Thalidomide destabilizes cyclooxygenase-2 mRNA by inhibiting p38 mitogen-activated protein kinase and cytoplasmic shuttling of HuR

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Abstract

We investigated the effect of thalidomide on transcriptional and post-transcriptional cyclooxygenase-2 (COX-2) expression, including a pathway leading to COX-2 mRNA destabilization. We found that thalidomide inhibited the interleukin-1β (IL-1β)-mediated induction of COX-2 protein and mRNA in Caco-2 cells. Transient transfection with a COX-2 promoter construct demonstrated that thalidomide did not affect IL-1β-induced transcriptional activation of COX-2, although it did decrease the stability of COX-2 mRNA and suppress IL-1β-induced cytoplasmic shuttling of an mRNA stabilizing protein, HuR. Thalidomide also suppressed IL-1β-induced p38 mitogen-activated protein kinase (MAPK) activation, while a p38 MAPK inhibitor destabilized COX-2 mRNA and the cytoplasmic shuttling of HuR induced by IL-1β. These data suggest that one of the molecular mechanisms of thalidomide may be destabilization of COX-2 mRNA through inhibition of cytoplasmic shuttling of HuR and p38 MAPK.

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1. Introduction

Thalidomide (N-phthalimidoglutarimide) was introduced as a sedative and anti-nausea medication, but its use was discontinued because it was linked to serious congenital birth defects. Nevertheless, thalidomide is now recognized as a clinically effective drug for its anti-angiogenic and anti-inflammatory properties. Diseases including inflammatory bowel disease, erythema nodosum leprosum, rheumatoid arthritis, multiple myeloma, and cancer are currently being treated with thalidomide, although its mechanism of action remains unclear. The anti-angiogenic properties (D’Amato et al., 1994; Kenyon et al., 1997) and T-cell co-stimulatory activity (Barlett et al., 2004) of thalidomide have both been demonstrated as anti-cancer mechanisms of the drug, which has been reported to inhibit basic fibroblast growth factor (bFGF)-induced angiogenesis (D’Amato et al., 1994) and tumor growth in animals (Verheul et al., 1999) and human cancer patients (Little et al., 2000; Singhal et al., 1999). The potent anti-inflammatory activity of thalidomide has been shown to suppress lipopolysaccharide-induced production of tumor necrosis factor-α (TNF-α) (Sampaio et al., 1991; Tavares et al., 1997) and interleukin-12 (IL-12) (Corral et al., 1999; Moller et al., 1997).

Transcription factor NFκB, a critical regulator of inflammatory genes and cell proliferation, is inhibited by thalidomide (Keifer et al., 2001). We have also previously reported that thalidomide inhibits interleukin-1β (IL-1β)-induced interleukin-8 (IL-8) production, nuclear translocation of NFκB, and degradation of inhibitor of NFκB (IκB) α (Jin et al., 2002). In addition, thalidomide inhibits lipopolysaccharide-mediated induction of cyclooxygenase-2 (COX-2), decreasing the stability of COX-2 (Fujita et al., 2001). Despite these findings, the mechanism of thalidomide’s effects on the expression of inflammatory and angiogenic molecules is not fully understood.

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Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to prostaglandin endoperoxide and has two isoforms, which are constitutively expressed COX-1 (DeWitt et al., 1990; Yokoyama and Tanabe, 1989) and mitogen-inducible COX-2 (Hla and Neilson, 1992; Jones et al., 1993). COX-2 is over-expressed in sites of inflammation and many types of tumor tissue (Eberhart et al., 1994; Kargman et al., 1993; Thomas et al., 2000; Yokoyama and Tanabe, 1989) and mitogen-conditioned medium (Hla et al., 1999; Smith et al., 2000). Several mechanisms are involved in regulating COX-2 expression and involve multiple mechanisms in different cell types and conditions (Hla et al., 1999; Smith et al., 2000). Regulation of COX-2 expression is complex and appears to involve multiple mechanisms in different cell types and conditions (Hla et al., 1999; Smith et al., 2000). 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 (Inoue et al., 1994; Inoue and Tanabe, 1998; Morris, 1996; Sirosis and Richards, 1993; Thomas et al., 2000; Xie et al., 1994; Yamamoto et al., 1995). COX-2 expression is also regulated at the post-transcriptional level of mRNA instability (Faour et al., 2001; Lasa et al., 2000). The 3′-untranslated region of COX-2 mRNA contains multiple copies of adenylyl- and uridylyl-rich (AU-rich) elements composed of the pentameric “AUUUUA” mRNA instability motif, which is a target for rapid mRNA degradation and has been implicated in determining the instability of COX-2 mRNA (Caput et al., 1986; Shaw and Kamen, 1986). It is also known that some proteins specifically bind to the ARE region and regulate mRNA stability. Among these, Hu antigen R (HuR), a mammalian homologue of the Drosophila embryonic lethal abnormal vision (ELAV)-like family of mRNA binding proteins, is a relatively well-characterized molecule associated with stabilization and shuttling of several target mRNAs containing AU-rich element II (Peng et al., 1998) between the nucleus and cytoplasm. Furthermore, signaling pathways elicited by extracellular stimuli could mediate the induction of COX-2 expression at post-transcriptional levels. Among the multiple signaling pathways involved in the induction of COX-2, p38 mitogen-activated protein kinase (MAPK) is well established as an important pathway in the post-transcriptional regulation of COX-2 mRNA stability (Jang et al., 2000; Lasa et al., 2000; Ridley et al., 1998).

In this study, we investigated whether thalidomide could suppress COX-2 expression at the transcriptional and post-transcriptional levels, and destabilize COX-2 mRNA through control of p38 MAPK and HuR.

2. Materials and methods

2.1. Cell culture

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

2.2. Materials

Thalidomide was purchased from Sigma (St. Louis, MO) and dissolved in sterile DMSO. IL-1β was obtained from R&D (Minneapolis, MN), while HuR-specific antibody and phospho-p38 MAPK specific antibody were purchased from Santa Cruz (Santa Cruz, CA). Lipofectamine-plus reagents were from Gibco-BRL (Rockville, MD), luciferase assay kits from Promega (Mannheim, Germany), and COX-2 promoter deletion constructs from Professor Won Jae Lee (Ewha Womans University, Seoul, Korea).

2.3. Transfection and luciferase assays

Caco-2 cells were plated on 6-well plates, grown to 60% confluence, and transfected with the Lipofectamine-Plus reagent and 0.5 μg of COX-2 promoter-Luc reporter plasmids harboring serial deletions. The medium was changed to complete medium after 6 h of transfection and then, after 18 h, 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 8 h. Cells were harvested and lysed in lysis buffer containing 25-mM Tris–phosphate (pH 7.8), 2-mM DTT, 2-mM 1,2-diaminocyclohexane-N,N,N′,N′,-tetraacetic acid, 10% glycerol, and 1% Triton X-100 for 5 min at room temperature. Luciferase activity was measured in a 20-μl cellular extraction using a luciferase assay system (Promega) and MicroLumat LB 96P luminometer (EG&G Berthold, Australia). All transfections were normalized for β-gal expression.

2.4. 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 MgCl2, 1-mM EDTA, 0.5-mM dithiothreitol (DTT), 1-mM phenylmethanesulfonyl fluoride (PMSF), 20-μg/ml leupeptin, 5-μg/ml pepstatin, and 0.5% NP-40. Lysates were incubated on ice for 30 min before centrifugation at 12,000 g for 10 min. The supernatants containing total cell proteins were transferred to clean tubes, and the proteins were diluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Following SDS-PAGE, proteins were electrophoretically transferred to 0.2-μm PVDF membranes (Millipore, Bedford, MA) during 1 h at 350 mA using a Tank transfer system (Bio-Rad, CA). The membranes lodging the transferred proteins were blocked with 5% non-fat milk in TBST (0.1-M Tris, pH 7.4, 0.9% NaCl, 0.05% Tween 20) for 30 min at room temperature, probed with specific primary antibody, and visualized by enhanced chemiluminescence with horseradish peroxidase (HRP)-conjugated secondary antibody.

2.5. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

5 × 106 cells per sample were collected at the indicated time points and frozen at −80 °C. Total RNA was extracted using
TRIzol reagent (Gibco-BRL) as per manufacturer’s instructions. RNA was simultaneously prepared from all samples in each experiment, quantitated, and used as template (10 μg) for reverse transcription (Promega). Ten percent of the resulting cDNA from each sample was subjected to 30 cycles of PCR consisting of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C in a 20-μl reaction mixture in a PCR-premix kit (Bioneer, Seoul, Korea). The PCR primers for COX-2 were 5′-CAG-CAAATCCTTGCTGTTCC-3′ for the forward primer and 5′-TGGGCAAAGAATGCAAACATC-3′ for the reverse primer.

2.6. Measurement of mRNA degradation

Caco-2 cells were stimulated with IL-1β (1 ng/ml) for 6 h, followed by a 30 min pretreatment with 1-mM thalidomide and SB20358 in order to measure the extent of mRNA degradation. Actinomycin D (5 μg/ml) was then added to the medium and incubation continued for the indicated times. Cells were harvested, and RT-PCR for COX-2 mRNA was performed. (B) Relative levels of COX-2 mRNA expression were determined by densitometric scanning of the bands and normalized to β-actin mRNA. Amounts are expressed as a relative percentage prior to the addition of actinomycin D. Values are presented as means±S.D. of four independent trials.

⁎ corresponds to P<0.05 versus treatment with IL-1β alone.

2.7. Immunofluorescence stain

Caco-2 cells (1×10⁵) were seeded in 4-chamber slides. Cells were incubated with or without thalidomide (1 mM) and/or IL-1β (1 ng/ml) for 6 h. After stimulation for 1 h, cells were fixed in 4% paraformaldehyde at 4 °C for 10 min. After three washes with PBS, cells were permeabilized using a 1:1 mixture of acetone and methanol for 1 min and washed a further three times with PBS. Anti-HuR antibodies (1:150) were added for overnight incubation at 4 °C. After three PBS washes, FITC-conjugated secondary antibodies were added (1:300) for 1 h at

CAATCCTTGCTTGCC-3′ for the forward primer and 5′-TGGGCAAAGAATGCAAACATC-3′ for the reverse primer.

Fig. 1. Thalidomide inhibits IL-1β-induced COX-2 mRNA and protein expression in Caco-2 cells. (A) Caco-2 cells were pre-treated with various concentrations of thalidomide (0, 0.1, 0.2, 0.5, 1.0 mM) for 30 min and then treated with 1 ng/ml of IL-1β. After 16 h, whole-cell proteins were obtained and Western blot was performed for COX-2 and β-actin using specific antibodies. (B) Caco-2 cells were pre-treated with thalidomide (1 mM) or the p38 MAPK inhibitor SB203580 (10 μM) for 30 min and then treated with 1 ng/ml of IL-1β for 6 h. Total RNA was isolated and examined by RT-PCR for COX-2 mRNA.

Fig. 2. Thalidomide does not affect IL-1β-induced transcriptional activation of COX-2 in Caco-2 cells. Caco-2 cells were transiently transfected with COX-2 promoter deletion constructs (d440, full construct; d1650, 2-kb site deletion; d1800, all cis element deletion) and a β-gal construct. Cells were pre-treated with or without 1 mM thalidomide for 30 min and then stimulated with 1 ng/ml IL-1β for 6 h. Luciferase activities were measured in cell lysates and normalized to β-gal activities. Values are presented as means±S.D. of three independent trials.

Fig. 3. Thalidomide and p38 MAPK inhibitor decrease IL-1β-induced stability of COX-2 mRNA. (A) Caco-2 cells were pre-treated with or without 1 mM thalidomide or SB203580 for 30 min and stimulated with 1 ng/ml IL-1β for 6 h. Actinomycin D (ActD, 5 μg/ml) was then added to the medium and incubation continued for the indicated times. Cells were harvested, and RT-PCR for COX-2 mRNA was performed. (B) Relative levels of COX-2 mRNA expression were determined by densitometric scanning of the bands and normalized to β-actin mRNA. Amounts are expressed as a relative percentage prior to the addition of actinomycin D. Values are presented as means±S.D. of four independent trials.

* corresponds to P<0.05 versus treatment with IL-1β alone.
room temperature. After final washing with PBS, slides were scanned using a confocal microscope (TCSNT, LEICA, Switzerland).

2.8. Statistical analyses

Each data value is expressed as the mean ± S.D. Data were analyzed by Student’s t-test for multiple variable comparison. A P value of less than 0.05 is considered significant.

3. Results

3.1. Thalidomide inhibits the IL-1β-induced expression of COX-2 mRNA and protein

We determined the effects of thalidomide on COX-2 gene expression at the mRNA and protein expression levels. RT-PCR and Western blot analysis showed that thalidomide inhibits COX-2 protein expression in a dose-dependent manner (Fig. 1A), and thalidomide and the p38 MAPK inhibitor SB203580 effectively suppress the IL-1β-induced expression of COX-2 mRNA (Fig. 1B).

3.2. Thalidomide does not inhibit the IL-1β-induced transcriptional activation of the COX-2 gene

As thalidomide suppressed IL-1β-induced NFκB activation in our previous work (Jin et al., 2002), we supposed that thalidomide may inhibit COX-2 gene expression through NFκB suppression. We investigated the role of NFκB and the effect of thalidomide on COX-2 transcriptional activity using a reporter assay and a deletion series of COX-2 promoter constructs. Deletion of NFκB sites in the COX-2 promoter constructs induced significant suppression of the transcriptional activity of the COX-2 promoter. Conversely, thalidomide did not affect IL-1β-induced transcriptional activation of the COX-2 promoter (Fig. 2). Our findings suggest that thalidomide suppresses the expression of COX-2 mRNA and protein without inhibiting COX-2 gene transcription.

3.3. Thalidomide and the p38 MAPK inhibitor SB203580 decrease the IL-1β-induced stability of COX-2 mRNA

We next investigated the effect of thalidomide on COX-2 post-transcriptional activity. Measuring mRNA degradation by RT-PCR after actinomycin D treatment, we found that pre-treatment with thalidomide induced more rapid degradation of COX-2 mRNA than did IL-1β treatment alone (Fig. 3). In addition, because the activation of p38 MAPK stabilizes mRNA, we tested the effect of pre-treatment with a p38 MAPK inhibitor, SB203580, as had been done with thalidomide. As shown in Fig. 3, SB203580 increased COX-2 mRNA degradation as thalidomide had. We suggest that thalidomide suppresses COX-2 expression via mRNA degradation.

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destabilization, which is related to the inhibition of p38 MAPK activation.

3.4. Thalidomide suppresses IL-1β-induced p38 MAPK activation

In order to show that thalidomide inhibits mRNA stabilization through p38 MAPK inhibition, we examined the effect of thalidomide on p38 MAPK activity by Western blotting. As shown in Fig. 4, IL-1β-induced phosphorylation of p38 MAPK was suppressed by thalidomide in a dose-dependent manner. Thus, we suppose that thalidomide inhibits COX-2 mRNA stabilization through p38 MAPK inhibition.

3.5. Thalidomide and the p38 MAPK inhibitor SB203580 suppress IL-1β-induced cytoplasmic localization of HuR

To investigate the effect of thalidomide on HuR, an mRNA stabilizing protein, we examined subcellular localization of HuR by immunofluorescence staining. As shown in Fig. 5, IL-1β treatment induced a shift in HuR expression from the nucleus to the cytoplasm, with maximal shift at 60 min. Pretreatment with thalidomide or SB203580 blocked IL-1β-induced cytoplasmic translocation of HuR, suggesting that thalidomide may inhibit the cytoplasmic translocation of HuR through inhibition of p38 MAPK activation.

4. Discussion

Although thalidomide is known to have anti-inflammatory and anti-cancer effects (Stephens and Fillmore, 2000) and its clinical role is expanding, the molecular mechanisms of the drug are not clearly known. Our interest in the action mechanism of thalidomide motivated our previous work showing that it inhibits IL-1β-induced IL-8 production and suppresses IL-1β-induced NFκB activation by inhibiting the NFκB-inducing kinase (NIK) downstream pathway (Jin et al., 2002). It has also been demonstrated that inhibition of NFκB activity by thalidomide occurs through the suppression of IκB kinase activity (Keifer et al., 2001). Based on these findings, we 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 carcinogenesis.

Our first inquiry was whether thalidomide suppressed COX-2 expression, and our investigation showed that thalidomide inhibits IL-1β-induced expression of COX-2 mRNA and protein. Considering the presence of NFκB binding sites in the promoter region of the COX-2 gene, we attempted to demonstrate the effect of thalidomide on transcription of the COX-2 gene using transient transfection of Caco-2 cells with COX-2 promoter constructs. We found that IL-1β-induced transcriptional activation of COX-2 was unaffected by thalidomide, even though thalidomide did suppress the stability of COX-2 mRNA. In our experimental setting, the concentration of thalidomide to inhibit the IL-1β-induced COX-2 expression is relatively high. Therefore, we did the same experiments using RAW 264.7 cells, inflammatory cell line, which showed that 25–50 μM of thalidomide was enough to inhibit the IL-1β-induced COX-2 induction (data not shown). We think that the concentration of thalidomide to suppress the COX-2 induction depends on the cell types and the inflammatory cells may be more sensitive than epithelial cells in the effect of thalidomide on COX-2 expression.

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 (Jin et al., 2002; Keifer et al., 2001), 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-cancer effects of thalidomide.

The suppression of COX-2 mRNA stability by thalidomide is consistent with previous observations that thalidomide decreased the stability of TNF-α mRNA and COX-2 mRNA (Fujita et al., 2001; Moreira et al., 1993). It is well documented that mRNA stability is an important factor in controlling gene expression (Caponigro and Parker, 1996; McCarthy, 1998), and, although constitutive transcription of COX-2 may initiate unregulated expression of the enzyme in colon neoplasm (Kutchera et al., 1996), growing evidence implicates mRNA stability and translational efficiency as central controls in COX-2 expression (Caput et al., 1986; Ristimaki et al., 1994). Some studies have shown that a major regulatory point of COX-2 gene expression occurs at the post-transcriptional level, with this control mediated by the ARE-containing 3′-untranslated region of COX-2 mRNA (Dixon et al., 2000; Sheng et al., 2000).

The AU-rich elements are well-characterized mRNA target sequences that interact with various RNA binding proteins and signaling pathways for post-transcriptional regulation of gene expression. AU-rich elements 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 (Anant et al., 2001; Cok et al., 2003; Dixon et al., 2001; Lasa et al., 2000). HuR is the best characterized AU-rich element-binding protein, and its target mRNAs, including TNFα, vascular endothelial growth factor (VEGF), p21, and COX-2, have been reported (Brennan and Steitz, 2001). Over-expression of HuR increases the lifetime of many AU-rich element-containing mRNAs, which suggests that HuR binding stabilizes mRNAs (Fan and Steitz, 1998; Levy et al., 1998; Wang et al., 1998, 2000). It is also possible, however, 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 (Dixon et al., 2001).
Another interesting feature of HuR is that it contains sequences that allow its shuttling between the nucleus and cytoplasm (Fan and Steitz, 1998). It has been suggested that HuR binds mRNAs in the nucleus and escorts them to the cytoplasm, where HuR protects them from degradation (Gallouzi et al., 2000). Several cellular stresses, including UV irradiation, heat shock, amino acid deprivation, and inhibition of protein synthesis, increase the cytoplasmic concentration of HuR, indicating that this shift plays a role in the regulation of mRNA stability (Brennan and Steitz, 2001). In fact, it has been shown that the increased cytoplasmic concentration of HuR in UV-treated cells correlates well with increased AU-rich element-mediated mRNA stability (Wang et al., 2000). From our immunofluorescence study using anti-HuR antibodies, we found that IL-1β-induced translocation of HuR from the nucleus to cytoplasm was blocked by thalidomide. This suggests that thalidomide may inhibit the RNA-stabilizing effect of HuR by reducing cytoplasmic expression of the protein.

Some authors have reported that COX-2 mRNA stability is regulated by p38 MAPK in some cell types and conditions (Dean et al., 1999; Frelval et al., 2003; Lasala et al., 2001; Ridley et al., 1998). Others have provided evidence of p38 MAPK-dependent COX-2 mRNA stabilization operating through the p38 MAPK substrate, MAPK-activated protein kinase-2, in sequence specific manner (Lasala et al., 2000). Recently, using cDNA array, it has been demonstrated that both the p38 MAPK-dependent and -independent signaling are associated with the stability of a lot of AU-rich element-containing transcripts including cytokines (Frelval et al., 2003). Furthermore, it has been shown that anti-inflammatory glucocorticoid such as dexamethasone destabilizes COX-2 mRNA by inhibiting p38 MAPK (Lasala et al., 2001). Here, we 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 (Sheng et al., 2000, 2001; Xu et al., 2000). Further assessment of these pathways as target molecules of thalidomide’s effects is needed.

Until now, although thalidomide has 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.

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