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Anisomycin induces COX-2 mRNA expression through p38^{MAPK} and CREB independent of small GTPases in intestinal epithelial cells

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Abstract

Cyclooxygenase (COX)-2 expression in intestinal epithelial cells is associated with colorectal carcinogenesis. COX-2 expression is induced by numerous growth factors and gastrointestinal hormones through multiple protein kinase cascades. Here, the role of mitogen activated protein kinases (MAPKs) and small GTPases in COX-2 expression was investigated. Anisomycin and sorbitol induced COX-2 expression in non-transformed, intestinal epithelial IEC-18 cells. Both anisomycin and sorbitol activated p38^{MAPK} followed by phosphorylation of CREB. SB202190 and PD169316 but neither PD98059 nor U0126 blocked COX-2 expression and CREB phosphorylation by anisomycin or sorbitol. *Clostridium difficile* toxin B inhibition of small GTPases did not affect anisomycin-induced COX-2 mRNA expression or phosphorylation of p38^{MAPK} and CREB but did inhibit sorbitol-dependent COX-2 expression and phosphorylation of p38^{MAPK} and CREB. Angiotensin (Ang) II-dependent induction of COX-2 mRNA and induced phosphorylation of p38^{MAPK} and CREB were inhibited by toxin B. Reduction of CREB protein in cells transfected with CREB siRNAs inhibited anisomycin-induced COX-2 expression. These results indicate that activation of p38^{MAPK} signaling is sufficient for COX-2 expression in IEC-18 cells. Ang II and sorbitol require small GTPase activity for COX-2 expression via p38^{MAPK} while anisomycin-induced COX-2 expression by p38^{MAPK} does not require small GTPases. This places small GTPase activity down-stream of the AT_1 receptor and hyperosmotic stress and up-stream of p38^{MAPK} and CREB.

Keywords: Cyclooxygenase-2; p38^{MAPK}; CREB; Anisomycin; Gene expression

1. Introduction

Prostaglandins (PGs) play a fundamental role in a broad range of physiological processes [1–5]. PGs, which are produced from arachidonic acid by cyclooxygenases (COX, EC 1.14.99.1), are implicated in pathological processes including cancer [6–8]. COX-2 is rapidly induced in response to pro-inflammatory cytokines, tumor promoters, and growth factors and is over-expressed in cancers of the colon [9–12]. Inhibition of COX-2 activity by nonsteroidal anti-inflammatory drugs has been associated with chemo-protective effects on colon cancer [4].

Cellular proliferation, lineage-specific differentiation, migration, and cell death of epithelial cells of the intestinal mucosa are a tightly regulated process that is modulated by broad spectrum of regulatory peptides [13–15], but the signal transduction pathways involved remain incompletely understood. The non-transformed epithelial IEC-18 cells derived from rat intestinal crypt [16] has been a useful model to examine intestinal epithelial migration, differentiation, proliferation and COX-2 expression [17–21]. These cells are undifferentiated as judged by morphologic and functional criteria, they resemble stem cells and thus may serve as a model to study the crypt stem cell [22]. The importance of examining this compartment is highlighted by reports suggesting that colorectal adenoma arise from

Abbreviations: PG, prostaglandin; COX, cyclooxygenase; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; GPCR, G-protein coupled receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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mutant cells from the crypt cell compartment involving aberrant COX-2 expression [20,21].

Recently, we showed agonist-induced prostaglandin production in IEC-18 cells by Angiotensin (Ang) II and EGF through distinct mitogen-activated protein kinase (MAPK) signaling cascades [23]. The three MAPK subfamilies, including p42/44-MAPK/extracellular regulated kinase-1 and-2 (ERK1/2), $p38^{MAPK}$, and c-Jun N-terminal kinase (JNK)[24], have been implicated in the induction of COX-2 expression by G-protein coupled receptors [25]. Specifically, we have shown in IEC-18 cells, COX-2 induction by Ang II and EGF through activation of $p38^{MAPK}$ and ERK signaling pathways, respectively, that converged onto a *cis*-acting ATF/CRE transcriptional element on the proximal COX-2 promoter [23]. The specific transcription factors that mediate MAP kinase induced COX-2 expression in IEC-18 cells have not been elucidated. Additionally, agonist-dependent COX-2 induction required Rho family, small GTPases but it is unclear whether MAP kinases are upstream or downstream of Rho GTPases.

In this study, we activate MAP kinase signaling using anisomycin and sorbitol to elucidate signaling pathways that lead to COX-2 expression in the absence of cell surface receptor activation [26–30]. We report, for the first time, that anisomycin and sorbitol, individually, directly induce COX-2 expression in IEC-18 cells via $p38^{MAPK}$ and CREB phosphorylation. Inhibition of Rho family GTPases by *Clostridium difficile* toxin B blocked both Ang II- and sorbitol-dependent COX-2 expression and phosphorylation of $p38^{MAPK}$ and CREB. Toxin B did not block anisomycin-dependent phosphorylation of either $p38^{MAPK}$ or CREB, or induction of COX-2 expression, indicating that Rho family GTPases are up-stream of $p38^{MAPK}$/ CREB signaling and down-stream of the AT_1 receptor and osmotic stress.

2. Materials and methods

2.1. Reagents

Anisomycin, sorbitol, angiotensin II, PD98059 and U0126 were purchased from Sigma (St. Louis, MO). *Clostridium difficile* Toxin B, SB202190, PD169316 and JNK inhibitor II (SP600125) were purchased from EMD Biosciences, Inc. (San Diego, CA). IEC-18 cell line was purchased from ATCC (Manassas, VA). Stock cultures of these cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics-antimycotic (Invitrogen, Carlsbad, CA) in a humidified 10% CO_2 atmosphere at 37 °C.

2.2. Isolation of RNA and Taqman analysis

Total RNA was harvested using Trizol reagent (Invitrogen) according to manufacturer's instructions. The relative levels of COX-2 mRNA to GAPDH mRNA was measured using Real-time RT-PCR, Taqman Assay (Applied Biosystems, Inc., Foster City, CA). Rat COX-2 Taqman primer set (Fig. 1A) was synthesized by MWG Biotech (High Point, NC). GAPDH mRNA was measured using the rodent GAPDH Taqman primer set (Applied Biosystems, Inc.). Dual reaction mixtures were made using the Reverse Transcriptase qPCR master mix (Eurogentec North America, Inc., San Diego, CA), 25 ng of sample RNA and either the COX-2 or the GAPDH primer-probe sets. Assays were run on an ABI 7700 using the settings of 30 m at 48 °C; 10 m at 95 °C; and (40X) 15 s at 95 °C, 60 s at 60 °C at either the Human Genetics Core Facility, UCLA or the Division of Pulmonary and Critical Care Medicine, UCLA. The change in COX-2 mRNA relative to GAPDH mRNA was calculated from the measured C_T value using the formula, fold RNA induction $=2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T = \Delta C_{T_f} - \Delta C_{T_i}$, $\Delta C_{T_f} = C_{T_{fCOX2}} - C_{T_{fGAPDH}}$ and $\Delta C_{T_i} = C_{T_{iCOX2}} - C_{T_{iGAPDH}}$.

2.3. RNA interference

Smartpool siRNA targeted to rat CREB (NM 031017) was purchased from Dharmacon (Lafayette, CO). IEC-18 cells were transfected with either CREB specific or random siRNA using a multiporator (Eppendorf, Westbury, NY). Cells were resuspended in hypoosmolar buffer (Eppendorf) at 2×10^6 cells/ml and aliquoted into sterile tubes (0.4 ml). After 15 m, siRNA was added and the cells transferred to a 2-mm electrocuvette. The cells were treated with 320 V, 100 μs, $n=2$ and then allowed to recover for 5 m. The cells were plated onto 35 mm dishes with complete media (2 ml).

2.4. Western blot analysis

Confluent IEC-18 cells grown on 35-mm dishes were incubated in Opti-MEM (18 h) prior to stimulation with agonists. The cells were washed twice (PBS) prior to lysis with 2× LDS-NuPAGE sample buffer (Invitrogen, Carlsbad, CA). Equal volumes of protein extract were run on 4–12% Nu-PAGE acrylamide gels (Invitrogen) and then electroblotted onto PVDF paper (Millipore, Bedford, MA). Membranes were blocked in 5% nonfat milk in Tris-buffered saline (TBS) for 1 h. Membranes were incubated overnight with antibodies, 1:1000 dilution in TBS, 0.05% Tween-20 (TBST). The membrane was washed 3 times using TBST and then incubated with Horseradish peroxidase-conjugated IgG antibody (1:5000; Amersham Biosciences, Piscataway, NJ) for 1 h. Immunoreactive bands were detected using Enhanced Chemiluminance (Amersham Biosciences). Antibodies specific for ERK 1/2, phospho-ERK, $p38^{MAPK}$, phospho-$p38^{MAPK}$, phospho-JNK, phospho-CREB and CREB were purchased from Cell Signaling (Beverly, MA).

2.5. $p38^{MAPK}$ kinase assay

Confluent, serum starved IEC-18 cells on 100 mm dishes were treated with anisomycin. At the indicated times,