European Journal of Pharmacology 702 (2013) 158–164

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

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

Pulmonary, gastrointestinal and urogenital pharmacology

Purinergic P2Y1 receptor signaling mediates wound stimuli-induced cyclooxygenase-2 expression in intestinal subepithelial myofibroblasts

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ARTICLE INFO

Article history:
Received 27 June 2012
Received in revised form
9 January 2013
Accepted 16 January 2013
Available online 31 January 2013

Keywords:
Purinergic
Cyclooxygenase
Myofibroblast
Wound healing

ABSTRACT

Intestinal subepithelial myofibroblasts (ISMFs) are crucial for barrier formation against inflammatory stimuli. Physical injury induces cyclooxygenase-2 (COX-2) expression, which accelerates wound healing by ISMFs. However, the mechanism of COX-2 induction remains unclear. Physically damaged cells release ATP. Here, we investigate the role of ATP-purinergic signaling in wound-induced COX-2 induction in ISMFs. By 24 h post-injury, bovine ISMFs had migrated to and closed the wounded area. A COX inhibitor, indomethacin or a purinergic P2 receptor antagonist, suramin, inhibited wound healing. However, additional treatment with indomethacin did not influence wound healing in suramin-treated ISMFs. RT-PCR showed an increase in COX-2 mRNA expression 2 h post-injury, which was inhibited by suramin. These results suggest that ATP mediates wound-induced COX-2 elevation. We next assessed the contribution of various purinergic receptors in COX-2 induction. An ATP analog, ATPγS and a purinergic P2Y1, 11–13 receptors agonist, ADP, were among the agents tested which increased COX-2 expression. ATPγS-induced COX-2 mRNA expression was suppressed by suramin or a purinergic P2Xs, P2Y1, 4, 6, and 13 receptors antagonist, PPADS. These data suggest the involvement of G_q-coupled purinergic P2Y1 receptor or G_i-coupled purinergic P2Y13 receptor in COX-2 induction. U73122, an inhibitor of phospholipase C, which is a downstream signal of G_q protein, showed suppression of COX-2 mRNA expression. However, pertussis toxin, a G_i inhibitor, did not show suppression. We also revealed that inhibitors of p38 MAPK and PKC inhibited ATPγS-induced COX-2 mRNA expression. Collectively, purinergic P2Y1 receptor signaling mediates wound-induced COX-2 expression through p38 MAPK and PKC pathways in ISMFs.

1. Introduction

Intestinal subepithelial myofibroblasts (ISMFs) are located under the epithelium on the luminal side of the intestinal wall. They have been thought to act as a barrier against bacterial infection (Blikslager et al., 1997; Furuya and Furuya, 2007). Inflammation can result in damage to ISMFs and epithelial cells increasing the risk of infectious disease (Francoeur et al., 2009). This makes it important to investigate and understand the mechanism of ISMF repair after such damage.

Cyclooxygenase (COX) is an enzyme which produces prostaglandins (PGs) including PGE_2 by metabolizing arachidonic acid, and is known as one of the tissue repair factors (Peskar, 2005; Radi and Khan, 2005). COX has two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in many types of cells and contributes to the maintenance of homeostasis. COX-2 expression is induced by inflammatory stimuli. We have shown previously that a response to the action of a wound is elevation of COX-2 expression and subsequent PGE_2 secretion by ISMFs contributing to the promotion of wound closure (Iwanaga et al., 2012). Another group has shown that PGE_2 derived from ISMFs also accelerates wound healing by intestinal epithelial cells (Shao et al., 2006). Moreover, in a murine colitis model, COX-2-dependent PGE_2 production stimulates intestinal mucosal restitution (Takeuchi et al., 2010). Those studies suggest the importance of COX-2-PGE_2 signaling in maintaining mucosal barrier function. However, it is unclear how the action of a wound elevates COX-2 expression in ISMFs.

Nucleotides such as ATP work not only as intracellular sources of energy, but can also act as extracellular signaling molecules including as neurotransmitters (Burnstock, 2006). Extracellular ATP acts by binding to the purinergic P2 receptor. Purinergic P2 receptor itself is classified into two subtypes: ionotrophic receptor P2X (P2X1-7) and G-protein-coupled receptor P2Y (P2Y1, 2, 4, 6, and 11–14) (Abbracchio et al., 2006; Gunosewoyo and Kassiou, 2010).

Our work involves the role of nucleotides as mediators of inflammation. Dying or damaged cells release an amount of

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http://dx.doi.org/10.1016/j.ejphar.2013.01.025

nucleotides into the extracellular space and this release can result in an increased rate of inflammation (Bours et al., 2006). In line with this report, several groups have shown that ATP induces elevated levels of inflammatory mediators including COX-2 and PGs in both macrophages and intestinal epithelial cells (Degagne et al., 2009; Ulmann et al., 2010). Others have reported that ATP accelerates wound healing of corneal epithelial cells or airway epithelial cells (Boots et al., 2009; Boucher et al., 2007). Thus extracellular nucleotides can be assumed to play important roles in both inflammation and tissue repair.

We hypothesize here that extracellular nucleotides mediate wound-induced elevation of COX-2 expression levels in ISMFs. We have shown that ATP enhances COX-2 expression in ISMFs via purinergic P2Y1 receptor signaling, and that this was mediated through p38 MAPK and PKC pathways.

2. Materials and methods

2.1. Materials

The following reagents were used in the experiments: adenosine-5′-(γ-thio)-triphosphate sodium salt (ATPγS), α,β-methylene-adenosine 5′-triphosphate sodium salt (α,β-methylene-ATP), pyridoxalphosphate-6-azophenyl-2′,4′-disulfonate (PPADS), suramin sodium salt, uridine 5′-diphosphate sodium salt (UDP), uridine-5′-(γ-thio)-triphosphate sodium salt (UTPγS), indomethacin, 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA); Pertussis toxin (PTX) (Kaketusken, Kumamoto, Japan); 2-(2′-amino-3′-methoxyphenyl) oxanaphthalen-4-one (PD98059), 1-(6-((3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione, (U-73122), 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole (PD169316), 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (bisindolylmaleimide I) (Calbiochem, Darmstadt, Germany); antimycotic–antibiotic (Invitrogen, Carlsbad, CA, USA); 8-phenyltheophylline (MP Biomedicals, Inc., Santa Ana, CA, USA); Anthra [1,9-cd]pyrazol-6(2H)-one (SP600125), Triton X-100 (Wako pure chemical industries Ltd., Osaka, Japan); PGE_2 (Cayman Chemical, Ann Arbor, MI); adenosine 5′-diphosphate sodium salt (ADP) (Yamasa, Chiba, Japan); random RT-primer, ReverTra Ace (Toyobo Engineering, Osaka, Japan); EX Taq (Takara Bio Inc., Otsu, Japan); Fetal bovine serum (FBS, Lot No.8M0055) (Nichirei Biosciences Inc., Tokyo, Japan); TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA); 3-[(4-tert-butylphenyl)sulfonyl] propenenitrile (BAY-11–7082) (Santa Cruz Biotechnology, Inc., CA, USA); and PGE_2 Quantikine kit (R&D Systems, Minneapolis, MN, USA). The following antibodies were used: mouse anti-β-actin antibody (Sigma), rabbit anti-COX-2 (Cayman Chemical), goat anti-rabbit IgG Alexa 568 dye conjugates (Invitrogen), goat anti-mouse IgG IRdye 700 antibody, and goat anti-rabbit IgG IRdye 800 antibody (LI-COR Biosciences, Lincoln, NE, USA).

2.2. Isolation of ISMFs

Bovine colon was dissected and the smooth muscle layer was detached from the mucosal layer. The mucosal layer was then treated with 1 mM EDTA at 37 °C for 1.5 h to remove epithelial cells. Epithelial cell-free mucosa was then cultured in Dulbecco's modified Eagle's medium containing 10% FBS and 1% antimycotic–antibiotic (final concentration: 250 ng/ml amphotericin B, 100 U/ml penicillin and 100 μg/ml streptomycin). When the ISMFs reached confluence, the epithelial-free mucosa was removed and the cells were passaged. ISMFs isolated from multiple animals were used for each experiment at passages 3–7. Cells were starved 24 h before use.

2.3. Wound healing assay

Confluent ISMFs in a 60 mm round culture dish were pretreated with each inhibitor for 3 h. Ten longitudinal lines intersecting with ten transverse lines were then scratched onto the cell surface using a 250-μl pipette tip. Three fields, in the center of which were intersected, were then photographed using a Digital Sight camera system (Nikon Instruments, Tokyo, Japan) attached to a phase contrast microscope. 24 h after making the scratch, the same areas were photographed again. Wounded areas were determined using the NIH ImageJ software. The amount of wound healing was expressed as a percentage according to the following formula: {(initial wounded area, agents added)−(resulting wounded area, agents added)}/{(initial wounded area, no agents added)−(resulting wounded area, no agents added)}.

2.4. Reverse transcript-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells employing TRI Reagent according to the manufacturer's protocol. The first strand of cDNA was synthesized using random 9-mer oligonucleotide primers and ReverTra Ace at 30 °C for 10 min, 42 °C for 1 h, 99 °C for 5 min and 4 °C for 5 min. PCR amplification was conducted using Ex Taq DNA polymerase and synthetic gene-specific primers for COX-2 (expected product size=213 base pairs, forward primer: CCAGAGCTCTTCCTCCTGTG, reverse primer: AAGCTGGTCCTCGTTCAAAA) and GAPDH (expected product size=220 base pairs, forward primer: CAGGGCTGCTTTTAATTCTG, reverse primer: AGCACCAGCATCACCCCACT). PCR was performed using 24 cycles. Each cycle consisted of 10 s at 98 °C, 30 s at 56 °C for COX-2 or 60 °C for GAPDH, and 60 s at 72 °C. The PCR products were electrophoresed on 2% agarose gel containing 0.2 μg/ml ethidium bromide. The bands were visualized using a Gel Doc EZ Imager (BioRad, Hercules, CA, USA).

2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde at room temperature for 5 min, followed by the treatment with 0.1% Triton X-100 and 3% bovine serum albumin in phosphate buffered saline at room temperature for 30 min. Cells were treated with rabbit anti-COX-2 antibody (1:100 dilution) overnight at 4 °C. Then, cells were treated with goat anti-rabbit IgG Alexa Fluor 568 antibody (1:200 dilution) at room temperature for 3 h. Nuclei were stained with DAPI (1 μg/ml) at room temperature for 5 min. The images were photographed using an Eclipse E800 fluorescence microscope (Nikon, Tokyo, Japan).

2.6. Western blot analysis

ISMFs were treated with homogenizing buffer (pH8.3) containing 100 mM NaF, 60 mM β-glycerophosphate, 2 mM EGTA, 1 mM Na_3VO_4, 0.5% Nonidet P-40 substitute, 0.2% Sodium dodecyl sulfate, 50 mM Tris–HCl, 1 mg/ml pefabloc SC (protease inhibitor) and 1 mg/ml Roche's complete protease inhibitor cocktail. After incubated at 4 °C for 30 min, homogenized cells were centrifuged at 20000 × *g* for 15 min at 4 °C, and supernatant was collected. The blots on polyvinylidene difluoride membranes were blocked in wash buffer containing 3% bovine serum albumin at room temperature for 30 min. The membranes were labeled with mouse anti-β-actin antibody (1:1000 dilution) or rabbit anti-COX-2 antibody (1:200 dilution) overnight at 4 °C. The membranes were treated with goat anti-mouse IgG IRdye 700 antibody (1:10,000 dilution) and goat anti-rabbit IgG IRdye 800 antibody (1:10,000 dilution) at room temperature for 30 min. The bands were detected and quantified with Odyssey system (LI-COR