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Up-regulation of early growth response gene 1 (EGR-1) via ERK1/2 signals attenuates sulindac sulfide-mediated cytotoxicity in the human intestinal epithelial cells

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to relieve pain and inflammation and have also received considerable attention because of their preventive effects against human cancer. However, the drug application is sometimes limited by the severe gastrointestinal ulcers and mucosal complications. In the present study, NSAID sulindac sulfide was investigated for the cytotoxic injury in the intestinal epithelial cells in association with an immediate inducible factor, early growth response gene 1 (EGR-1). Previously we reported that sulindac sulfide can suppress tumor cell invasion by inducing EGR-1. Extending the previous study, EGR-1 induction by sulindac sulfide was observed both in the non-transformed and transformed human intestinal epithelial cell lines. In terms of signaling pathway, ERK1/2 MAP kinases and its substrate Elk-1 transcription factor were involved in the sulindac sulfide-induced EGR-1 gene expression. Moreover, sulindac sulfide stimulated the nuclear translocation of the transcription factor EGR-1, which was also mediated by ERK1/2 signaling pathway. The roles of EGR-1 signals in the apoptotic cell death were assessed in the intestinal epithelial cells. Suppression of EGR-1 expression retarded cellular growth and colony forming activity in the intestinal epithelial cells. Moreover, induced EGR-1 ameliorated sulindac sulfide-mediated apoptotic cell death and enhanced the cellular survival. Taken all together, sulindac sulfide activated ERK1/2 MAP kinases which then mediated EGR-1 induction and nuclear translocation, all of which played important roles in the cellular survival from NSAID-mediated cytotoxicity in the human intestinal epithelial cells, implicating the protective roles of EGR-1 in the NSAID-mediated mucosal injuries.

Keywords: EGR-1; NSAID; ERK1/2 MAP kinase; Apoptosis; Intestinal epithelial cells; Ulcer

Introduction

Cyclooxygenase (COX)-inhibiting nonsteroidal anti-inflammatory drugs (NSAIDs) have been administered to inhibit tumor growth since some major COX metabolites are involved in the tumor progression. Moreover, some lines of evidence suggested that NSAIDs modulate tumor growth by cyclooxygenase-independent signaling pathways (Brown and DuBois, 2005; Kashfi and Rigas, 2005; Grosch et al., 2006). For example, NSAIDs induce apoptosis-inducing or growth-arresting proteins such as NSAID-activated gene-1 (NAG-1) and p21 protein (Baek et al., 2005; Lee et al., 2006).

Despite of the anti-tumor activity of NSAIDs, they can cause the cytotoxic effects on the normal gastrointestinal epithelial

Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; EGR-1, early growth response gene 1; MAP kinase, mitogen activated protein kinase; ERK, extracellular signal-regulated protein kinase; BFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; TSP-1, thrombospondin 1; NAG-1, NSAID-activated gene-1; COX, cyclooxygenase; siRNA, small interference RNA; 7-AAD, 7-Amino-actinomycin D; PTEN, phosphatase and tensin homolog deleted on chromosome ten; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; TBST, Tris-buffered saline plus Tween 0.05%; RT-PCR, reverse transcription-polymerase chain reaction; MTT, thiazolyl blue tetrazolium bromide; DMSO, dimethyl sulfoxide.

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tissues. NSAIDs have been well recognized to cause severe ulcers in the upper gastrointestinal (GI) tracts (Morris and MacKenzie, 1991; Chan, 2006). These untoward effects in the GI tract include the increased mucosal permeability, mucosal inflammation, anemia and occult blood loss, malabsorption, protein loss, ileal dysfunction, diarrhea, mucosal ulceration, strictures due to diaphragm disease as well as active bleeding and perforation. Moreover, with the recent increased use of sustained release or enteric coated formulation of NSAIDS, NSAID-induced damage to the distal GI tract became more frequent than previously recognized. It is possible that these modified release formulations may increase the exposure of active drug to the mucosa distally to the duodenal bulb, and thereby increase toxicity to distal GI regions where the effects are difficult to monitor (Davies, 1999).

Body has developed early defense mechanisms against the ulcerative stresses via eliciting the acute expression of immediately expressed proteins such as early growth response gene 1 (EGR-1) or stress kinases in the epithelium (Wong et al., 2000; Braddock, 2001). EGR-1 is immediately induced after acute injury and serves to promote tissue repair and regeneration. The protein product of EGR-1 (also known as knox24, zif268, and Tis8) is a Cys2-His2-typed zinc-finger transcription factor implicated in the regulation of a number of genes involved in inflammation, differentiation, growth and development (Khachigian and Collins, 1997; Murphy et al., 2004). The ulcerative injuries of GI tracts invoke a wide range of molecular and physiological healing processes that are coordinated in a temporally specific manner by the innate mediators such as the growth factors and transcriptional factors. In terms of wound healing process, MAP kinase signaling cascade and its multiple downstream targets have been broadly investigated (Dieckgraefe and Weems, 1999; Wong et al., 2000; Szabo et al., 2001). MAP kinases also can act on EGR-1 protein which mediates the production of the protective soluble factors after injury. Suppression of EGR-1 thus aggravates experimental duodenal ulcers, most likely through the transcriptional inhibition of the beneficial growth factors such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) (Szabo et al., 2000; Braddock, 2001; Khomenko et al., 2006).

Recently, we reported that NSAID-induced EGR-1 in the tumor cells, which contributed to the tumor invasion (Moon et al., 2005). To investigate the roles of EGR-1 in the intestinal injuries, we turned to a new hypothesis that up-regulated EGR-1 can alleviate NSAID-induced cytotoxic injury in the intestinal epithelial cells. We also investigated the signaling pathway in up-regulation of EGR-1 and its nuclear translocation by sulindac sulfide, an active metabolite of NSAID sulindac sulfoxide. The study will provide a promising molecular evidence for the role of EGR-1 in the intestinal epithelium insulted by chemical stresses.

Materials and methods

Cell culture conditions and reagents. Human intestinal epithelial cell lines, intestine 407, HCT-116 and HT-29 were purchased from American Type Culture Collection (Rockville, MD) and maintained in RPMI medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma Chemical Company, St. Louis, MO), 50 unit/ml penicillin (Sigma Chemical Company, St. Louis, MO), and 50 μg/ml streptomycin (Sigma) in a 5% CO_2 humidified incubator at 37 °C. Intestine 407 cells were additionally supplemented with non-essential amino acids (Invitrogen). Cell number and viability were assessed by trypan blue (Sigma) dye exclusion using a hemacytometer. Sulindac sulfide and MAPK inhibitors were purchased from Calbiochem (EMD Biosciences, Inc., La Jolla, CA).

Construction of plasmids. The full length EGR-1 cDNA inserted into the pcDNA3.1/neo expression vector (pEGR-1) was described previously (Baek et al., 2003). CMV-driven small interference RNA (siRNA) expression vector was constructed by inserting the hairpin siRNA template into *pSilencer 4.1-CMV-neo* vector (Ambion Inc., Austin, TX). The empty vector and siEGR-1 insert-containing vector were named as pSilence and pSiEGR1 respectively. Insert EGR-1 siRNA (Dharmacon, Lafayette, CO) was targeting the sequence, AAGTTACTACCTC TTATCCAT. The luciferase constructs containing EGR-1 5′-untranscribed region (−1260/+35) in pGL3 basic vector were kindly provided from Dr. Seung Joon Baek (University of Tennessee). The reporter plasmid was generated as the following procedure. After PCR of the promoter region with *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA), the fragment was cloned into the TA vector (Invitrogen), sequenced, and sub-cloned into the pGLBasic3 vector.

Western immunoblot analysis. Levels of protein expression were compared using Western immunoblot analysis using rabbit polyclonal anti-human actin antibody (Santacruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-p-MAPKs, anti-Elk, anti-Egr-1 and anti-p-Elk antibody (Cell Signaling Technology, Beverly, MA). Cells were washed with ice-cold phosphate buffer, lysed in boiling lysis buffer [1% (w/v) SDS, 1.0 mM sodium ortho-vanadate, and 10 mM Tris, pH 7.4], and sonicated for 5 s. Lysates containing proteins were quantified using BCA protein assay kit (Pierce, Rockford, IL). Fifty micrograms of protein was separated by Bio-Rad gel mini electrophoresis. Proteins were transferred onto PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and the blots were blocked for 1 h with 5% skim milk in Tris-buffered saline plus Tween 0.05% (TBST) and probed with each antibody for 2 h at room temperature or overnight at 4 °C. After three times washing with TBST, blots were incubated with horseradish-conjugated secondary antibody for 1 h and washed with TBST three times. Protein was detected by ECL Chemiluminescent substrate (Amersham Pharmacia Biotech, Piscataway, NJ).

Traditional reverse transcription-polymerase chain reaction (RT-PCR). RNA was extracted with RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA (100 ng) from each sample was transcribed to cDNA by BD Sprint PowerScript (Clontech, Mountain View, CA). The amplification was performed with Takara HS ExTaq DNA Polymerase (Takara Bio Inc., Shiga, Japan) in Mycycler Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) using the following parameters: denaturation at 94 °C for 2 min and 25 cycles of reactions of denaturation at 98 °C for 10 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 45 s. An aliquot of each PCR product was subjected to 1.2% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide. The 5′ forward and 3′ reverse-complement PCR primers for amplification of each gene were as follows: human Egr-1 (5′-CAGTGGCC-TAGTGAGCATGA-3′ and 5′-CCGCAAGTGGATCTTGGTAT-3′) and human GAPDH (5′-TCAACGGATTTGGTCGTATT-3′ and 5′-CTGTGGTCATGA-GTCCTTCC-3′).

Transient and stable transfection. Cells were transfected with mixture of plasmids using Trans-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. For transfection of the luciferase reporter gene, a mixture of 1.5 μg firefly luciferase reporter and 0.15 μg renilla luciferase, pRL-null vector (Promega, Madison, WI) per 4.5 μl of Trans-LT1 reagent was applied for a 6 well culture plate. For the luciferase assay, at 18 h after transfection, cells were exposed to chemicals for the next 24 h and lysed for dual-luciferase reporter assay system (Promega, Madison, WI). All transfection efficiency was maintained at around 50 to 60%, which was confirmed with pMX-enhanced GFP vector. To create pSilence and pSiEGR1-expressing stable cell lines, cells were transfected using Trans-LT1 reagent. After 48 h, the cells were subjected to