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Lung inflammation caused by adenosine-5′-triphosphate is mediated via Ca^{2+}/PKCs-dependent COX-2/PGE_2 induction

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ABSTRACT

Up-regulation of cyclooxygenase (COX)-2 and prostaglandin E_2 (PGE_2) are implicated in lung inflammation. Adenosine 5′-triphosphate (ATP) has been shown to act via activation of P2 purinoceptors, leading to COX-2 expression in various inflammatory diseases. The mechanisms of ATP-induced COX-2 expression and PGE_2 release remain unclear. We showed that pretreatment with the inhibitors of P2 receptors (PPADS and Suramin), Gq protein (GPA2A), phosphatidylcholine-phospholipase C (PC-PLC; D609), phosphoinositide-phospholipase C (PI-PLC; ET-18-OCH_3), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; KN62), protein kinase C (PKC; Gö6976, Ro-318220, GF109203X, and rottlerin), MEK1/2 (PD98059), p38 MAPK (SB202190), and nuclear factor-kappaB (NF-κB; Bay11-7082) and the intracellular calcium chelator (BAPTA/AM) or transfection with siRNAs of these molecules and $cPLA_2$ reduced ATPγS-induced COX-2 expression or PGE_2 production in A549 cells. In addition, ATPγS-induced elevation of intracellular Ca^{2+} concentration was attenuated by PPADS, Suramin, D609, or ET-18-OCH_3. ATPγS-induced p38 MAPK, p42/p44 MAPK, and NF-κB p65 activation were inhibited by Gö6976, Ro-318220, GF109203X, or rottlerin. ATPγS also induced $cPLA_2$ phosphorylation and activity, which were reduced via inhibition of P2 receptors, PKCs, p38 MAPK, and p42/p44 MAPK. ATPγS-induced $cPLA_2$ expression was inhibited by SB202190, PD98059, or Bay11-7082. In the in vitro study, we established that ATPγS induced PGE_2 generation via a $cPLA_2$/COX-2-dependent pathway. In the in vivo study, we found that ATPγS induced COX-2 mRNA expression in the lungs and leukocyte (mainly eosinophils and neutrophils) count in bronchoalveolar lavage (BAL) fluid in mice via a P2 receptors-dependent signaling pathway. We concluded that ATPγS may induce lung inflammation via a $cPLA_2$/COX-2/PGE_2-dependent pathway.

1. Introduction

Lung inflammation is a pivotal event in the pathogenesis of chronic obstructive pulmonary disease (COPD) and asthma (Lin et al., 2007). Cyclooxygenases (COXs) are responsible for the formation of prostaglandins (PGs), which are involved in inflammatory responses (Li et al., 2011). COX-2 is primarily an inducible isoform whose expression can be up-regulated by cytokines, mitogens, and endotoxins in many cell types (Li et al., 2011). It is highly expressed in inflamed tissues and believed to produce PGs involved in inflammatory processes (Carey et al., 2003). Moreover, the physiological relevance of the purinergic signaling network for airway defenses is emerging through cumulating reports of abnormal adenosine 5′-triphosphate (ATP) and adenosine levels in the airway secretions of patients with asthma and COPD. The consequences for airway defenses range from abnormal clearance responses to the destruction of lung tissue by inflammation (Esther et al., 2011). Thus, to clarify the mechanisms of COX-2 induction by ATP in lung epithelium was recognized as a new therapeutic approach in the management of respiratory diseases.

ATP transports chemical energy within cells, is produced by cellular respiration and is used by enzymes and structural proteins in many cellular processes (Mortaz et al., 2010). Extracellular ATP is an important mediator of intercellular communication via the activation of purinergic P2X and P2Y receptors mediated through ion channels and GTP binding protein coupled receptors, respectively (Polosa and Blackburn, 2009). Growing evidence indicates the involvement of ATP and purinoceptors in the pathogenesis of lung diseases (Polosa and Blackburn, 2009; Mortaz et al., 2010). ATP

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has been shown to induce COX-2 expression (Lin et al., 2009, 2012), and then causes the inflammatory responses. However, the mechanisms by which ATP induced COX-2 expression in A549 cells are not completely understood.

Activation of P2Y receptors can modulate cellular function via change in the activity of secondary signaling components, including phospholipase C (PLC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), mitogen-activated protein kinases (MAPKs), and NF-κB (Schafer et al., 2003, 2006). Both MAPKs and NF-κB have also been shown to induce COX-2 expression and inflammatory responses in RAW 264.7 macrophages (Zhou et al., 2008). Phosphorylation of MAPKs or NF-κB is mediated via activation of PLC, increased intracellular Ca^{2+} concentration, and activation of protein kinase C (PKC) in response to ATP or lipopolysaccharide in various cell types (Zhou et al., 2006; Katz et al., 2006; Scodelaro et al., 2007). Thus, whether ATP induces COX-2 expression in A549 cells via these signaling pathways, including PLC, Ca^{2+}, PKCs, MAPKs, and NF-κB is investigated in this study.

Several studies have revealed that increase in $cPLA_2$ enzyme activity plays a key role in the regulation of cellular responses. It is found that arachidonic acid (AA) stimulates COX-2 expression in renal cells of streptozotocin-diabetic rats (Li et al., 2005). In human pancreatic cancer cells, VEGF production is regulated by an AA-dependent PGE_2 release manner (Eibl et al., 2003). The release of AA is chiefly controlled by $cPLA_2$ hydrolytic activity which is regulated by intracellular Ca^{2+} and MAPKs (Tucker et al., 2009; Isenovic et al., 2009), suggesting that exposure of cells to ATP, Ca^{2+} and MAPKs may promote AA release via up-regulation of $cPLA_2$ activity. Newly released AA may further impact on COX-2 gene expression and tissue inflammation.

In addressing these questions, the experiments were performed to investigate the roles of P2 receptors, PLC, Ca^{2+}, PKC, MAPKs, or NF-κB in ATP-induced activation of $cPLA_2$ and expression of COX-2 in A549 cells. ATPγS is an ATP analogue and often considered as a nonhydrolyzable substrate for ecto-ATPases. In this study, we made an attempt to differentiate the signaling pathways of ATP-induced $cPLA_2$ activation and COX-2 expression. Thus, we need to use the steady ATPγS escaping from hydrolysis to investigate the mechanisms of ATP-regulated COX-2 expression in A549 cells. We found that activation of NF-κB and $cPLA_2$ mediated through a PLC/Ca^{2+}/PKC/MAPKs cascade was required for maximal induction of COX-2 expression in ATPγS-challenged A549 cells. We also demonstrated that ATPγS-induced COX-2 expression and leukocytes recruitment in lung tissues were significantly attenuated by the inhibitor of $cPLA_2$ or COX-2 in animal models, suggesting that ATP promotes lung inflammation via increased expression of inflammatory proteins.

2. Materials and methods

2.1. Materials

Antibodies against COX-2, Gq-coupled receptor, Gi-coupled receptor, CaMKII, PKCα, PKCμ, PKCι, PKCδ, p42, p38, p65, and $cPLA_2$ were from Santa Cruz (Santa Cruz, CA). Anti-phospho-p42/p44 MAPK, anti-phospho-p38 MAPK, anti-phospho-p65, and anti-phospho-$cPLA_2$ antibodies were from Cell Signaling (Danver, MA). Adenosine 5′-O-(3-thiotriphosphate) (ATPγS), Suramin, PPADS, GP antagonist-2 (GPA2), GP antagonist-2A (GPA2A), D609, ET-18-OCH_3, BAPTA/AM, KN62, Ro-318220, rottlerin, Gö-6976, GF109203X, PD98059, SB202190, SP600125, Bay11-7082, NS-398, and AACOCF3 were from Biomol (Plymouth Meeting, PA). All other reagents were from Sigma (St. Louis, MO).

2.2. Human alveolar epithelial cell carcinoma (A549) culture

A549 cells, a human alveolar epithelial cell carcinoma (type II alveolar epithelial cells), were purchased from the American Type Culture Collection (Manassas, VA) and cultured in DMEM/F-12 supplemented with 10% FBS and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin, and 250 ng/ml fungizone) at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Western blot

Growth-arrested A549 cells were incubated with ATPγS at 37 °C for the indicated time intervals. The cells were washed, scraped, collected, and centrifuged at 45000 × *g* at 4 °C for 1 h to yield the whole cell extract, as previously described (Lin et al., 2007). Samples were denatured, subjected to SDS-PAGE using a 12% running gel, and transferred to nitrocellulose membrane. Membranes were incubated with an anti-COX-2 antibody for 24 h, and then incubated with an anti-mouse horseradish peroxidase antibody for 1 h. The immunoreactive bands were detected by ECL reagents.

2.4. Real-time RT-PCR

Total RNA was extracted using TRIzol reagent. mRNA was reverse-transcribed into cDNA and analyzed by real-time RT-PCR. Real-time PCR was performed using SYBR Green PCR reagents (Applied Biosystems, Branchburg, NJ) and primers specific for COX-2 and GAPDH mRNAs. The levels of COX-2 expression were determined by normalizing to GAPDH expression.

2.5. Measurement of PGE_2 production and $cPLA_2$ activity

To determine the levels of PGE_2 and activity of $cPLA_2$, A549 were treated with ATPγS. The levels of PGE_2 and activity of $cPLA_2$ were determined with a PGE_2 enzyme immunoassay kit (Cayman Chemical) and a $cPLA_2$ activity assay kit (Cayman Chemical) according to the manufacturer's instructions.

2.6. COX-2 promoter assay

Gene expression is derived from gene activation through various transcription activator and co-activator. To investigate ATPγS-induced COX-2 gene activity, COX-2-luci plasmid was cloned with −459 to +9 of human COX-2 promoter region into a pGL3-basic vector. COX-2-luci plasmid was transfected into A549 together with β-galactosidase plasmid using the Lipofectamine reagent according to the manufacturer's instructions. To assess promoter activity, after treatment with ATPγS, cells were collected and disrupted by sonication in a lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Firefly luciferase activities were standardized for β-galactosidase activity.

2.7. Transient transfection with siRNAs

Human siRNAs of scrambled, G-coupled receptor Gq subunit, G-coupled receptor Gi subunit, CaMKII, PKCα, PKCμ, PKCι, PKCδ, p42, p38, p65, and $cPLA_2$ were from Sigma (St. Louis, MO). Transient transfection of siRNAs (100 nM) was performed using a Lipofectamine™ RNAiMAX reagent according to the manufacturer's instructions.