molecules.[22,23] The regulation of NFκB activity is thought to provide significant meaning to understanding the mechanism of immune and inflammatory responses.

Previous results indicate that thalidomide inhibits the NFκB activity through suppression of IκB kinase activity.[24] In our previous report, we also confirmed that thalidomide suppresses the IL-1β-induced NFκB activation in colon cancer cells and demonstrated the anti-inflammatory action of thalidomide through NFκB inhibition.[25] In addition, thalidomide inhibits lipopolysaccharide-mediated induction of cyclooxygenase (COX)-2, decreasing the stability of COX-2.[26] Despite these findings, the mechanism of thalidomide's effects on the expression of inflammatory and angiogenic molecules is not fully understood.

Cyclooxygenase which catalyzes the conversion of arachidonic acid to prostaglandin endoperoxide has two isoforms, which are constitutively expressed COX-1[27,28] and mitogen-inducible COX-2.[29,30] COX1 is a constitutively present enzyme and its products are thought to be important in gastric and renal homeostasis.[31,32] In contrast, COX-2 is induced by inflammatory mediators and has

been linked to inflammation, fever, pain and a number of cancers.[32,33] Recently, many reports showed that COX-2 was overexpressed in sites of inflammation and in many types of tumor tissues.[34,35] Therefore, the regulation of COX-2 expression is an important pharmacological target for treatment and prevention of inflammation and cancer. Regulation of COX-2 expression is complex and appears to involve multiple mechanisms in different cell types and conditions.[36,37] Studies on the transcriptional regulation of COX-2 genes have led to the identification of a number of transcriptional factors that are mediated through specific *cis*-acting elements. Several transcription factors, including NFκB, NF-IL6, CCAAT-enhancer binding protein (CEBP), and cyclic AMP-response element-binding protein (CREBP), have been shown to act as positive regulatory elements for COX-2 transcription in different cell types .[38-44]

In the human COX-2 gene, the nucleotide sequence of the 5'-flanking region contains a canonical TATA box and consensus sequences of the NFκB in the 275-bp region upstream from the transcriptional start site.[45] According to many reports, these NFκB binding sites are necessary to COX-2 expression but they act differently up to cell type or mediator.[43-44,46-47] Some reports identified that COX-2 gene expression is regulated at the level of transcriptional and post-transcriptional mechanisms.[36-37, 48] In eukaryotic organism, post transcriptional gene regulation occurs through alteration in mRNA stability.[49] The COX-2 expression is also regulated at the post-transcriptional level[48, 50-51] and especially, AU-rich elements in 3'-UTR of COX-2 mRNA appears to be involved in the determination of mRNA stability.[52-54]

Recently, signaling pathways including p38 MAPK and RNA binding proteins, such as HuR, have been reported as ARE-related mRNA stabilizing factors. HuR, a member of Hu family of RNA binding proteins, is known to bind ARE and stabilize

several inducible mRNAs.[51, 55-57] However, little is known about the exact identities and functional roles of mRNA stability-related signaling pathways and RNA binding proteins.

In this study, we investigated a molecular action mechanism of thalidomide through NFκB mediated transcription and post-transcriptional regulation of COX-2 expression. In particular, we examined the effects of thalidomide on COX-2 mRNA stability and its stabilizing factors.

II. MATERIALS AND METHODS

1. Cell culture

The colon cancer cell line, Caco-2 cells were cultured in RPMI1640 medium supplemented with 100U/㎖ penicillin A, 100U/㎖ streptomycin and 10% heat-inactivated fetal bovine serum. Cells were maintained at 37℃ in a humidified incubator containing 5% CO2.

2. Materials

Thalidomide was purchased from Sigma (St.Louis, MS, USA) and dissolved in sterile DMSO. IL-1β was provided from R&D (Minneapolis, MN, USA). HuR specific antibody and phospho-p38 MAPK specific antibody were purchased from Santacruz (Santa Cruz, CA, USA). Lipofectamin-plus reagents were purchased from Gibco-BRL (Rockville, MD, USA). Luciferase assay kits were provided from Promega (Mannheim, Germany). Expression vector of NIK and NIK mutant were presented from Dr. David Wallach (Weizmann Institute of Science, Israel) and COX-2 promoter deletion construct were presented from Dr. WJ Lee (Ewha Woman's University, Seoul, Korea).

3. Transfection and Luciferase Assays

A. Transfection : Caco-2 cells were plated on 6-wells plate, grown to 70% confluence, and transfected with 0.5 μg of 3κB site-Luciferase reporter plasmid or 0.5 μg of COX-2 promoter-Luc reporter plasmids harboring serial deletions by Lipofectamine-Plus reagent. After transfection at the indicated time, the medium was changed to fresh and complement medium.

B. Luciferase assay : After 18hr, the medium was changed to medium containing inhibitors or control medium. After 30 min of incubation, cells were stimulated with 1 ng/ml of IL-1β and allowed to incubate for designated time. Cells were harvested and lysed in lysis buffer containing 25mM Tris-phosphate (pH 7.8), 2mM 1,2-diaminocyclohexane- N,N,N-tetraacetic acid, 2mM DTT, 10% glycerol, and 1% Triton X-100 for 5 min at room temperature. Luciferase activity was measured in a 20 ㎕ of cellular extraction using a luciferase assay system (Promega, Mannheim, Germany) and MicroLumat LB 96P luminometer (EG&G Berthold, Australia). All transfection were normalized for β-gal expression.

4. EMSA (Electrophoretic Mobility Shift Assay)

10 μg of nuclear protein was incubated for 30 min at 37 ℃ in binding buffer (10 mM HEPES, pH 7.6, 5 mM MgCl2, 60 mM KCl, 1 mM DTT, 5% glycerol, and 5 mg/ml heparin) with 5 pmol of labeled RNA probe in a final volume of 20 ㎕. For supershift experiments, binding mixtures included 1 ug of affinity-purified IgG raised against NFκB(Santa Cruz Biotechnology). Loading buffer containing 80% glycerol and 0.1% bromphenol blue in 50 mM Tris-Cl, pH 7.5, was added, and samples were electrophoresed (250 V for 2.5 hr) on 4% polyacrylamide gels (pre-run for 1 hr at 250 V) containing 44 mM Tris-Cl, pH 8.3, 44 mM boric acid, 1 mM EDTA, 4%

acrylamide-bisacrylamide (29:1), and 2.5% glycerol. EMSAs were visualized by autoradiography.

5. ELISA (Enzyme Linked ImmunoSorbent Assay)

IL-8 production was detected by sandwich ELISA. Microtiter plates (Costar, Coarning ,NY, USA) were coated with 100 ㎕/well of anti-IL-8 monoclonal antibody in PBS(137mM NaCl, 2.7mM KCl, 10 mM Na_2HPO_4, pH 7.4, 2 mM KH_2PO_4) at room temperature for 18hr. After washing with PBS for three times, 2% BSA was added for 1hr and cells were washed with PBS. Sample and recombinant IL-8 were diluted in PBS containing 0.5% Tween-20. To each well, diluted standard or sample(100㎕/㎖) were added and incubated at room temperature for 2hr. Bound IL-8 were detected after subsequent incubations with biotinylated antibody and Streptavidin-Alkaline phosphatase. After washing, color was detected with ELISA Amplification System (GIbco-BRL, Rockville, MD, USA) and absorbance was determined at 490nm. IL-8 concentration was determined by interpolation of their absorbance from the standard curve. The relative ratio is determined as compared with IL-1β stimulation.

6. Immunofluorescence Stain

Caco-2 cells (1×10^5) were seeded in 4-chamber slides. Cells were incubated with or without thalidomide (1mM) and/or IL-1β (1ng/ml). After stimulation for 1hr, cells were fixed in 4% paraformaldehyde at 4℃ for 10 min. After washing with PBS for three times, cells were permeabilized using a 1:1 mixture of aceton/methanol for 1 min and washed a furthwr three times with PBS. Anti-p65 antibody or HuR antibody was treated (1:150) at 4℃ for overnight. After three times washing with PBS, FITC-conjugated secondary antibody was added (1:300) for 1hr at room temperature. After final washing with PBS, cells were scanned using a confocal

microscope (TCSNT, LEICA, Switzerlan).

7. Western blot

For the extraction of whole-cell lysates, cells were suspended in lysis buffer containing 50 mM Tris.Cl (pH 7.4), 100 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 20 ㎍/㎖ leupeptin, 5 ㎍/㎖ pepstatin, and 0.5% NP-40. The lysates were incubated on ice for 30 min and centrifuged at 12,000 g for 10 min. The supernatants containing total cell proteins were transferred into a new tube. Proteins were diluted in SDS-PAGE loading buffer. Following SDS-PAGE, the proteins were electrophoretically transferred to 0.2 μm PVDF membranes (Millipore, Bedford, MA, USA) for 1hr at 350 mA using a Tank transfer system (Bio-rad, CA, USA). The membranes loading the transferred proteins were blocked with 5% non-fat milk in TBST (0.1M Tris, pH7.4, 0.9% NaCl, 0.05% Tween 20) for 30 min at room temperature. The membranes were probed with specific primary antibody and visualized by enhanced chemiluminescence with HRP-conjugated secondary antibody.

8. Nuclear and Cytoplasmic Extracts

A. Cytoplasmic protein extraction : For extracting cytosolic protein, cells were lysed in buffer containing 10 mM HEPES (pH 8.0), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 20 ㎍/㎖ leupeptin, 50 ㎍/㎖pepstatin, and 0.5% NP-40. The lysates were incubated on ice for 5 min and centrifuged at 2000 g for 1 min. The supernatants contain cytoplasmic proteins and the pellets were used for nuclear extraction.

B. Nuclear protein extraction : The pellets resuspended in buffer containing 10 mM HEPES (pH 8.0), 20% glycerol, 420 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 20 ㎍/㎖ leupeptin, 50㎍/㎖ pepstatin, 1 mM PMSF and 0.1% NP-40 and kept on

ice for an additional 30 min. After centrifugation at 12,000 g for 10 min, supernatant containing nuclear protein was obtained.

9. RNA extraction and RT-PCR

A. RNA extraction : 5 X 10^6 cells per sample were collected at the indicated time points and frozen at -80℃. Total RNA was extracted using TRIzol reagent (Gibco-BRL, Rockville, MD, USA) as described in the manufacturer's specifications. RNA was simultaneously prepared from all samples in each experiment, quantitated, and used as template for reverse transcription.

B. RT-PCR : 10 μg of total RNA from each sample were transcribed reversely using the reverse transcriptase kit (Promega, Mannheim, Germany). Ten percent of the resulting cDNA from each sample was subjected to 30 cycles of PCR consisting of 1 min at 94℃, 1 min at 58℃ and 1 min at 72℃ in 20 μl reaction mixture in PCR-premix kit (Bionier, Dajeon, Korea). The PCR primers for COX-2 were 5'- CAGCAA ATCCTTGCTGTTCC-3' for the forward primer and 5'-TGGGCAAAGAATGCAAACATC-3' for the reverse primer.

10. Measurement of mRNA degradation

Caco-2 cells were stimulated with IL-1β (1 ng/ml) for 6 hr, followed by pretreatment with 1mM thalidomide and 10 μM SB203580 for 30 min in order to measure the extent of mRNA degradation. Actinomycin D (5 g/ml) was then added, followed by further incubation for 0, 15, 30, 60, or 120 min. Total RNA was prepared, and mRNA was measured as above. All data were normalized to the housekeeping genes of β-actin.

III. RESULTS

1. IL-1β induces NFκB transcriptional activation

First of all, this research confirmed the IL-1β-induced transcriptional activation of NFκB by reporter gene assay. As shown in figure 1, 50U/ml of IL-1β induced a 10 folds increase in luciferase activity compared with cells not exposed to IL-1β and IL-1β induced NFκB transcriptional activation in dose dependent manner (Fig. 1).

2. Thalidomide inhibits NFκB transcriptional activation by IL-1β

To determine if thalidomide inhibits NFκB transcriptional activity, transient transfection assays were performed using a luciferase reporter plasmid containing three NFκB binding sites. 50U/ml of IL-1β induced a 6.32 fold increase in luciferase activity compared with cells not exposed to IL-1β. Treatment of cells with thalidomide at the time of IL-1β induction led to the suppression of NFκB transcriptional activation in dose dependent manner. 1mM thalidomide suppressed luciferase activity to about 47% levels from that of the treatment of IL-1β alone (Fig. 2). These results demonstrate that thalidomide inhibits the ability of NFκB to activate gene expression.

3. Thalidomide Inhibits IL-8 production by IL-1β

To determine whether thalidomide inhibits NFκB dependent gene transcription, IL-8 productions were measured in cell cultured medium when Caco-2 cells were pre-treated with thalidomide at indicated dose for 30 min followed by exposure to 50U/ml of IL-1β for 2hr. The media were collected and subjected to IL-8 ELISA. The treatment of thalidomide inhibited IL-1β-induced IL-8 production in a

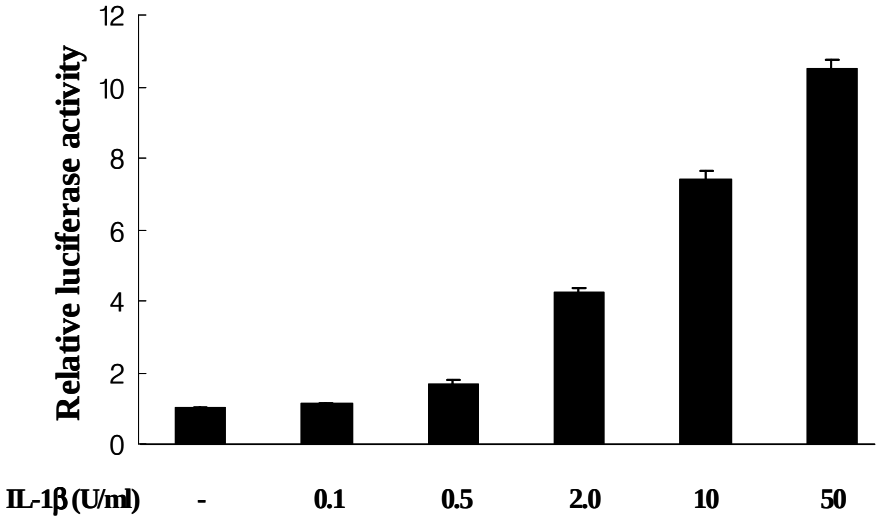


Figure 1. Induction of NFκB transcriptional activation by IL-1β

Caco-2 cells were transfected with an HIV-1 long terminal repeat luciferase construct containing three NFκB binding sites. After 4 hr, media were changed with fresh media and cells were incubated for 18hr. After that, cells were treated for 4 hr with IL-1β at the indicated dose. Cell extracts were harvested and luciferase activity was determined. The data are representative of five independent experiments.

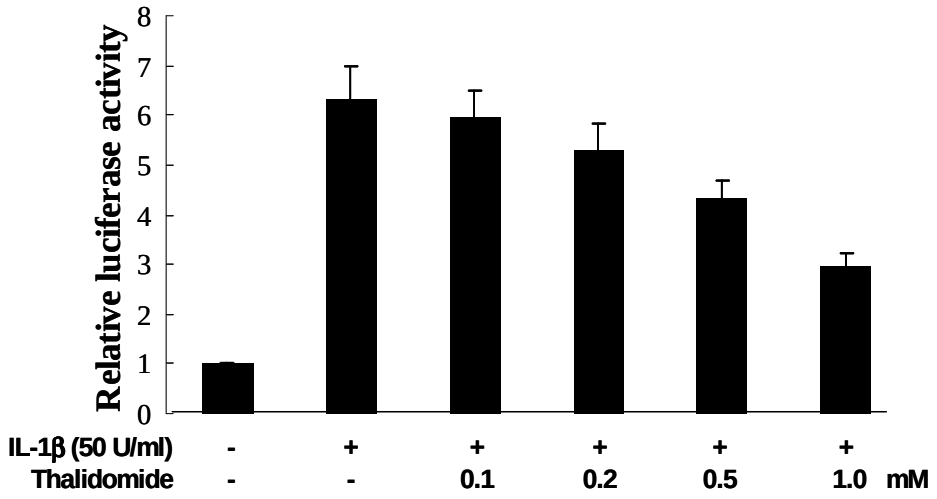


Figure 2. Inhibition of IL-1β -induced NFκB transcriptional activation by thalidomide

Caco-2 cells were transfected with an HIV-1 long terminal repeat luciferase construct containing three NFκB binding sites. After 4 hr, media were changed with fresh media and cells were incubated for 18 hr. After that, cells were pretreated with thalidomide at the indicated dose for 30 min and then incubated with IL-1β for 4 hr. Cell extracts were harvested and luciferase activity was determined. The data are representative of five independent experiments.

dose-dependent manner. Based on the average of three independent experiments, IL-1

β-induced IL-8 production was inhibited by about 30% when 1mM thalidomide was added to cells (Fig. 3). This data indicates that thalidomide blocks not only NFκB transcriptional activity but also NFκB dependent gene expression.

4. Thalidomide inhibits NFκB DNA binding by IL-1β

Caco-2 cells were preincubated for 30 min with different concentrations of thalidomide and treated with IL-1β for 30 min at 37°C, and then nuclear extracts were prepared and assayed for NFκB activation by EMSA. As shown in Fig. 4A, thalidomide inhibited IL-1β-mediated NFκB activation in a dose-dependent manner, with maximum inhibition occurring at 1mM Thalidomide by itself did not activate NFκB.

Because NFκB is a family of proteins, various combinations of Rel/NFκB protein can constitute an active NFκB heterodimer that binds to a specific sequence in DNA. To show that the retarded band visualized by EMSA in IL-1β-treated cells was indeed NFκB, nuclear extracts were incubated from IL-1 β-activated cells with antibody to either the p50 (NFκB1) or the p65 (RelA) subunit of NFκB. Antibody against p65 shifted the band to a higher molecular mass (Fig. 4B), but antibody against p50 did not shifted the band, thus suggesting that the IL-1β-activated complex consisted of p65 subunits. Excess unlabeled NFκB (100-fold) caused complete disappearance of the band.

5. Thalidomide Inhibits NFκB nuclear translocation by IL-1β

In unstimulated cells, NFκB dimers are kept as inactive complexs in the

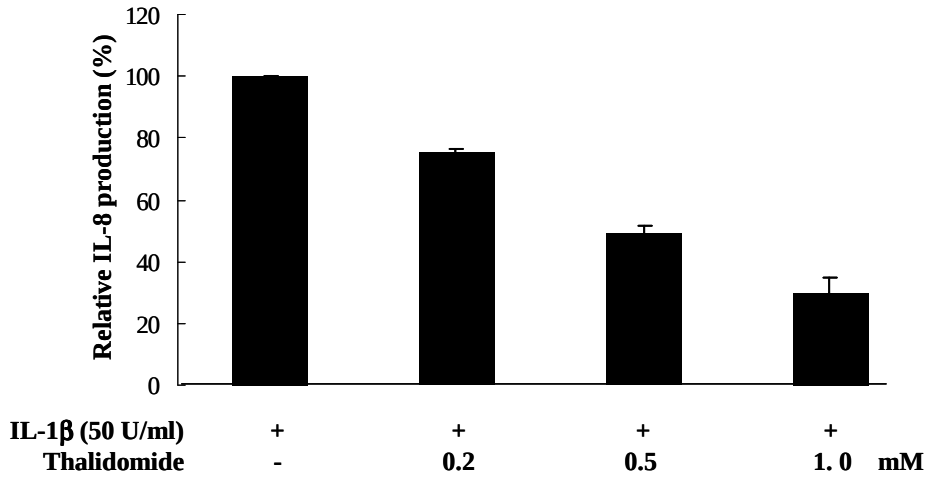


Figure 3. Inhibition of IL-1β-induced IL-8 production by thalidomide

Caco-2 cells were pretreated with thalidomide at the indicated dose for 30 min and then treated with IL-1β for 2 hr. Culture media were collected and used for the detection of IL-8 production. IL-8 production was determined by IL-8 ELISA assay. The data are representative of five independent experiments.

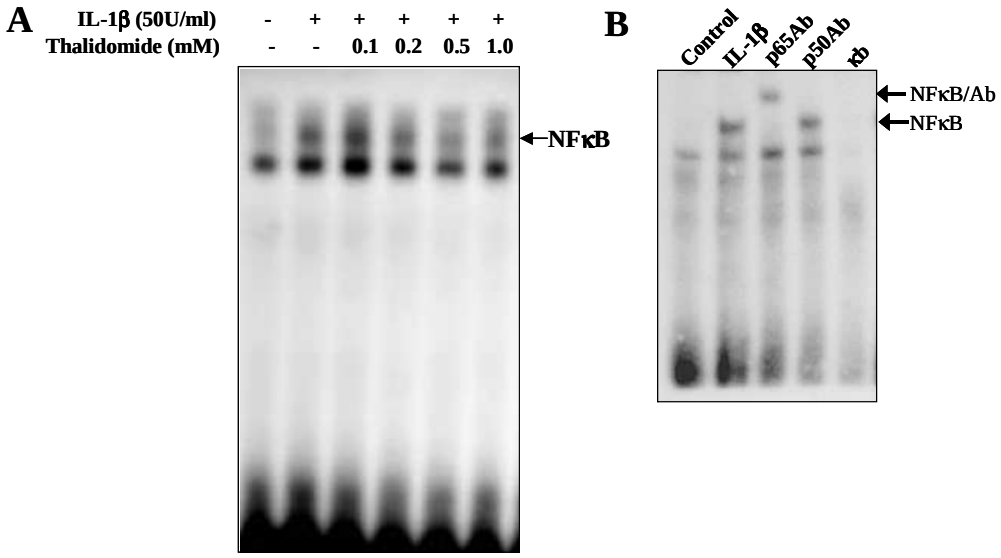


Figure 4. Inhibition of IL-1β-induced NFκB DNA binding activity by thalidomide

A. The effect of different concentrations of thalidomide on IL-1β-dependent NFκB activation. Caco-2 cells were preincubated at 37°C for 30 min with thalidomide at the indicated dose and then treated with 1ng/ml of IL-1β for 30 min. After these treatments nuclear extracts were prepared and then assayed for NFκB. B. Nuclear extracts were prepared from untreated or IL-1β-treated Caco-2 cells, incubated for 30 min with different Abs or cold NFκB oligo probes, and then assayed for NFκB.

cytoplasm by inhibitory proteins such as IκBα and IκBβ. After cell stimulation, IκBs

are phosphorylated and degraded, and free dimmers translocate into the nucleus.

To investigate whether thalidomide inhibits nuclear translocation of p65, this reseach performed Western blot using the anti-p65 antibody. Nuclear and cytosolic proteins were examined separately. IL-1β treatment increased the amount of nuclear p65 and decreased the cytosolic amount of p65, and the pretreatment of thalidomide suppressed this change (Fig. 5). This reseach also confirmed the inhibition of nuclear translocation of p65-containing complexes by thalidomide using immunofluoresence staining in the same codition. In the unstimulated state, p65 localized exclusively to the cytoplasm (Fig. 6a). After stimulation with IL-1β, p65 was found in the nuleus (Fig. 6b). The staining pattern did not change upon treatment with thalidomide alone (Fig. 6c). However, treatment with IL-1β plus thalidomide resulted in exclusive cytoplasmic staining indistinguishable from that of unstimulated cells (Fig. 6d). Thease data indicate that thalidomide prevents nuclear translocation of p65.

6. Thalidomide Inhibits IκBα degradation by IL-1β

To investigate the upstream of NFκB translocation inhibited by thalidomide, IκBα degradation was examined following thalidomide treatment. Caco-2 cells were pretreated with thalidomide for 30 min at the indicated dose, and were subsequently stimulated with 50U/ml of IL-1β. Cells were harvested and cytoplasmic proteins were extracted and were analyzed by Western blotting using anti-IκBα antibody. When cells were incubated with 50U/ml of IL-1β, the amount of cytoplasmic IκBα was decreased compared with the result of no treatment. The treatment of thalidomide blocked IκBα degradation at dose dependently (Fig. 7). This data show that thalidomide blocks IL-1β-induced IκBα degradation and then NFκB translocation is

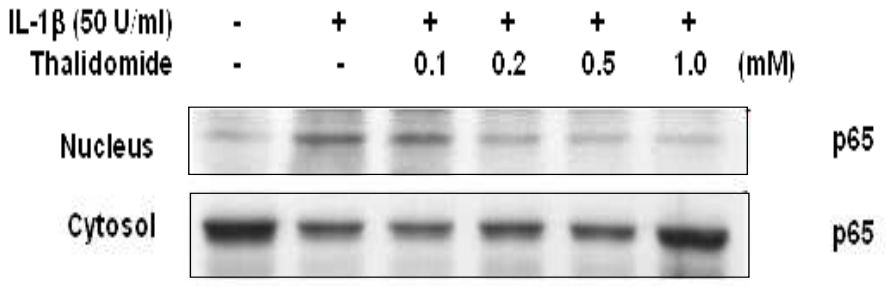


Figure 5. Inhibition of IL-1β-induced NFκB nuclear translocation by thalidomide

Caco-2 cells were pretreated with thalidomide at the indicated dose for 30 min and then treated with IL-1β for 30 min. And then, nuclear and cytosolic proteins were collected separately and subjected to Western blot using the anti-p65 antibody.

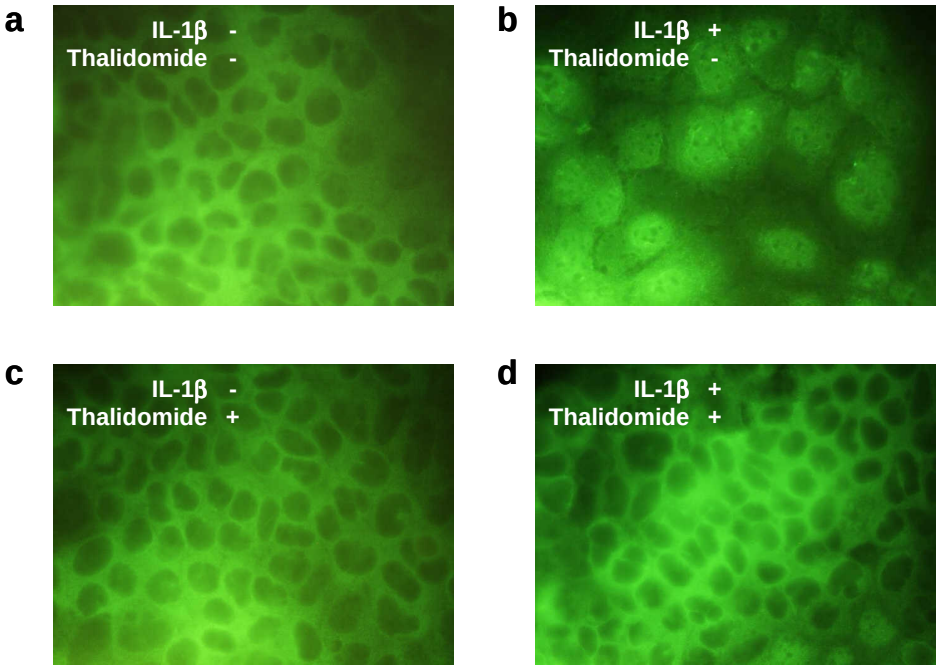


Figure 6. Inhibition of IL-1β-induced NFκB nuclear translocation by thalidomide

Caco-2 cells were left untreated (a), stimulated with IL-1β 50 U/ml (b), treated with thalidomide 1 mM alone (c), or pretreated with 1 mM thalidomide followed by stimulation with IL-1β 50U/ml (d). The intracellular location of NFκB was determined by immunofluorescence study using an anti-p65 antibody.

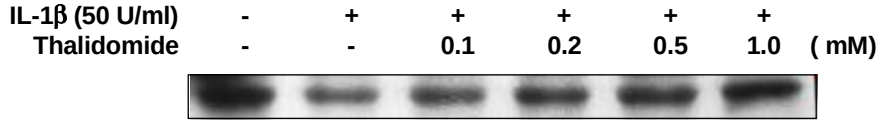


Figure 7. Inhibition of IL-1β-induced IκBα degradation by thalidomide.

Caco-2 cells were pretreated with thalidomide at the indicated dose for 30 min and then treated with IL-1β for 30 min. Equal amounts of cytoplasmic extracts were prepared and subjected to Western blot using a specific anti-IκBα antibody.

inhibited.

7. Thalidomide Inhibits NIK-induced NFκB activation

To identify the inhibitory target of thalidomide, this reseach investigated the effect of thalidomide on NIK, the IκBα upstream molecule, by using NIK expression vector. Caco-2 cells, which were all transfected with HIV-1 long terminal repeat luciferase construct containing three NFκB-binding sites, were co-transfected with empty vector (pcDNA), NIK expression vector (pcDNA-NIK(wt)) or mutant NIK expression vector (pcDNA-NIK(mut)), and incubated with or without thalidomide (1 mM) for 24 hr. After that, luciferase activity was measured. NIK expression vector induced NFκB transcriptional activation compared with empty vector or mutant NIK expression vector. Thalidomide inhibits NIK expression vector-induced NFκB transcriptional activation without change in empty vector or mutant NIK transfected cells (Fig. 8). These data indicate that thalidomide inhibits NIK dependent NFκB transcriptional activation.

8. Thaldomide inhibits COX-2 expression by IL-1β

This reseach also examined the effect of thalidomide on IL-1β-induced COX-2 expression. Western blot analysis showed that 1ng/ml of IL-1β induced the increase of COX-2 expression compared with the result of no treatment and treatment with thalidomide caused a dose-dependent decrease in IL-1β-mediated of COX-2 expression (Fig. 9).

9. Thaldomide inhibits induction of COX-2 mRNA by IL-1β

This reseach also investigated the effects of thalidomide on COX-2 mRNA

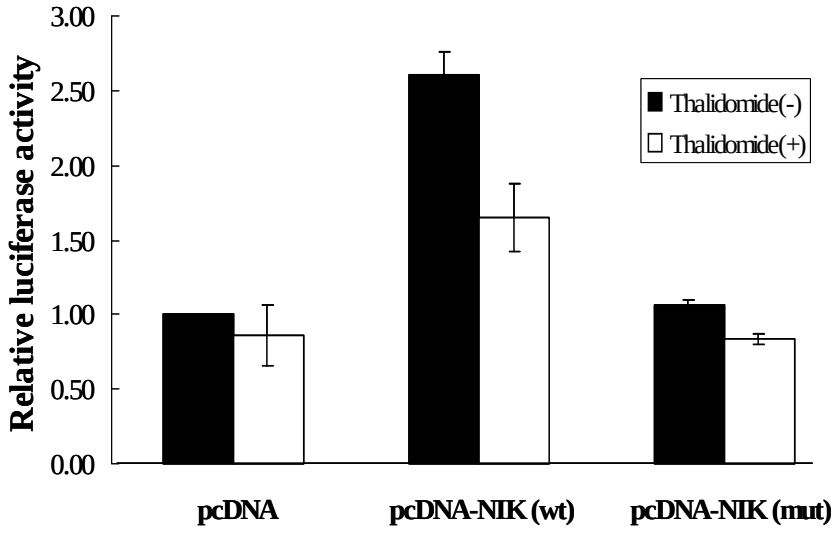


Figure 8. Inhibitory effect of thalidomide on NIK-induced NFκB transcriptional activation.

Caco-2 cells, which were all transfected with HIV-1 long terminal repeat luciferase construct containing two NFκB-binding sites, were cotransfected with empty vector (pc DNA), NIK expression vector (pcDNA-NIK(wt)) or mutant NIK expression vector (pcDNA-NIK(mut)), and incubated with or without thalidomide (1 mM) for 24 hr. After that, luciferase activity was measured. The data are representative of four independent experiments.

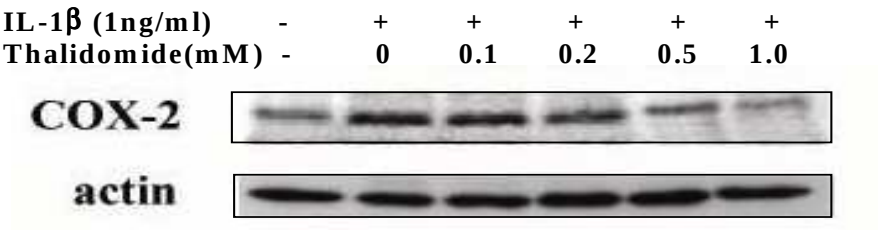


Figure 9. The inhibition of IL-1β induced COX-2 expression by Thalidomide

Cells were pre-treated before 30 min with thalidomide at the indicated dose and then were treated 1ng/ml of IL-1β. After 16hr, whole cell proteins were obtained and Western blot was performed for COX-2 and actin by specific antibody. Actin was used as internal control.

expression by RT-PCR. The same as Western blot, 1ng/ml of IL-1β induced

COX-2 mRNA expression compared with no treatmen and thalidomide effectively inhibited IL-1β -induced COX-2 mRNA synthesis (Fig. 10). As shown in figure 10, p38MAPK inhibitor, SB203580, suppressed IL-1β -induced COX-2 mRNA synthesis and these data were based on that p38 MAPK regulates mRNA stability. p38 MAPK was known for mRNA stabilizer and many reports showed that p38 MAPK can regulate mRNA.[51,57] Therefore, these suggest the possibility that thalidomide can be a regulator of mRNA stability in that the effect of thalidomide on COX-2 mRNA is like that of SB203580.

10. NFκB site is not essential for transcription of COX-2 by IL-1β

To further elucidate the mechanism responsible for changes in amounts of COX-2 protein, the transcriptional activity of COX-2 was investigated, especially in NFkB-mediated transcription. COX-2 expression is regulated through multiple pathways including NFκB, C/EBP transcription factors, and MAPK.[38,40,58-59] NF κB is thought to be a very important transcription factor on COX-2 expression. Our previous data showed that thalidomide suppressed NFκB activation through NIK dependent pathway. Thus, this reseach supposed that the suppression of COX-2 by thalidomide is caused to the inactivation of NFκB by thalidomide. First, this reseacher examined the act of NFκB site on COX-2 transcription. Caco-2 cells were transfected with COX-2 promoter deletion construct (d440 : full construct, d1650 : 2NFκB-site deletion, d1800 : all *cis* element deletion) and COX-2 transcription was measured by luciferase reporter assay and normalized by β -gal value. IL-1β induced the transcriptional activation of COX-2 in reporter assay using complete COX-2 promoter construct, but there was no remarkable decrease of IL-1β-induced luciferase

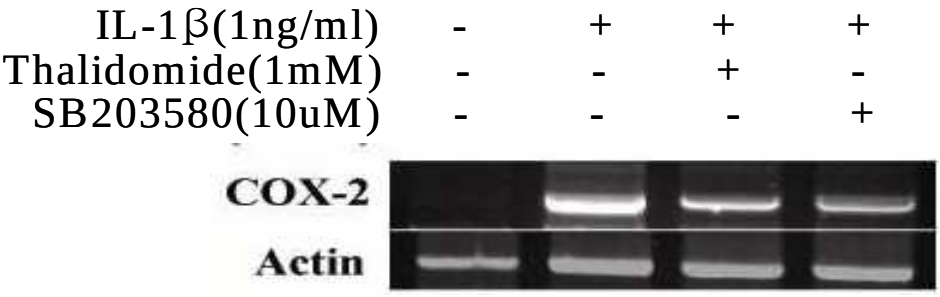


Figure 10. The suppression of IL-1β-induced COX-2 mRNA synthesis by thalidomide and SB203580, p38 MAPK inhibitor

Caco-2 cells were pre-treated with or without 1mM thalidomide and 10 μM SB203580, p38 MAPK inhibitor. After 30 min, 1ng/ml IL-1β was added to the media for 6hr. Total RNA was isolated and measured mRNA content by RT-PCR. β -actin were used internal control.

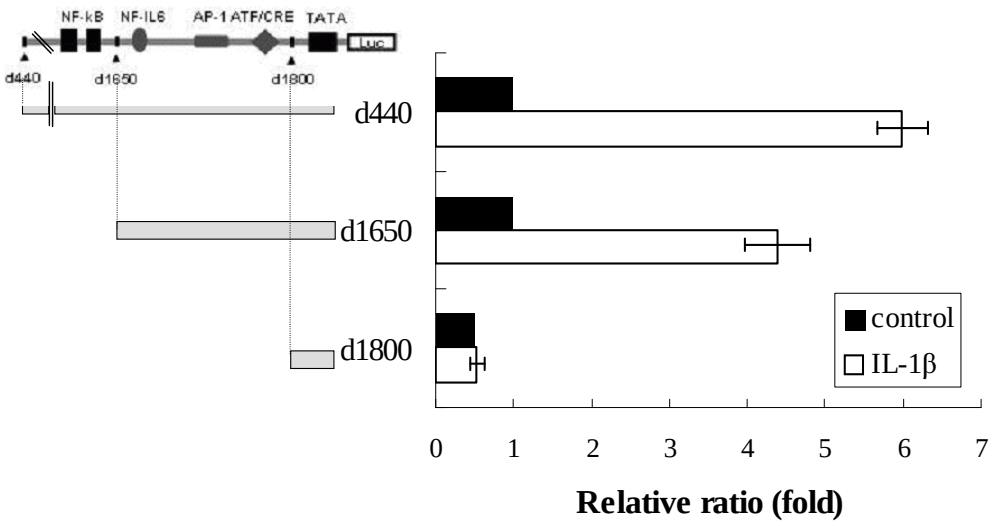


Figure 11. The role of NF-κB site on IL-1β -induced COX-2 transcritional activation

Caco-2 cells were transfected with COX-2 promoter deletion construct (d440 : full construct, d1650 : 2NFκb-site deletion, d1800 : all *cis* element deletion) and β-gal construct by lipofectamin. After transfection, cells were incubated with 1ng/ml of IL-1β for 6 hr. Finally total cell lysates were obtained and used in luciferase reporter assay and normalized by β -gal value. The data are representative of three independent experiments.

activity in reporter assay using NFkB-deleted COX-2 promoter construct (Fig. 11).

The deletion of NFκB caused to decrease of COX-2 transcription about 30%. These data suggest that NFκB site is required but is not essential to IL-1β-induced COX-2 expression in Caco-2 cells. These data agree with that induction of COX-2 by IL-1β is mediated partly by NFκB in colorectal cancer cells.[60,61]

11. Thalidomide dose not affect COX-2 transcription by IL-1β

This reseacher searched wether thalidomide affects the transcriptioal induction of COX-2 by IL-1β. This reseacher performed reporter gene assay using COX-2 promoter with various deletions. Thalidomide does not affect on COX-2 promoter-driven luciferase expression in both complete COX-2 promoter construct and NFκB-deleted COX-2 promoter construct (Fig. 12). These data suggest that thalidomide does not regulate the transcriptional activity of COX-2 gene as well as NFκB mediated COX-2 gene transcription. This reseacher determined that thalidomide suppressed the expression of COX-2 protein but did not inhibit COX-2 gene transcription.

12. Thalidomide and SB203580 destabilize COX-2 mRNA

Thalidomide had no effect on IL-1β-mediated induction of COX-2 transcription. Treatment with IL-1β stimulated COX-2 promoter activity, but this effect was not suppressed by thalidomide. Post-transcriptional gene regulation contains mRNA copy number and mRNA half-life time. To examine the effect of thalidomide on the post-transcriptional regulation of COX-2 mRNA, this reseacher investigated the effects of thalidomide on the stabilization of COX-2 mRNA.

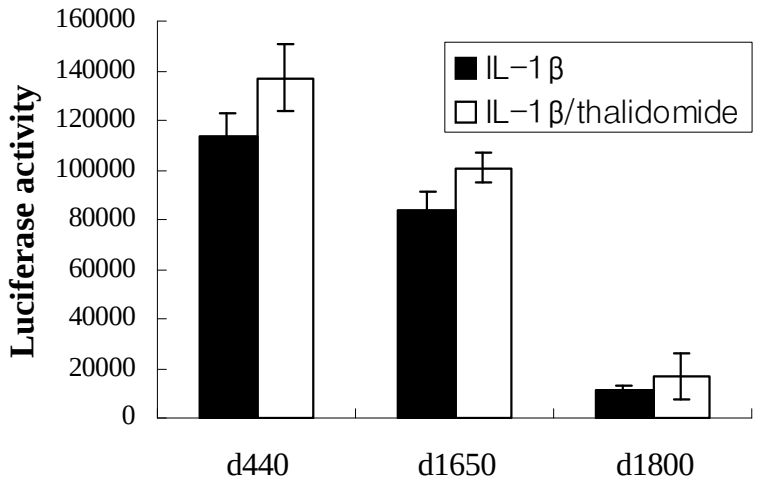


Figure 12. The effect of thalidomide on IL-1β-induced transcription of COX-2

Caco-2 cells were transfected transiently with COX-2 promoter deletion costruct (d440 : full construct, d1650 : 2NFκb-site deletion, d1800 : all *cis* element deletion) and β-gal construct by lipofectamin. Cells were pre-treated with 1mM thalidomide for 30 min and incubated with 1ng/ml of IL-1β for 6 hr. Total cell lysates were used in luciferase reporter assay and normalized by β-gal value. The data are representative of three independent experiments.

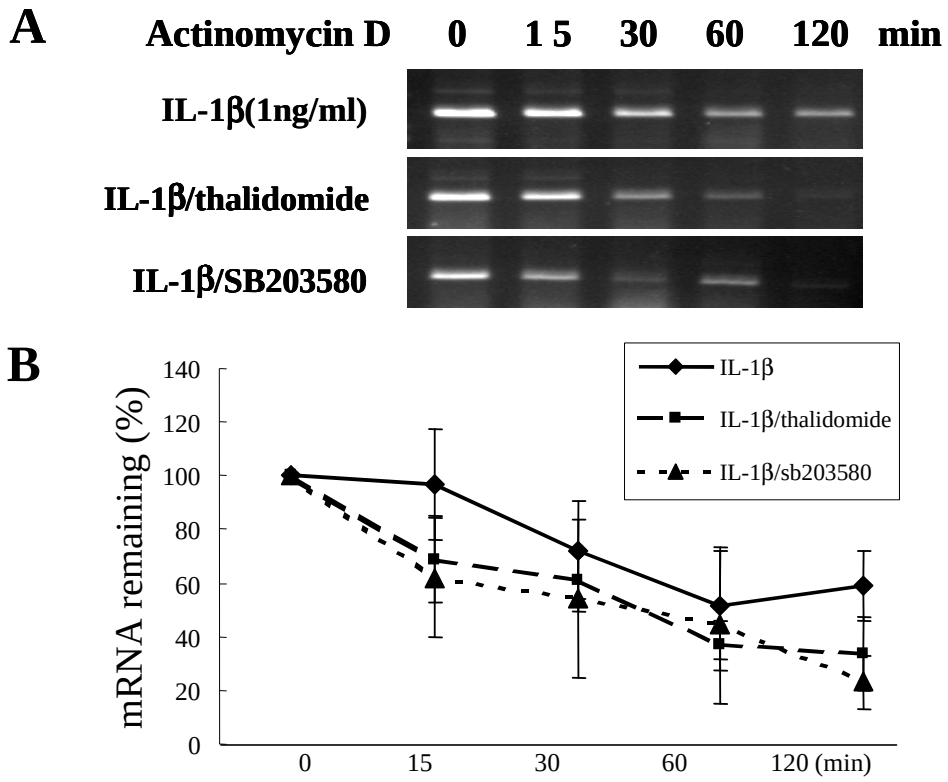


Figure 13. The destabilization of COX-2 mRNA by thalidomide and p38 MAPK

Caco-2 cells were pre-treated with or without 1mM thalidomide and SB203580. After 30 min, IL-1β (1ng/ml) was added to the media for 6 hr. After that, Actinomycin D (5 g/ml) was added to the media for the indicated time, and COX-2 mRNA levels were analyzed by RT-PCR (A). Relative levels of COX-2 mRNA expression were determined by densitometric scanning of the bands and normalized to the β-actin signal (B). The data are representative of five independent experiments.

This reseacher measured the rates of degradation of COX-2 mRNA after

treatment with IL-1β or IL-1β plus thalidomide or SB203580. Cells were treated with IL-1β or IL-1β plus thalidomide for 4hr, then transcription was stopped with the addition of actinomycin D. RNA was isolated for 15, 30, 60 and 120 min after treatment with actinomycin D and subjected to RT-PCR. As shown in figure 13, treatment with thalidomide in IL-1β-treated cells induced more rapid degradation of COX-2 mRNA than IL-1β treatment alone. In addition, because the activation of p38 MAPK stabilizes mRNA, this reseacher tested the effect of pre-treatment with a p38 MAPK inhibitor, SB203580, as had been done with thalidomide. Like thalidomide, SB203580 increased COX-2 mRNA degradation (Fig. 13). This reseacher suggest that thalidomide suppresses COX-2 expression via mRNA destabilization, which is related to the inhibition of p38 MAPK activation.

13. Thaldomide inhibits p38 MAPK activation by IL-1β

This reseach showed that p38 MAPK activation is also related with IL-1β-induced COX-2 mRNA stabilization. To know the inhibitory mechanism of thalidomide on mRNA stabilization, this reseacher investigated whether there is the relation between thalidomide and p38 MAPK. This reseacher performed Western blot using specific antibody against phospho-p38 MAPK to examine the effect of thalidomide on IL-1β-induced p38 MAPK activation. p38 MAPK was activated by IL-1β and its phosphorylation was suppressed by thalidomide in a dose-dependent manner. We also examined at the same time the effect of thalidomide on ERK and JNK activation. Thalidomide did not suppress the other MAPK activation, ERK and JNK (Fig. 14). Therefore, we identified that thalidomide inhibits COX-2 mRNA stabilization through p38 MAPK suppression.

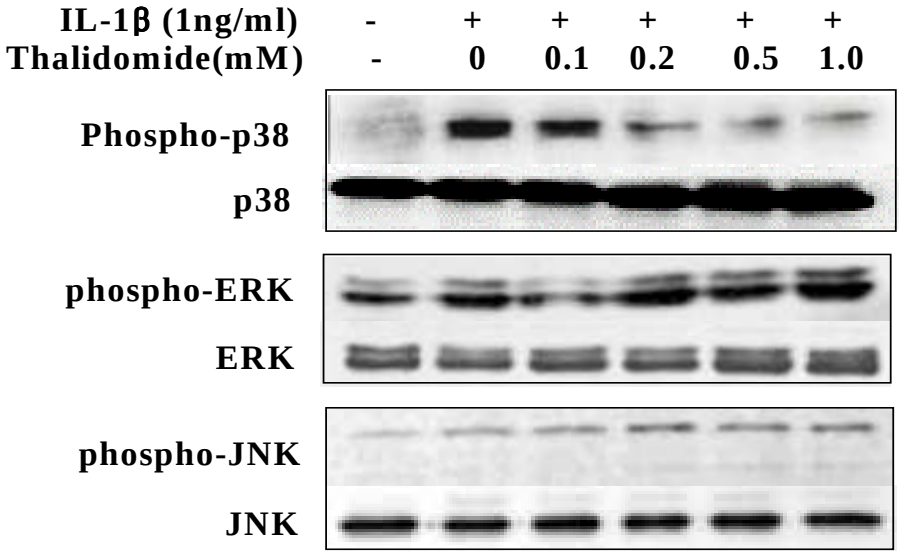


Figure.14. The inhibition of IL-1β induced activation of p38 MAPK by thalidomide

Cells were treated 1ng/ml of IL-1β. 30min before IL-1β treatment, cells were pre-treated with thalidomide. After 30 min, whole cell proteins were obtained and Western blot analysis was performed using specific antibodies against phospho-p38 MAPK (*upper panels*) and control antibodies that recognize these kinases regardless of their phosphorylation status (*lower panels*). At the same condition, Western blot analysis was performed using specific antibodies against phospho-ERK or phospho-JNK.

14. Thalidomide dose not affect HuR Expression by IL-1β

A RNA-binding protein, HuR, is also known to be an important mRNA stabilizing protein. This researcher examined whether thalidomide affects on total HuR expression by Western blot using specific HuR antibody. As shown in Figure 15, IL-1β as well as thalidomide dose not affect on HuR total expression.

15. Thalidomide and SB203580 alter the subcellular localization of HuR

HuR is also known to be a nuclear-cytoplasmic shuttling protein. HuR binds to mRNA in nucleus and then translocates cytoplasm with mRNA. So HuR can protect mRNA from nuclease. Therefore, to identify the effect of thalidomide on HuR subcellular localization, this researcher performed the immunofluorescence study using anti-HuR antibody. HuR localized in nucleus at the unstimulated condition. but, when it was stimulated by various factor, it shuttled in cytoplasm. Thalidomide and p38 inhibitor, SB203580 blocked the alteration of HuR localization (Fig. 16). This event occurred very early and then recovered. With these results, this research suggested that thalidomide suppressed HuR shuttling to cytoplasm and then mRNA cannot be protected from nuclease.

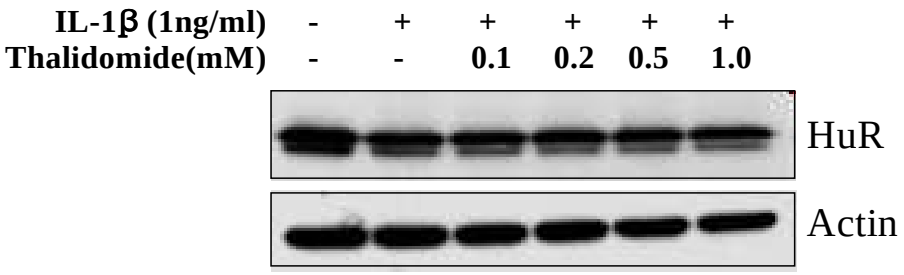
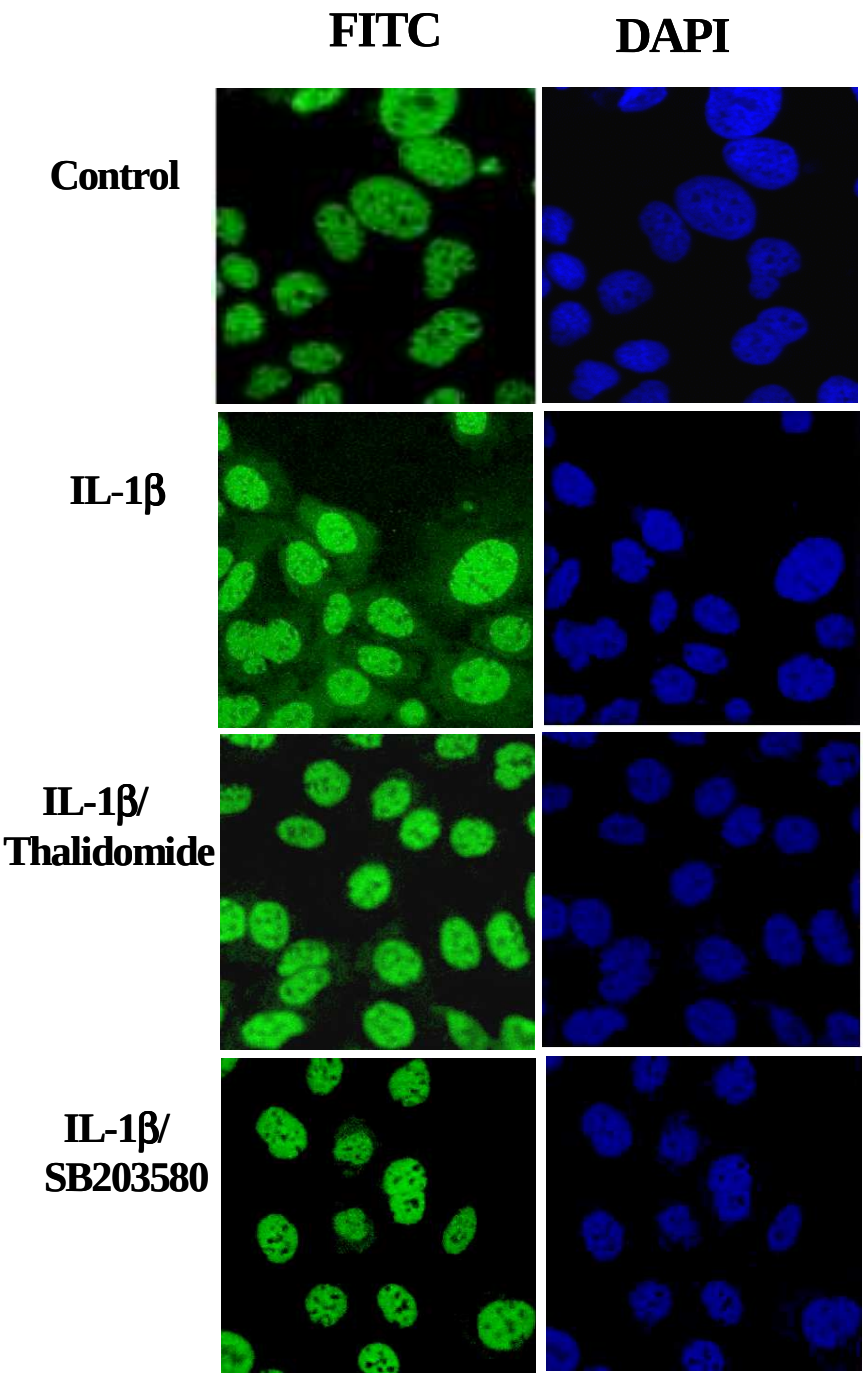


Figure 15. The effect of thalidomide on expression of total HuR expression by IL-1β

Caco-2 cells were pre-treated with or without at the indicated dose of thalidomide for 30 min prior to stimulation with IL-1β (1 ng/ml) for 8 hr. Total proteins were extracted and then HuR was detected by Western blot using specific antibody for HuR.

Figure 16. The alteration of subcellular localization of HuR by thalidomide and SB203580

Caco-2 cells were left untreated or stimulated with 1ng/.ml of IL-1β for 1hr. 1 mM thalidomide and 10μM SB203580 were pretreated for 30 min. The intracellular location of HuR was determined by immunofluorescence staining using specific antibody for HuR antibody and FITC conjugated antibody. Nucleus were stained with DAPI (5 ug/ml).


FITC
DAPI
Control
IL-1β
IL-1β/
Thalidomide
IL-1β/
SB203580

IV. DISCUSSION

Thalidomide has known for anti-inflammatory and anti-angiogenic drug.[62] Despite its clinical role is expanding, its internal mechanism was not known clearly. This researcher has concerned about thalidomide's target mechanism and has defined the molecular mechanism of thalidomide, especially in the NFκB pathway.[25] Our report showed that thalidomide suppressed IL-1β induced NFkB activation by the inhibition of NIK downstream pathway. This research showed that thalidomide inhibited NFκB mediated gene transcription through IL-8 production. Angiogenic factors such as IL-8 are transcriptionally regulated by NFκB, and TNF-α-dependent angiogenesis requires the activation of NFκB for the expression of IL-8.[17] This result explains the anti-angiogenic effect of thalidomide through inhibition of IL-8 expression which is required in neovascularization and agrees that thalidomide is being used as a cancer therapy partly based on its ability to inhibit neovascularization.[16,63]

In most cell types, NFκB is kept as an inactive complex in the cytoplasm bound to its inhibitor protein, IκBα. Upon activation IκBα rapidly degrades, and free NFκB dimmers translocate to the nucleus and activate target genes.[64] Both TNF-α and IL-1β activate NFκB through the induction of IKK.[19,65-66] IKK activation results in the phosphorylation of IκBα on serine residues 32 and 36, which ultimately leads to the degradation of this inhibitor.[67,68] Because thalidomide is capable of inhibiting DNA binding activity, as well as inhibiting the transcription potential of NFκB, this researcher determined whether thalidomide affected on the regulation of the NFκB inhibitory protein, IκBα. Our result shows that thalidomide blocks IκBα degradation in a dose-dependent maner. This data means that thalidomide blocks IκBα degradation and then NFκB translocation. Therefore,

these results indicate that the target of thalidomide for NFκB inhibition is IKK which phosphorylates and then degrades of IκBα. Our results agree with the latest report that thalidomide inhibits IKK activity.[24]

Based on these findings, this researcher hypothesized that inhibition of NFκB could be essential in the mechanistic role of thalidomide and thus investigated the effect of thalidomide on COX-2 expression, which is important in both inflammation and oncogenesis.

COX-2 expression is a rate-limiting step in colon cancer carcinogenesis. Therefore, the regulation of COX-2 expression contributes to suppression of inflammation and oncogenesis. In this research, thalidomide suppressed COX-2 protein expression and mRNA expression. To know the mechanism of regulation of COX-2 expression, this researcher also investigated the effect of thalidomide on transcriptional activity and posttranscriptional activity of COX-2. This research finally identified that NFκB was not essential in COX-2 gene transcription and thalidomide did not affect on COX-2 gene transcription, regardless of NFκB. Our results also suggest that thalidomide suppresses COX-2 expression through destabilization of COX-2 mRNA.

Regulation of COX-2 expression is complex and appears to involve multiple mechanisms in different cell types and conditions. Although the promoter regions of the COX-2 gene contain binding sites for several transcription factors, including thalidomide-inhibited NFκB[24], inhibition of COX-2 expression by thalidomide was not associated with inhibition of NFκB under our experimental conditions. It is possible that the inhibitory effect of thalidomide on the NFκB pathway is associated with the regulation of other cytokines or growth factors rather than COX-2 gene regulation and also that the IL-1β-induced transcriptional activation of the COX-2

gene is more dependent on activation of other transcriptional factors than on activation of NFκB. Further studies are needed to determine whether the different conditions in cell types and stimuli for induction of cytokines give rise to the different targets for the anti-inflammatory and anti-angiogenic effects of thalidomide.

The suppression of COX-2 mRNA stability by thalidomide is consistent with previous observations that thalidomide decreases the stability of TNF-α mRNA and COX-2 mRNA.[26,69] It is well documented that mRNA stability is an important factor in controlling gene expression,[70,71] and, although constitutive transcription of COX-2 may initiate unregulated expression of the enzyme in colon neoplasm,[34] growing evidence implicates mRNA stability and translational efficiency as central controls in COX-2 expression. [72,73]

Previous reports showed that a major regulatory point of COX-2 gene expression occurs at the post transcriptional level, and this control is mediated by the ARE-containing 3'UTR of the COX-2 mRNA.[53,54] Regulation of mRNA is dependent on both *cis* elements in the RNA and trans-acting factors. The best characterized *cis* element is the AU-rich element (ARE) within the 3' untranslated region (3'UTR) and target for rapid RNA degradation.[74,75] Based on their sequences and their effects on mRNA stability, three classes of AREs have been defined. Class I AREs contain scattered copies of the AUUUA sequence within a U-rich region. Class II AREs contain overlapping AUUUA motifs within a U-rich region. Class III AREs do not contain the AUUUA motif, but they are U-rich sequences.[74] AREs have been shown to be recognized by such RNA-binding proteins as HuR, AUF1, CUBP2, TIA-1, TIAR, and hnRNP, which can promote or suppress the stability of mRNAs. [51, 76-78]

This researcher focused our attention on HuR because this is the best

characterized ARE-binding protein, and its target mRNAs, including TNF, VEGF, p21, and COX-2, have been reported.[74] Over-expression of HuR increases the lifetime of many ARE-containing mRNAs, which suggests that HuR binding stabilizes mRNAs. [58,59,79] However, it is also possible that HuR over-expression stabilizes mRNAs by sequestering other proteins that decrease mRNA stability. Furthermore, altered expression of HuR promotes COX-2 expression in colon cancer cells, suggesting that dysregulation of these RNA-stabilizing factors can lead to over-expression of carcinogenic proteins.[78]

An interesting feature of HuR is that it contains sequences that allow shuttling between the nucleus and the cytoplasm.[56] It has been suggested that HuR binds mRNAs in the nucleus and then escorts the mRNAs to the cytoplasm where HuR protects them from degradation.[75] Several cellular stresses, including UV irradiation, heat shock, and inhibition of protein synthesis increase the cytoplasmic concentration of HuR suggesting that this shift plays a role in the regulation of mRNA stability.[74] We identify that thalidomide blocks the shuttling of HuR from nucleus to cytosol and this causes to destabilize COX-2 mRNA.

Some reports have showed that COX-2 mRNA stability is regulated by p38 MAPK in human monocytes and in HeLa cells.[80,81] Others have provided evidence of p38 MAPK-dependent COX-2 mRNA stabilization, operating through the p38 MAPK substrate, APK-2, and possibly mediated in part by the phosphorylation of the small heat shock protein hsp27.[51]

The p38-dependent stabilization of mRNA is sequence specific, as 3' UTR sequences derived from c-myc or TNFα destabilize the β-globin reporter transcript, but do not confer responsiveness to the p38 pathway [51]. Using a similar system, it has

recently been demonstrated that the p38 pathway regulates the stability of reporter transcripts containing IL-6, Il-8, c-fos, and GM-CSF AREs.[82] Thus, p38 is able to regulate the stability of a subset of mRNAs containing class I AREs or class II AREs, including COX-2 mRNA [51].

Here, this research showed that thalidomide inhibits IL-1β-induced p38 MAPK activation and that the p38 MAPK inhibitor SB203580 decreases COX-2 mRNA stability and blocks the IL-1β-induced translocation of HuR. In addition to p38 MAPK, other signaling pathways such as MAPK1/2, Ras, and the PI3K/Akt/PKB (protein kinase B) signaling pathway are known to lead to post-transcriptional regulation of COX-2 mRNA stability.[54,83,84] But, in our result, thalidomide did not inhibit IL-1β-induced ERK and JNK activation. Therefore, this research suggest that thalidomide blocks p38 MAPK mediated COX-2 mRNA stabilization.

Until now, although thalidomide had been shown to suppress the expression of COX-2 and TNF-α through the decreased stability of their mRNAs, the drug's mechanism involving RNA-binding proteins and signaling pathways has been unknown. Our findings demonstrate that inhibition of p38 MAPK and HuR translocation could be important mechanisms related to the destabilization of COX-2 mRNA by thalidomide.

V. CONCLUSION

Thalidomide has been shown to have both anti-inflammatory and anti-oncogenic properties. But, its action mechanism is poorly understood. This research present here our investigation of the molecular mechanism of thalidomide activity, particularly in NFκB and COX-2, a critical regulator of immune and inflammatory response.

In this results, thalidomide inhibited both IL-1β-induced IL-8 production and IL-1 β-induced NFκB dependent luciferase activity. In addition, nuclear translocation of NFκB and degradation of IκB after IL-1β stimulation were blocked by thalidomide. Thalidomide inhibits NIK dependent NFκB transcriptional activation. To sum up, these results indicate that anti-inflammatory property for thalidomide may be based on its ability to inhibit NFκB activation through suppression of NFκB activation through NIK dependent pathway.

This research also examined the effect of thalidomide on COX-2 expression which is overexpressed in cancer and chronic inflammatory disease because its regulation is essential in the suppression of tumor and inflammation. NFκB is thought to be a very important transcription factor on COX-2 expression and IL-1β -induced NFκB activation is inhibited by thalidomide. Therefore, we investigated the role of NFκB and the effect of thalidomide on COX-2 transcriptional activity. NFκB binding site is partially worked and not essential in IL-1β mediated COX-2 transcription. Although thalidomide did not inhibit the IL-1β-induced COX-2 transcription, it suppressed the induction of COX-2 protein and mRNA by IL-1β. Furthermore, thalidomide decreased the IL-1β-induced COX-2 mRNA stability in actinomycin D-treated experiments, and also significantly reduced IL-1β-induced p38 MAPK activation. And thalidomide suppresses changing subcellular localization of

HuR which is a mRNA stabilizing protein and nuclear shuttling protein. These data indicate that thalidomide downregulates COX-2 expression through p38 MAPK suppression and alteration of HuR localization.

Conclusively this research suggest that thalidomide contributes to the well-known anti-inflammatory and anti-angiogenic effects through the suppression of NFκB activation and destabilization of COX-2 mRNA by inhibiting p38 MAPK and changing HuR subcellular localization.

REFERENCES

1. Lenz WA. Short history of thalidomide embryopathy. Teratology 1988; 38(3):203-215.
2. Mcbride WG. Thalidomide embryopathy. Teratology. 1977;16(1):79-82.
3. Hauschild A, Kroeger H, Mitchison NA, Ugrinovic S, Zwingenberger K. Thalidomide therapy of established collagen-induced arthritis (CIA) not accompanied by an evident Th2 shift. Clin Exp Immunol.1997;108:428-431.
4. Oliver SJ, Freeman SL, Corral LG, Ocampo CJ, Kaplan G. Thalidomide analogue CC1069 inhibits development of rat adjuvant arthritis. Clin Exp Immunol.1999;118:315-321.
5. Sampaio EP, Kaplan G, Miranda A, Nery JA, Miguel CP, Viana SM *et al.* The influence of thalidomide on the clinical and immunologic manifestation of erythema nodosum leprosum. J Infect Dis. 1993;168:408-414.
6. Eisen T, Boshoff C, Mak I, Sapunar F, Vaugha MM, Pyle L, *et al.* Continuous low dose Thalidomide: a phase II study in advanced melanoma, renal cell, ovarian and breast cancer. Br J Cancer. 2000;82:812-817.
7. Calabrese L, Fleischer AB. Thalidomide: current and potential clinical applications. Am J Med. 2000;108:487-495.
8. Minchiton AI, Fryer KH, Wendt KR, Clow KA, Hayes MM. The effect of thalidomide on experimental tumors and metastases. Anticancer Drugs. 1996;7(3):339-343.
9. Rovelli A, Arrigo C, Nesi F, Balduzzi A, Nicolini B, Locasciulli A, *et al.* The role of thalidomide in the treatment of refractory chronic graft-versus-host disease following bone marrow transplantation in children. Bone Marrow Transplant. 1998;21(6):577-581.

10. Sauer H, Gunther J, Hescheler J, Wartenberg M. Thalidomide inhibits angiogenesis in embryoid bodies by the generation of hydroxyl radicals. Am J Pathol. 2000;156(1):151-158.

11 Zwingenberger K, Wnendt S. Immunomodulation by thalidomide: systematic review of the literature and of unpublished observations. J Inflamm 1995;46:177-211.

12. Sampaio EP, Sarno EN, Galilly R, Coh ZA, Kaplan G. Thalidomide selectively inhibits tumor necrosis factor alpha production by stimulated human monocytes. J Exp Med. 1991;173(3):699-703.

13. Tavares JL, Wangoo A, Dilworth P, Marshall B, Kotecha S, Shaw RJ. Thalidomide reduces tumour necrosis factor-alpha production by human alveolar macrophages. Respir Med. 1997;91(1):31-39.

14. Moller DR, Wysocka M, Greenlee BM, Ma X, Wahl L, Flockhart DA, *et al*. Inhibition of IL-12 production by thalidomide. J Immunol. 1997 ;159(10):5157-5161.

15 Corral LG, Haslett PA, Muller GW, Chen R, Wong LM, Ocampo CJ, *et al*. Differential cytokine modulation and T cell activation by two distinct classes of thalidomide analogues that are potent inhibitors of TNF-alpha. J Immunol. 1999;163(1):380-386.

16. D'Amato RJ, Loughnam MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci U S A. 1994;91(9):4082-4085.

17 Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, Suzuki H, *et al*. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. Mol Cell Biol. 1997l;17(7):4015-4023.

18. Joussen AM, Germann T, Kirchhof B. Effect of thalidomide and structurally related compounds on corneal angiogenesis is comparable to their teratological potency. Graefe's Arch. Clin. Exp. Ophthalmol. 1999;237: 952-961.

19. Mercurio F, Manning AM. Multiple signals converging on NF-kappaB. Curr Opin Cell Biol. 1999;11(2):226-232.

20. Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS Jr. NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol. 1999;19(8):5785-5799.

21. Mayo MW, Baldwin AS. The transcription factor NF-kappaB: control of oncogenesis and cancer therapy resistance. Biochim Biophys Acta. 2000 ;1470(2):M55-62

22 Grilli M, Chiu S, Lenardo.MJ. Nf-kB and Rel-participants in a multiform transcriptional regulatory system. Int.Rev Cytol. 1993;143:1-62.

23. Baeuerle P, Baltimore D. The physiology of NF-kB transcription factor. 1991 :409-432.

24. Jayne A. Keifer, Denis C, Guttridge, Brian P, Ashburner, *et al*. Inhibition of NF-kappa B activity by thalidomide through suppression of IkappaB kinase activity. J Biol Chem. 2001;276(25):22382-22387.

25. Jin SH. Kim TI., Han DS., Shin SG, Kim WH. Thalidomide suppresses the interleukin 1beta-induced NFkappaB signaling pathway in colon cancer cells. Ann N Y Acad Sci. 2002;973:414-418.

26. Fujita J, Mestre JR, Zeldis JB, Subbaramaiah K, Dannenberg AJ. Thalidomide and its analogue inhibit lipopolysaccharide-mediated induction of cyclooxygenase-2. Clin Cancer Res. 2001;7: 3349-3355.

27. Dewitt DL, EI-Harith EA, Kraemer SA, Andrews MJ, Yao EF, Armstrong RL, *et al*. The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. J Biol Chem. 1990;265(9):5192-5198.

28. Yokoyama C, Tanabe T. Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme. Biochem Biophys Res Commun. 1989;165(2):888-894.

29. Hla T, Neilson K. Human cyclooxygenase-2 cDNA. Proc Natl Acad Sci U S A. 1992;89(16):7384-7388.

30. Jones DA, Carlton DP, McIntyre TM, Zimmerman GA, Prescott SM. Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines. J Biol Chem. 1993;268(12):9049-9054.

31. smith WL, Langenbach R. Why there are two cyclooxygenase isozymes. J Clin Invest. 2001;107(12):1491-1495.

32. Brock TG, McNish RW, Peters-Golden M. Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E2. J Biol Chem. 1999;274(17):11660-11666.

33. Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. J Clin Invest. 2001;108(1):15-23.

34. Kutchera W, Jones DA, Matsunami N, Groden J, McIntyre TM, Zimmerman GA, *et al*. Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: evidence for a transcriptional effect. Proc Natl Acad Sci U S A. 1996;93(10):4816-4820.

35. Eberhart CE, Coffey RJ, Radhika A, Giadiello FM, Ferrenbach S, Dubois RN.

Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology. 1994;107(4):1183-1188.

36. Smith WL, DeWitt DL, Garavito RM Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem. 2000;69:145-82.

37. Hla T, Bishop-Bailey D, Liu CH, Schaefers HJ, Trifan OC. Cyclooxygenase-1 and -2 isoenzymes. Int J Biochem Cell Biol. 1999;31(5):551-557.

38. Caivano M, Gorgoni B, Cohen P, Poli V. The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta transcription factors. J Biol Chem. 2001;276(52):48693-48701.

39. Inoue H, Nanayama T, Hara S, Yokoyama C, Tanabe T. The cyclic AMP response element plays an essential role in the expression of the human prostaglandin-endoperoxide synthase 2 gene in differentiated U937 monocytic cells. FEBS Lett. 1994;350:51-54.

40. Mestre JR, Mackrell PJ, Rivadeneira DE, Stapleton PP, Tanabe T, Daly J M. Redundancy in the signaling pathways and promoter elements regulating cyclooxygenase-2 gene expression in endotoxin-treated macrophage/monocytic cells. J Biol Chem. 2001;276(6):3977-3982.

41. Xie W, Fletcher BS, Anderson RD, Herschman HR. V-src induction of the TIS10/PGS2 prostaglandin synthase gene is mediated by an ATF/CRE transcription response element. Mol Cell Biol 1994;14:6531-6539.

42. Thomas B, Berenbaum F, Humbert L, Bian H, Bereziat G, Crofford L, *et al*. Critical role of C/EBPdelta and C/EBPbeta factors in the stimulation of the cyclooxygenase-2 gene transcription by inerleukin-1 beta in articular chondrocytes. Eur J Biochem. 2000;267:6798-6809.

43. Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcriptional role of nuclear factor κB and nuclear factor interleukin-6 in the tumor necrosis factor α -dependent induction of cyclooxygenase-2 in MC 3T3-E1 cells. J Biol Chem. 1995; 270:31315-31320.

44 Inoue H, Tanabe T. Transcriptional role of the nuclear factor kappa B site in the induction by lipopolysaccharide and suppression by dexamethasone of cyclooxygenase-2 in U937 cells. Biochem Biophys Res Commun. 1998;244(1):143-148.

45. Kosaka T, Miyata A, Ihara H, Hara S, Sugimoto T, Takeda O, *et al*. Charaaterization of the human gene(PTGS2) encoding prostaglandin-endoperoxide synthase 2. Eur J Biochem 1994;221:889-897.

46. Schmedtje JF J, Ji YS, Liu WL, Dubois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. J Biol Chem. 1997;272(1):601-608.

47. Newton R, Hart LA, Stevens DA, Bergmann M, Donnelly LE, Adcock I M, *et al*. Effect of dexamethasone on interleukin-1beta-(IL-1beta)-induced nuclear factor-kappaB (NF-kappaB) and kappaB-dependent transcription in epithelial cells. Eur J Biochem. 1998;254(1):81-89.

48.Zhang Z, Sheng H, Shao J, Beauchamp RD, DuBois RN. Posttranscriptional regulation of cyclooxygenase-2 in rat intestinal epithelial cells. Neoplasia. 2000;2(6):523-530.

49. Sachs AB. Messenger RNA degradation in eukaryotes. Cell. 1993;74(3):413-421.

50. Faour WH, He Y, He QW, de Ladurantave N, Quintero M, Mancini A, *et al*. Prostaglandin E_2 Regulates the Level and Stability of Cyclooxygenase-2

mRNA through Activation of p38 Mitogen-activated Protein Kinase in Interleukin-1β-treated Human Synovial Fibroblasts. J Biol Chem. 2001;276: 31720-31731.

51. Lasa M, Mahtani KR, Finch A, Brewer G, Saklatvala J, Clark AR. Regulation of Cyclooxygenase 2 mRNA Stability by the Mitogen-Activated Protein Kinase p38 Signaling Cascade. Mol Cell Biol. 2000;20:4265-4274.

52. Shaw G, Kamen RA. Conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell. 1986;46(5):659-667.

53 Dixon DA, Kaplan CD, Mclntyre TM, Zimmerman GA, Prescott SM. Post-transcriptional control of cyclooxygenase-2 gene expression. The role of the 3'-untranslated region. J Biol Chem. 2000;275(16):11750-11757.

54 Sheng H, Shao J, Dixon DA, Williams CS, Prescott SM, DuBois RN, *et al.* Transforming growth factor-beta1 enhances Ha-ras-induced expression of cyclooxygenase-2 in intestinal epithelial cells via stabilization of mRNA. J Biol Chem. 2000;275(9):6628-6635.

55. Peng SS., Chen CY, Xu N, Shyu AB. RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. EMBO J. 1998;17(12):3461-3470.

56. Fan XC, Steitz JA HNS, a nuclear-cytoplasmic shuttling sequence in HuR. Proc Natl Acad Sci USA. 1998;95(26):15293-15298.

57. Wang W, Furneaux H, Cheng H, Caldwell MC, Hutter D, Liu Y, *et al.* HuR regulates p21 mRNA stabilization by UV light. Mol Cell Biol. 2000;20(3):760-769.

58. Wu D, Marko M, Claycombe K, Paulson KE, Meydani SN.

Ceramide-induced and age-associated increase in macrophage COX-2 expression is mediated through up-regulation of NF-kappa B activity. J Biol Chem. 2003;278(13):10983-10992.

59. Yeo SJ, Gravis D, Yoon JG, and Yi AK. Myeloid differentiation factor 88-dependent transcriptional regulation of cyclooxygenase-2 expression by CpG DNA: role of NF-kappaB and p38. J Biol Chem. 2003;278(25):22563-22573.

60. Liu W, Reinmuth N, Stoeltzing O, Parikh AA, Tellez C, Williams S, *et al*. Cyclooxygenase-2 is up-regulated by interleukin-1 beta in human colorectal cancer cells via multiple signaling pathways. Cancer Res. 2003;63(13):3632-3636.

61. Charalambous MP, Maihofner C, Bhambra U, Lightfoot T, Gooderham N J. Upregulation of cyclooxygenase-2 is accompanied by increased expression of nuclear factor-kappa B and I kappa B kinase-alpha in human colorectal cancer epithelial cells. Br J Cancer. 2003;88(10):1598-1604.

62. Stephens TD, Fillmore BJ. Hypothesis: thalidomide embryopathy-proposed mechanism of action. Teratology. 2000;61(3):189-195.

63. Kenyon BM, Browne F, D'Amato RJ. Effects of thalidomide and related metabolites in a mouse corneal model of neovascularization. Exp Eye Res. 1997;64(6):971-978.

64. Siebenlist UG, Franzoso, Brown K. Structure, regulation and function of NF-kB. Annu Rev. cell Biol 1994;10:405-455.

65. May MJ, Ghosh S. Signal transduction through NF-kappa B. Immunol Today. 1998;19(2):80-88.

66. DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A

cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature. 1997;388(6642):548-554.

67. Whiteside ST, Israel A. I kappa B proteins: structure, function and regulation. Semin Cancer Biol. 1997;8(2):75-82.

68. Karin M. The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. J Biol Chem. 1999;274(39):27339-27342.

69. Moreira AL, Sampaio EP, Zmuidzinas A, Frindt P, Smith KA, Kaplan G. Thalidomide exerts its inhibitory action on TNFby enhancing mRNA degradation. J Exp Med 1993;177:1675-1680.

70. Caponigro G, Parker R. Mechanisms and control of mRNA turnover in Saccharomyces cerevisiae. Microbiol Rev. 1996;60:233-249.

71. McCarthy JE. Posttranscriptional control of gene expression in yeast. Microbiol Mol Biol Rev. 1998;62:1492-1553.

72. Caput D, Beutler B, Hartog K, Thayer R, Brown-shimer S, Cerami A. Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. Proc Natl Acad Sci USA. 1986;83(6):1670-1674.

73. Ristimaki A, Garfinkel S, Wessendorf J, Maciag T, Hla T. Induction of cyclooxygenase-2 by interleukin-1. J. Biol. Chem. 1994;269:11769-11775.

74. Brennan CM, Steitz JA. HuR and mRNA stability. Cell Mol Life Sci. 2001;58(2):266-277.

75. Gallouzi IE, Brennan CM, Stenberg MG, Swanson MS, Eversole A, Maizels N, et al. HuR binding to cytoplasmic mRNA is perturbed by heat shock. Proc Natl Acad Sci USA. 2000;97(7):3073-3078.

76. Cok SJ, Acton SJ, Morrison AR. The proximal region of the 3'-untranslated

region of cyclooxygenase-2 is recognized by a multimeric protein complex containing HuR, TIA-1, TIAR, and the heterogeneous nuclear ribonucleoprotein U. J. Biol. Chem. 2003;278: 36157-36162.

77. Anant S, Henderson JO, Mukhopadhyay D, Navaratnam N, Kennedy S, Min J, Davidson NO. Novel role for RNA-binding protein CUGBP2 in mammalian RNA editing.CUGBP2 modulates C to U editing of apolipoprotein B mRNA by interacting with apobec-1 and ACF, the apobec-1 complementation factor. J Biol Chem. 2001;276: 47338 47351.

78. Dixon DA, Tolley ND, King PH, Nabors LB, McIntyre TM, Zimmerman GA, Prescott SM. Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. J Clin Invest. 2001;108: 1657-1665.

79. Levy NS, Chung S, Furneaux H, Levy AP. Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J Biol Chem. 1998;273(11):6417-6423.

80. Dean JL, Brook M, Clark AR, Saklatvala J. p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. J Biol Chem. 1999;274(1):264 -269.

81. Ridley SH, Dean JL, Sarsfield SJ, Brook M, Clark AR, Saklatvala J. A p38 MAP kinase inhibitor regulates stability of interleukin-1-induced cyclooxygenase-2 mRNA. FEBS Lett. 1998;439(1-2):75-80.

82. Whitmarsh AJ, Yang SH, Su MS, Sharrocks AD, Davis RJ. Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. Mol Cell Biol. 1997;17(5):2360-2371.

83. Xu K, Robida AM, Murphy TJ. Immediate-early MEK-1-dependent stabilization of rat smooth muscle cell cyclooxygenase-2 mRNA by Galpha(q)-coupled receptor signaling. J Biol Chem 2000;275:23012-23019.

84. Sheng H, Shao J, DuBois RN. K-Ras-mediated increase in cyclooxygenase-2 mRNA stability involves activation of the protein kinase B1. Cancer Res. 2001;61:2670-2675.

ABSTRACT(IN KOREAN)

Thalidomide의 작용기전: IL-1β에 의한 NFκB 활성화와 cyclooxygenase-2 mRNA 안정화의 억제

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진 수 현

진정제이며 구토 억제제인 thalidomide는 부작용으로 인하여 그 사용이 중지되었으나 항염증및 항종양 효과가 알려지면서 다시 그 유용성이 알려지고 있다. Thalidomide의 항염증성 효과는 사이토카인 발현을 억제하는 것과 관련이 있고 항종양적 효과는 신생혈관생성을 억제함으로써 나타나는 것으로 알려져 있다. 그러나 thalidomide의 세포내 표지물과 작용기전은 완전히 이해되고 있지는 않다. NFκB와 cyclooxygenase (COX)-2 는 면역 및 염증반응과 암화과정의 결정적인 조절인자로 알려져 있다. 따라서 본 연구에서는 thalidomide의 항염증 및 항종양효과의 분자적 기전을 NFκB 신호전달계, NFκB에 의해 매개되는 COX-2 발현, 그리고 COX-2 발현의 후전사적 단계를 중심으로 조사하였다.

본 연구 결과에서 thalidomide는 IκB kinase 활성을 억제하며, NIK 의존적인 신호전달계 억제를 통하여 NFκB 활성을 억제하였다. 이러한 결과는 thalidomide의 작용효과가 NFκB 활성을 억제할 수 있는 능력에 기초를 두고 있다는 사실을 제시한다

Prostaglandin의 유도합성 효소인 COX-2는 여러 종양과 만성적 염증질병

에서 과발현되어 있으므로 COX-2 발현의 조절은 종양과 염증을 억제함에 있어서 중요한 요인이된다. COX-2 발현에는 NFκB를 포함한 여러 전사인자와 함께 전사 후 단계의 조절이 중요한 조절인자로 작용한다.

따라서 본 연구의 다음 단계로서 thalidomide에 의한 COX-2 발현 조절에서 앞선 결과인 NFκB 억제작용과 관련이 있는지 알아보고 전사 후 단계에서 RNA 결합 단백질인 HuR과 MAPK 활성의 관련성을 조사하였다. COX-2 promoter deletion series를 이용한 reporter assay실험을 통해서 thalidomide가 COX-2 전사조절에는 영향을 주지 않음을 확인하였다. 그러나 후전사적 단계에서, thalidomide 와 p38 MAPK 억제제인 SB203580는 actinomycin D 처리 후 관찰한 COX-2 mRNA의 분해를 증가시켰으며, thalidomide는 또한 p38 MAPK 활성을 억제하였다. 그리고 thalidomide와 p38 MAPK 억제제 처리는 mRNA 안정화 단백질인 HuR의 세포내 위치를 변화시켜 세포질로의 이동을 억제하였다. 이러한 결과는 thalidomide가 p38 MAPK 활성억제와 HuR 발현위치의 변화를 통해서 COX-2 발현을 조절할 수 있음을 제시한다. 이상과 같이 thalidomide에 의한 NFκB와 COX-2에 대한 작용은 각각 전사 및 전사 후 단계에서 독립적으로 작용하는 기전으로서 thalidomide의 항염증 및 항종양 효과로서 광범위한 작용기전의 특성을 이해하는데 도움을 줄 것이다.

핵심되는 말 : Thalidomide, Cyclooxygenase-2(COX-2), NFκB, MAPK, HuR
신생혈관생성, 염증, mRNA 안정화