Toxicology and Applied Pharmacology 231 (2008) 260–266

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Regulation of cyclooxygenase-2 expression by cAMP response element and mRNA stability in a human airway epithelial cell line exposed to zinc

Weidong Wu [a,b,*], Robert A. Silbajoris [c], Dongsun Cao [a], Philip A. Bromberg [a,d], Qiao Zhang [e], David B. Peden [a,b,d], James M. Samet [c]

[a] Center for Environmental Medicine, Asthma and Lung Biology, University of North Carolina, Chapel Hill, NC, USA
[b] Department of Pediatrics, University of North Carolina, Chapel Hill, NC, USA
[c] Human Studies Division, National Health Effects and Environmental Effects Research Laboratory, Research Triangle Park, NC, USA
[d] Department of Medicine, University of North Carolina, Chapel Hill, NC, USA
[e] College of Public Health, Zhengzhou University, Zhengzhou, China

ARTICLE INFO

Article history:
Received 11 January 2008
Revised 26 March 2008
Accepted 18 April 2008
Available online 29 April 2008

Keywords:
Zinc
Cyclooxygenase
Airway epithelial cell
Cyclic AMP response element
mRNA stability

ABSTRACT

Exposure to zinc-laden particulate matter in ambient and occupational settings has been associated with proinflammatory responses in the lung. Cyclooxygenase 2-derived eicosanoids are important modulators of airway inflammation. In this study, we characterized the transcriptional and posttranscriptional events that regulate COX-2 expression in a human bronchial epithelial cell line BEAS-2B exposed to Zn^{2+}. Zn^{2+} exposure resulted in pronounced increases in COX-2 mRNA and protein expression, which were prevented by pretreatment with the transcription inhibitor actinomycin D, implying the involvement of transcriptional regulation. This was supported by the observation of increased COX-2 promoter activity in Zn^{2+}-treated BEAS-2B cells. Mutation of the cAMP response element (CRE), but not the κB-binding sites in the COX-2 promoter markedly reduced COX-2 promoter activity induced by Zn^{2+}. Inhibition of NFκB activation did not block Zn^{2+}-induced COX-2 expression. Measurement of mRNA stability demonstrated that Zn^{2+} exposure impaired the degradation of COX-2 mRNA in BEAS-2B cells. This message stabilization effect of Zn^{2+} exposure was shown to be dependent on the integrity of the 3′-untranslated region found in the COX-2 transcript. Taken together, these data demonstrate that the CRE and mRNA stability regulates COX-2 expression induced in BEAS-2B cells exposed to extracellular Zn^{2+}.

Published by Elsevier Inc.

Introduction

Cyclooxygenase (COX) is a heme-containing enzyme that catalyzes two sequential enzymatic reactions: the bis-oxygenation of arachidonic acid leading to the production of prostaglandin G2 (PGG2) and the reduction of 15-hydroperoxide of PGG2, leading to the formation of PGH2, a precursor of all PGs, thromboxanes, and prostacyclins, in concert with a series of cell-specific isomerases (Smith et al., 2000). Three COX isoforms, COX-1, COX-2, and COX-3, have been identified in mammals (Chandrasekharan et al., 2002). COX-1 is expressed constitutively in most tissues and appears to be responsible for the production of PGs that modulate physiological functions. COX-3 is an alternatively spliced form of COX-1, expressed primarily in brain and heart as a constitutive enzyme. In contrast, COX-2 is expressed at low or undetectable levels in most tissues and cells under basal conditions, but is rapidly inducible by a variety of stimuli such as lipopolysaccharide (LPS), inflammatory cytokines, growth factors, ultraviolet radiation, and chemicals (Fu et al., 1990; Zhang et al., 1998; Subbaramaiah et al., 2000; Chang et al., 2003; Huh et al., 2003).

The COX-2 gene is mapped to human chromosome 1q25.2-q25.3, approximately 8.3 kb in length with 10 exons, and is transcribed as a 4.4 kb mRNA (Tanabe and Tohnai, 2002). The human COX-2 5′-flanking region contains a canonical TATA box and several functionally important enhancer elements including a cyclic AMP response element (CRE), E box and activator protein 1 (AP-1) regulatory element complex situated very close to TATA, a CCAAT/enhancer binding protein (C/EBP) site and two κB sites (Tazawa et al., 1994). The proinflammatory stimuli can induce binding of different transcription factors to their specific DNA-binding sites in a cell type- and stimulus-

Abbreviations: COX, cyclooxygenase; LPS, lipopolysaccharide; CRE, cyclic AMP response element; AP-1, activator protein 1; C/EBP, CCAAT/enhancer binding protein; NF-IL-6, nuclear factor interleukin-6; ATF, activating transcription factor; CREB, CRE-binding protein; USF-1, upstream stimulatory factor 1; UTR, untranslated region; ARE, AU-rich element; PM, particulate matter; KBM, keratinocyte basal medium; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMA, phorbol 12-myristate 13-acetate; MAPKs, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase; ERK, extracellular-signal regulated kinase; TIA-1, T-cell intracellular antigen 1; TIAR, TIA-1 related protein; HuR, Hu antigen R; hnRNP U, heterogeneous nuclear ribonucleoprotein U.

* Corresponding author. Center for Environmental Medicine, Asthma and Lung Biology, University of North Carolina at Chapel Hill, North Carolina 27599, USA. Fax: +1 919 966 9863.
E-mail address: Weidong_Wu@med.unc.edu (W. Wu).

0041-008X/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.taap.2008.04.012

specific fashion. The transcription factors that bind and activate COX-2 transcription involve C/EBPβ and C/EBPδ for the nuclear factor interleukin-6 (NF-IL-6) elements, AP-1, activating transcription factor (ATF) and CRE-binding protein (CREB) for the CRE element, and upstream stimulatory factor 1 (USF-1) for the E box (Murakami and Kudo, 2004). Posttranscriptional events also play an important role in modulating COX-2 mRNA levels (Dannenberg et al., 2005). The first 60 nucleotides of the 3′-untranslated region (UTR) of COX-2 mRNA are highly conserved and contain multiple copies of the regulatory sequence AUUUA. These well-known AU-rich elements (AREs), present within the 3′-UTRs of many proto-oncogene and cytokine mRNAs, confer posttranscriptional control of expression by acting as a mRNA instability determinant or as a translation inhibitory element that can affect both mRNA and protein translation (Caput et al., 1986; Xu et al., 1997). An ARE element within the 3′-UTR of COX-2 mRNA has been identified that can control both mRNA decay and protein translation (Dixon et al., 2000, 2001).

Increased COX-2 protein expression has been implicated in the pathogenesis of lung diseases characterized by chronic airway inflammation, including asthma, chronic bronchitis, cystic fibrosis, and bronchiectasis (Ermert et al., 1998; Oguma et al., 2002). Expression of the *COX-2* gene has been shown in human airway epithelial cells exposed to exogenous stimuli, such as air-borne residual oil fly ash (Samet et al., 2000), hydrochloric acid (Bonnans et al., 2006), peroxisome proliferator-activated receptor-gamma agonists (Patel et al., 2005), respiratory syncytial virus and *Streptococcus pneumoniae* infection (Liu et al., 2005; N'Guessan et al., 2006). Zinc (Zn) is an essential micronutrient involved in structural and regulatory cellular functions of a large number of proteins (Vallee and Falchuk, 1993). Zn is also a ubiquitous contaminant in ambient and occupational settings. It exists as a combustion-derived metal associated with ambient particulate matter (PM) and may contribute to the adverse health effects of ambient PM inhalation (Horner, 1996; Adamson et al., 2000). In this study, the regulation of COX-2 expression was studied in a human bronchial epithelial cell line BEAS-2B exposed to Zn^{2+}. We report here that Zn^{2+} exposure increases COX-2 expression through the CRE site located in the COX-2 promoter region and stabilization of COX-2 mRNA.

Methods

Materials and reagents. American Chemical Society-grade zinc sulfate, Triton X-100, and polyacrylamide were purchased from Sigma Chemical Co. (St. Louis, MO). SDS-PAGE supplies such as molecular mass standards and buffers were from Bio-Rad (Richmond, CA). Anti-human COX-2 polyclonal antibody was obtained from Cayman Chemical (Ann Arbor, MI). β-actin antibody was purchased from USBiological (Swampscott, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Actinomycin D (Act D), Bay11-7082 and Dup-697 were purchased from EMD Biosciences, Inc. (San Diego, CA). FuGENE 6 transfection reagent was obtained from Roche Diagnostics Corporation (Indianapolis, IN). Chemiluminescence reagents were from Pierce Biotechnology (Rockford, IL).

Cell culture and in vitro exposure. BEAS-2B (subclone S6) cells were obtained from Drs. Curtis Harris and John Lechner (National Institutes of Health). The BEAS-2B cell line was derived by transforming human bronchial cells with an adenovirus 12-simian virus 40 construct (Reddel et al., 1988). BEAS-2B cells (passages 70–80) were grown on tissue culture-treated Costar plates in keratinocyte basal medium (KBM) supplemented with 30 μg/ml bovine pituitary extract, 5 ng/ml human EGF, 500 ng/ml hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, and 5 ng/ml insulin. Cells were placed in KBM (without supplements) for 20–22 h before further treatment.

A suspension of 50 mM zinc sulfate (Zn^{2+}) was prepared in water and used as a stock for dilution into KBM, as described previously.

Immunoblotting. BEAS-2B cells were treated with Zn^{2+}, washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors: 20 μg/ml leupeptin, 20 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, and 20 mM sodium fluoride). Cell lysates were subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose membrane. Membranes were blocked with 5% nonfat milk, washed briefly, incubated with primary antibody at 4 °C overnight, followed by incubating with corresponding HRP-conjugated secondary antibody for 1 h at room temperature. Immunoblot images were detected using chemiluminescence reagents and the Gene Gnome Imaging System (Syngene, Frederick, MD).

Real-time reverse transcriptase/polymerase chain reaction (RT-PCR). BEAS-2B cells grown to confluence were exposed to Zn^{2+}. Cells were washed with ice-cold PBS and then lysed with TRIZOL reagent (Invitrogen Corporation, Carlsbad, CA). Total RNA was isolated according to manufacturer-provided instructions. RNA (200 ng) was reverse transcribed into cDNA. Quantitative PCR was performed using Platinum Quantitative PCR SuperMix-UDG (Invitrogen Corporation, Carlsbad, CA) and an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). COX-2 mRNA levels were normalized using the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. Relative amounts of COX-2 and *GAPDH* mRNA were based on standard curves prepared by serial dilution of cDNA from human BEAS-2B cells. The following oligonucleotide primers and probes were employed: COX-2: 5′-GAA TCA TTC ACC AGG CAA ATT G-3′ (sense), 5′-TCT GTA CTG CGG GTG GAA CA-3′ (antisense), 5′-TCC TAC CAC CAG CAA CCC TGC CA-3′ (probe); *GAPDH*: 5′-GAA GGT GAA GGT CGG AGT C-3′ (sense), 5′-GAA GAT GGT GAT GGG ATT TC-3′ (antisense), 5′-CAA GCT TCC CGT TCT CAG CC-3′ (probe).

Site-directed mutagenesis. Mutations in three COX-2 promoter sites were generated by PCR with the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). Briefly, the PCR reaction was carried out in 50 μl of solution containing 50 ng of COX-2 promoter reporter constructs (pGL2COX-2-luc) as a template, 10 μM of mutagenic oligonucleotide primers, 100 μM dNTPs, 10 mM KCl, 6 mM $(NH4)_2SO_4$, 20 mM Tris–HCl, pH8.0, 2 mM $MgCl_2$, 0.1% Triton X-100, 10 μg/ml BSA and 2.5 units of *Pfu* Turbo DNA polymerase. The PCR scheme consists of 2 min incubation at 95 °C, followed by 18 cycles of 95 °C for 1 min, 55 °C for 1 min, 68 °C for 8 min, and a final incubation at 72 °C for 10 min. Then 10 units of *Dpn I* was added to the PCR solution and incubated for 30 min at 37 °C to degrade the parent template strand. The remaining plasmid DNA was used to transform *E. coli* XL1-Blue cells. These mutation sites included one CRE site (−62), and two NFκB-binding elements, NFκB-1, −449 and NFκB-2, −225. All mutation constructs were confirmed by sequencing. The following primers were used (the mutated bases are underscored) in the mutagenesis reactions: (a) COX-2 promoter CRE site, 5′-GAA ACA GTC ATT TGA GCT CAT GGG CTT GGT-3′; (b) COX-2 promoter NFκB-1 site, 5′-C GGC GGG AGA GGC GATTCG CTG CGC CCC CGG-3′; (c) COX-2 promoter NFκB-2 site, 5′-GAC AGG AGA GTG GCG ACT ACG CCC TCT GCT CC-3′.

mRNA decay assay. mRNA decay assay was conducted according to the previously published procedure. Specifically, BEAS-2B cells were treated with a combination of 20 ng/ml TNFα, 5 ng/ml IL-1β, and 10 ng/ml interferon γ (IFN γ) for 3 h to induce COX-2 mRNA expression. The medium was then decanted and cells were washed with PBS for three times. Fresh medium containing Act D (10 μg/ml) was replaced and incubated for 30 min before further treatment with PBS or 50 μM Zn^{2+}. At different time intervals (0, 1, 2, and 4 h), cells were collected for RNA preparation. RNA was examined by RT-PCR analysis. The levels of COX-2 mRNA were normalized for the intensity of the GAPDH signals as described previously.

Transient gene transfection. BEAS-2B cells were grown to 40–50% confluence prior to transfection with COX-2 promoter reporter constructs (pGL2COX-2-luc), the COX-2 promoter reporter constructs mutated at one CRE site or two NFκB-binding sites using FuGENE 6 transfection reagent, respectively, according to the manufacturer's instructions. pSV-β-galactosidase constructs were cotransfected. 24 h after transfection, cultures were incubated with KBM overnight. The cells were then treated with Zn^{2+} before being lysed with lysis buffer. Detection of luciferase and β-galactosidase activities was conducted using the Dual-Light chemiluminescent reporter gene assay system from Tropix and an AutoLumat LB953 luminometer (Berthold Analytical Instruments, Nashua, NH). Luciferase activity was estimated as luciferase count/β-galactosidase count.

Luciferase expression constructs ligated to the full-length COX-2 3′-UTR (luc+3′-UTR), the COX-2 ARE (luc+ARE), and luciferase constructs without a 3′-UTR (lucΔ3′-UTR) were obtained from Dr. Dan A. Dixon, University of Utah. Similarly, these constructs were cotransfected into BEAS-2B cells with pSV-β-galactosidase constructs as described previously. Luciferase activity was estimated as luciferase count/β-galactosidase count.

Electrophoretic mobility shift assay (EMSA). BEAS-2B cells were stimulated with 50 μM Zn^{2+} or 100 ng/ml TNFα for 2 h, respectively. Cells were washed twice with cold PBS. Nuclear proteins were extracted with a Nuclear/Cytosol Fractionation kit (Biovision Inc., Mountain View, CA). EMSA was conducted using an EMSA Gel-Shift kit (Panomics Inc., Fremont, CA) according to the manufacturer's instructions. Briefly, nuclear proteins (10 μg) were incubated with 2 μl of 5× binding buffer, 1 μl of poly d(I-C) (1 μg/ml), 1 μl of cold unlabeled or biotin-labeled CRE probe (10 ng/ml), and 5 μl of distilled water at room temperature for 30 min. After mixed with 1 μl of loading dye, the samples were subjected to electrophoresis in 0.5× TBE buffer. The protein–oligos complexes were transferred onto nylon membrane. The images were developed using the detection buffer and substrates provided by the manufacturer, and detected using chemiluminescence reagents and the Gene Gnome Imaging System as described previously. The cold probe and positive nuclear proteins were provided with the kit.

Measurement of prostaglandin E_2 (PGE_2). BEAS-2B cells grown to confluence were incubated with 50 μM Zn^{2+} for 6 h. PGE_2 in the supernatants of stimulated BEAS-2B cells was quantified by enzyme linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, MN) following the manufacturer's instructions. Meanwhile, Dup-697 (20 μM), the inhibitor of COX-2 enzyme activity, was added with Zn^{2+} to test its inhibitory effect on COX-2 activity.

Statistics. Data are presented as means ± SE. COX-2 mRNA and cotransfection data were evaluated using nonparametric paired *t* tests with the overall α level set at 0.05.