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Triptolide inhibits COX-2 expression by regulating mRNA stability in TNF-α-treated A549 cells

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ABSTRACT

Cyclooxygenase-2 (COX-2) over-expression is frequently associated with human non-small-cell lung cancer (NSCLC) and involved in tumor proliferation, invasion, angiogenesis and resistance to apoptosis. In the present study, the effects of triptolide on COX-2 expression in A549 cells were investigated and triptolide was found to inhibit TNF-α-induced COX-2 expression. In our further studies, it was found that triptolide decreased the half-life of COX-2 mRNA dramatically and that it inhibited 3′-untranslated region (3′-UTR) fluorescence reporter gene activity. Meanwhile, triptolide inhibited the HuR shuttling from nucleus to cytoplasm. After triptolide treatment, decreased COX-2 mRNA in pull-down experiments with anti-HuR antibodies was observed, indicating that the decreased cytoplasmic HuR is responsible for the decreased COX-2 mRNA. Taken together, our results provided evidence for the first time that triptolide inhibited COX-2 expression by COX-2 mRNA stability modulation and post-transcriptional regulation. These results provide a novel mechanism of action for triptolide which may be important in the treatment of lung cancer.

1. Introduction

Tripterygium wilfordii Hook.f, a member of the Celastraceae family of plants, has been used as a traditional Chinese medicine for centuries. Triptolide, a diterpenoid, was first isolated from *T.* wilfordii Hook.f and structurally characterized in 1972 [1] and has been used for the treatment of a variety of autoimmune and inflammatory diseases including rheumatoid arthritis [2]. Recent explorations of triptolide revealed many properties relevant to its anti-inflammatory and anticancer activities. Many in vitro and in vivo studies tried to elucidate the anticancer mechanism of triptolide; however, conclusions have been inconsistent. Triptolide exerts multiple effects on apoptosis [3], angiogenesis [4], metastasis [5] and drug-resistance [6]. For example, triptolide inhibits tumor metastasis, reducing basal and stimulated colon cancer cell migration and decreasing the expression of VEGF and COX-2 [7].

Cyclooxygenase (COX) is a rate-limiting enzyme that catalyzes the conversion of arachidonic acid to prostaglandins. COX-1 is constitutively expressed in most cells and tissues, but COX-2 is undetectable in most normal tissues and expressed in response to inflammatory stimuli, such as cytokines interleukin-1β and tumor necrosis factor-α (TNF-α) [8]. In normal cells, COX-2 mRNA is not only transcribed at a very low rate, but also degraded rapidly [9]. More recently, it has been found that COX-2 is elevated through both increased transcription rate and higher levels of mRNA stability [10] in tumor proliferation and invasion angiogenesis [11]. COX-2 is one of the targets under investigation for lung cancer therapy and chemoprevention [12]. Treatment with chemotherapeutic agents such as taxanes induces COX-2 expression [13]. A distinct benefit of combining COX-2 inhibitors with chemotherapy in patients is the possibility of limiting chemotherapy-induced COX-2 expression in tumor cells [14].

Many previous studies have documented that triptolide could reduce COX-2 expression. Triptolide has been shown to suppress the induction of COX-2 activity and PGE2 production in lipopolysaccharide (LPS)-treated microglia by suppressing the activity of NF-κB and JNK [15]. Triptolide also impairs dendritic cell migration by inhibiting COX-2 and CCR7 expression through NF-κB and phosphatidylinositol-3 kinase (PI3-K)/Akt pathway [16]. Previous papers have been focused on the transcriptional regulation of COX-2, which is mainly related to the NF-κB pathway. In this study, we investigated whether triptolide inhibits the proliferation of lung cancer cells by regulating the mRNA stability of COX-2. The mechanisms underlying COX-2 mRNA destabilization were further examined, and we found that triptolide destabilized COX-2 mRNA by decreasing the binding of the mRNA stabilization factor HuR to COX-2 mRNA. Our results provide the first evidence that triptolide can alter post-transcriptional mechanisms.

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2. Material and methods

2.1. Materials

Reagents were obtained as follows: PretoBlue™ Cell Viability Reagent and TRIzol were purchased from Invitrogen. Goat polyclonal mouse COX-2, mouse monoclonal HuR, lamin B and actin antibodies were from Santa Cruz Biotechnology, Inc. Recombinant human TNF-α was purchased from GIBCO and actinomycin D from Sigma.

2.2. Cell culture, TNF-α, and triptolide treatment

A549 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were starved for 24 h in a medium containing 0.1% FBS before treatment with triptolide. The concentration of DMSO in cell cultures was less than 0.1%.

2.3. Western blot analysis

Whole cell lysate was prepared using lysis buffer containing 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, and protease and phosphatase inhibitors. Nuclear and cytosolic extracts were isolated as previously described [17]. Proteins were resolved on 10% SDS–PAGE gels under reducing conditions followed by electrophoretic transfer onto nitrocellulose membranes. Immunoblots were incubated with primary antibodies against COX-2, HuR, lamin B or β-actin at 4 °C overnight. Immunoreactive bands were detected using HRP-conjugated secondary antibodies with Western Lightning Chemiluminescence Plus reagent.

2.4. RNA extraction and real-time PCR

Total cellular RNA was isolated from A549 cells with TRIzol. Total RNA (2.5 μg) was used for first-strand cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Fermentas). The mRNA of COX-2 in A549 cells was quantified using the following primers: forward primer 5′-CCC TGA GCA TCT ACG GTT TG-3′, reverse primer 5′-TCG CAT ACT CTG TTG TGT TCC-3′. Platinum SYBR Green qPCR Supermix (Invitrogen) was used as a fluorescent dye to detect the presence of double-strand DNA. The mRNA values for each gene were normalized to internal control β-actin mRNA with the following primers: forward primer 5′-GCG TGA CAT TAA GGA GAA G-3′, reverse primer 5′-GAA GGA AGG CTG GAA GAG-3′. The ratio of normalized mean value for each treatment group to vehicle control group (DMSO) was calculated.

2.5. mRNA stability assays

To determine COX-2 mRNA stability, A549 cells were pretreated with triptolide (50nM) for 1 h, then stimulated with TNF-α (10 ng/ml) for 4 h before actinomycin D (10 μg/ml) was added to stop new RNA synthesis. Total cellular RNA was isolated from the cells using TRIzol at 0, 0.5, 1, and 2 h after the addition of actinomycin D. Reverse transcription was performed to synthesize single-strand cDNAs using My iQ Cycler (Bio-Rad). The remaining mRNA levels of COX-2 were quantified by real-time quantitative PCR using SYBR Green dye.

2.6. Immunoprecipitation of mRNP complex and reverse transcription-PCR

To assess the association of endogenous HuR with COX-2 mRNAs, immunoprecipitation (IP) of HuR–mRNA complexes was performed as described. Briefly, after treatment with triptolide or TNF-α, cells were harvested and resuspended in polysome lysis buffer containing 100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.0), 0.5% Nonidet P-40 with 1 mM DTT, 100 U/ml RNaseOUT and protease inhibitors cocktails (Sigma). The protein G beads were coated with excess immunoprecipitating antibody (30 μg), using either a mouse mAb specific for HuR, or an IgG1 isotype control Ab on rotator at 4 °C overnight. Thirty microgram of cell lysate was added to the coated beads and incubated 4 h at room temperature, supplemented with 1% BSA. The beads were pulled down and the RNA in IP materials was separated. The RNA was reverse transcribed and used to detect the presence of COX-2 mRNA by real-time PCR. PCR products were visualized by 1.5% agarose gel electrophoresis.

2.7. COX-2 AU-rich element (ARE) and 3′-UTR GFP reporter gene assay

For reporter activity, plasmids (generous gift from Huiping Zhou, Richmond, VCU) containing 1947–2575 fragment of COX-2 3′UTR fused to the GFP gene (pEGFP-COX-2–3′-UTR-Fluo) were used. A549 cells were transfected using Lipofectin 2000 transfection reagent (Invitrogen) following the instructions of the manufacturer. Following transfection, we combined use of flow cytometry and G418 to screen the stable transformed cell lines. Fluorescence was measured using fluorescence plate reader and the following filter settings: excitation at 380 nm and emission at 510 nm. Fluorescence was corrected for background activity shown by cells transfected with the pEGFP C3 vector (Clontech) alone and for the cell number between individual experiments by cell protein content determined using the Bio-Rad protein assay system.

2.8. Immunofluorescence staining

To determine intracellular HuR protein localization, we seeded A549 cells directly on coverslips. After 24 h, cells were treated as indicated and fixed on the slides by incubation in 3.7% paraformaldehyde for 20 min at room temperature. Thereafter, cells were permeabilized in PBS containing 0.2% Triton X-100 for 3 min. Cells were incubated for 2 h with a 1/250 dilution of a mouse HuR Ab (Santa Cruz Biotechnology) at room temperature. After that, cells were incubated with a secondary goat anti-mouse Ab (1/1000) labeled with Alexa Fluor 488 (Invitrogen Life Technologies) for 2 h at room temperature. Then, cells were counterstained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; 1 μg/ml in PBS for 10 min). HuR localization was determined using fluorescence microscope (Olympus; IX71).

2.9. Statistical methods

Student's *t*-test was employed to analyze the differences between sets of data. Statistics were performed using GraphPad Prism (GraphPad, San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

3. Result

3.1. Triptolide inhibits TNF-α-induced COX-2 activation in a dose- and time-dependent manner

To determine the effect of triptolide on the expression of COX-2 genes, we performed Western and Real-time PCR analysis. First,