Adenosine triphosphate regulates NADPH oxidase activity leading to hydrogen peroxide production and COX-2/PGE₂ expression in A549 cells

Chih-Chung Lin,¹ I-Ta Lee,¹ Wan-Ling Wu,² Wei-Ning Lin,³ and Chuen-Mao Yang²

¹Department of Anesthesiology, Chang Gung Memorial Hospital at Lin-Kou and College of Medicine, Chang Gung University, Kwei-San, Tao-Yuan, Taiwan; and ²Department of Physiology and Pharmacology and Health Aging Research Center, College of Medicine, Chang Gung University, Kwei-San, Tao-Yuan, Taiwan; and ³Graduate Institute of Basic Medicine, Fu Jen Catholic University, Hsin-Chuang, New Taipei City, Taiwan

Am J Physiol Lung Cell Mol Physiol 303: L401–L412, 2012. First published July 6, 2012; doi:10.1152/ajplung.00090.2012.—Non-small cell lung carcinoma (NSCLC) is the most common cause of death due to cancer in the United States and worldwide (1). Clinical statistics reveal that the extent of COX-2 expression is associated with a lower overall survival rate and a diminished disease-free survival rate (22). COX is the rate-limiting enzyme for the production of eicosanoid prostaglandins and thromboxanes from free arachidonic acid (AA), which is generated from membrane phospholipids by phospholipase A₂. Currently, three COX isoenzymes have been identified, COX-1, COX-2, and COX-3 (32). The expression of COX-2 is transiently transcriptionally regulated by various cytokines and growth factors (20). Moreover, several studies have demonstrated the expression of COX-2 is also upregulated in response to extracellular nucleotides, such as ATP, ADP, UTP, or UDP in pathological status (6, 43). The main sources of extracellular nucleotides in lung include the stressed endothelial and epithelial cells, specialized compartments of nerve terminals, and activated platelets (30). Thus, extracellular nucleotides may function not only as an intracellular energy source but also as a mediator of inflammatory responses.

Extracellular nucleotides exert their bioactivities mainly through action on membrane P2 receptors. There are two types of P2 receptors, ionotropic P2X and metabotropic P2Y (37), which are ubiquitous in lung. It is found that extracellular ATP protects lung epithelial cells against ozone toxicity through P2Y receptor-mediated increase of protein kinase B (Akt) phosphorylation (2). Activation of P2 receptors also stimulates phosphorylation of p38 mitogen-activated protein kinase and thrombospondin-1 expression, which contributes to the synaptogenesis in astrocytes (38). Moreover, extracellular ATP can induce reactive oxygen species (ROS) production by a Ca²⁺-dependent activation of NADPH oxidases (15). However, whether extracellular nucleotides releasing from stressed resident tissues contributes to the expression of COX-2 remains unclear. Here, we showed that stimulation of A549 cells by adenosine 5”-O-(3-thiotriphosphate) (ATP₈S) led to an increase in COX-2 gene expression and prostaglandin E₂ (PGE₂) synthesis, revealed by Western blotting, RT-PCR, promoter assay, and enzyme-linked immunosorbent assay. In addition, ATP₈S induced intracellular reactive oxygen species (ROS) generation through the activation of NADPH oxidase. The increase of ROS level resulted in activation of the c-Src/epidermal growth factor receptor (EGFR)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/nuclear factor (NF)-κB cascade. We also found that activated Akt was translocated into the nucleus and recruited with NF-κB and p300 to form a complex. Thus, activation of p300 modulated the acetylation of histone H4 via the NADPH oxidase/c-Src/EGFR/P13K/Akt/NF-κB cascade stimulated by ATP₈S. Our results are the first to show a novel role of NADPH oxidase-dependent Akt/p65/p300 complex formation that plays a key role in regulating COX-2/PGE₂ expression in ATP₈S-treated A549 cells. Taken together, we demonstrated that ATP₈S stimulated activation of NADPH oxidase, resulting in generation of ROS, which then activated the downstream c-Src/EGFR/P13K/Akt/NF-κB/p300 cascade to regulate the expression of COX-2 and synthesis of PGE₂ in A549 cells. Understanding the regulation of COX-2 expression and PGE₂ release by ATP₈S on A549 cells may provide potential therapeutic targets of NSCLC.

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inflammation; signaling transductions; oxidative stress; cyclooxygenase-2; human

NON-SMALL CELL LUNG CARCINOMA (NSCLC) is the most common one of various types of lung cancers. During the processes of lung carcinogenesis, inflammation has been postulated to play a key role in these responses. It is found that there are constitutive high levels of inflammatory proteins, cyclooxygenase-2 (COX-2), and expression in human NSCLC compared with normal adjacent lung tissue (39). Despite various genetic alterations in lung cancer, the elevated expression of COX-2 is believed to be the central factor of cancer invasion, metastasis, angiogenesis, and decreased host immunity (27).
p300 pathway. These results provide new insights into the mechanisms of ATPγS action on A549 cells to regulate the expression of COX-2 and thus exaggerate the inflammation responses.

MATERIALS AND METHODS

Materials. Antibodies against p47phox, Gos, c-Src, EGFR, platelet-derived growth factor receptor (PDGFR), NOX1, Akt, p65, p300, and acetyl H4 were from Santa Cruz (Santa Cruz, CA). Human polyclonal antibody against COX-2 was from BD Transduction Laboratories (San Diego, CA). PhosphoPlus c-Src, EGFR, Akt, and p65 Ab kits were from Cell Signaling (Danvers, MA). ATPγS, LY-294002, AG-1478, AG-1296, diphenyleneiodonium chloride (DPI), apocynin (APO), PP1, GM-6001, Bay11–7082, and GR-343 were from Biomol (Plymouth Meeting, PA). Edaravone (MCI-186) was from Tocris Bioscience (Ellisville, MO). All other reagents were from Sigma (St. Louis, MO). CellROX Deep Red Reagent was from Invitrogen (Carlsbad, CA).

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% CO2 atmosphere.

Western blot analysis. Growth-arrested A549 cells were incubated with ATPγS at 37°C for the indicated time intervals. The cells were washed, scraped, and centrifuged at 45,000 g at 4°C for 1 h to yield the whole cell extract, as previously described (28). 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 enhanced chemiluminescence reagents.

RT-PCR analysis. Total RNA was isolated with Trizol according to the protocol of the manufacturer. The cDNA obtained from 0.5 μg total RNA was used as a template for PCR amplification as previously described (28). The primers used were as follows: β-actin: 5′-GAGCCCTAAGGGCAACCCTG-3′ (sense) and 5′-TTGCGTATAGAGGTC-3′ (antisense); COX-2: 5′-TGGCATAGAGGTC-3′ (sense) and 5′-GCAATCGGTTCTGATCTG-3′ (antisense).

Measurement of PGE2 production. To determine the level of PGE2, A549 were treated with ATPγS. The levels of PGE2 were determined in a PGE2 enzyme immunoassay kit (Cayman Chemical) according to the manufacturer’s instructions.

COX-2 promoter assay. Gene expression is derived from gene activation through various transcription activators and coactivators. To investigate ATPγS-induced COX-2 gene activity, COX-2-luc plasmid was cloned with −459 to +9 of human COX-2 promoter region into a pGL3-basic vector. COX-2-luc 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 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 with β-galactosidase activity.

Transient transfection with small-interfering RNAs. The small-interfering RNA (siRNA) duplexes corresponding to human NOX2, p47phox, c-Src, Akt, EGFR, PDGFR, p65, and p300 and scrambled siRNA were from Invitrogen. Transient transfection of siRNAs was carried out using Metafectene transfection reagent. siRNA (100 nM) was formulated with Metafectene transfection reagent according to the manufacturer’s instruction (Biontix Laboratories, Planegg/Martinsried, Germany).

Cell fraction isolation. A549 cells were harvested, sonicated for 5 s at output 1.5 with a sonicator (Misonix, Farmingdale, NY), and centrifuged at 8,000 rpm for 15 min at 4°C. The pellet was collected as the nuclear fraction. The supernatant was centrifuged at 14,000 rpm for 60 min at 4°C to yield the pellet (membrane fraction) and the supernatant (cytosolic fraction).

Measurement of intracellular ROS accumulation. CellROX Deep Red Reagent is a fluorogenic probe designed to reliably measure ROS in living cells. The cell-permeable CellROX Deep Red dye is nonfluorescent while in a reduced state and, upon oxidation, exhibits excitation/emission maxima at 640/665 nm. A549 cells were treated with ATPγS for the indicated time intervals, and CellROX Deep Red Reagent was added at a final concentration of 5 μM to the cells and then incubated for 30 min at 37°C. Subsequently, medium was removed, and the cells were washed three times with PBS. The resulting fluorescence was measured using a fluorescence microscope (Axiovert 200M; Zeiss).

Measurement of NADPH oxidase activity. The NADPH oxidase activity in intact cells was assayed by lucigenin chemiluminescence assay described by Edwards (14) and modified by Parinandi et al. (31). Cells grown on six-well culture plates, after exposure to ATPγS for the indicated times, were gently scraped and centrifuged at 400 g for 10 min at 4°C. The cell pellet was resuspended in 35 μl/vial of ice-cold RPMI-1640 medium ( Gibco-BRL, Grand Island, NY), and the cell suspension was kept on ice. To a final 200-μl volume of prewarmed (37°C) RPMI-1640 medium containing either NADPH (1 μM) or lucigenin (20 μM), 5 μl of cell suspension (0.2 × 105 cells) were added to initiate the reaction followed by immediate measurement of chemiluminescence in an Appliskan luminometer (Thermo) in out-of-coincidence mode. Appropriate blanks and controls were established, and chemiluminescence was recorded. Neither NADPH nor lucigenin enhanced the background chemiluminescence of lucigenin alone (30–40 counts/min). Chemiluminescence was measured continuously for 12 min, and the activity of NADPH oxidase was expressed as counts per million cells.

Immunofluorescence staining. Growth-arrested A549 cells were incubated with ATPγS for the indicated time intervals. After being washed twice with ice-cold PBS, cells were fixed, permeabilized, and stained using an anti-p65 antibody as previously described (26). The images were observed using a fluorescence microscope (Axiovert 200M; Zeiss).

Analysis of data. All of the data were estimated using the GraphPad Prism Program (GraphPad, San Diego, CA). Data were expressed as means ± SE and analyzed with a one-way ANOVA followed by Tukey’s post hoc test at a P < 0.05 level of significance. All of the experiments were performed at least three times.

RESULTS

ATPγS upregulates COX-2 expression. Nucleotides stimulate the release of AA, a rate-limiting step in PGE2 synthesis, and upregulate inducible COX expression (25). Thus, we investigated whether ATPγS could induce COX-2 expression. Moreover, we found that ATPγS induced COX-2 expression in a time- and concentration-dependent manner (Fig. 1A). ATPγS also induced COX-2 mRNA accumulation in a time-dependent manner (Fig. 1B). To further confirm the effect of ATPγS on COX-2 expression at the transcriptional level, COX-2 promoter luciferase gene expression was determined by COX-2 promoter assay. As shown in Fig. 1C, ATPγS stimulated COX-2 promoter activity in a time-dependent manner.

ATPγS induces COX-2 expression through NADPH oxidase/ROS. Several studies have suggested that elevated intracellular ROS may function as signaling components to mediate cell patholog-
p47phox regulatory subunit plays a critical role in acute activation of NADPH oxidase (Fig. 2, A and B). Application of edaravone, DPI, or APO markedly inhibited ATPγS-induced COX-2 mRNA expression and promoter activity (Fig. 2, A and B). The p47phox regulatory subunit plays a critical role in acute activation of NADPH oxidase (4). In addition, we have established that NOX2 is expressed in A549 cells determined by RT-PCR (data not shown). To further confirm that NADPH oxidase activation is involved in ATPγS-induced COX-2 expression, siRNAs of NOX2 and p47phox were used. As shown in Fig. 2D, transfection with either NOX2 siRNA or p47phox siRNA markedly inhibited ATPγS-induced COX-2 expression in A549 cells. Furthermore, ATPγS stimulated the translocation of p47phox from the cytosol to the membrane, which was blocked by pretreatment with APO (Fig. 2E). Similarly, NADPH oxidase activity was also increased in response to ATPγS (Fig. 2F). Finally, we demonstrated that ATPγS time-dependently induced ROS generation, which was reduced by transfection with siRNAs of NOX2 and p47phox or preincubation with edaravone, DPI, or APO (Fig. 2G). These data suggested that ATPγS stimulated NADPH oxidase/ROS-dependent COX-2 expression in A549 cells.

Involvement of c-Src in ATPγS-stimulated COX-2 expression. G protein-coupled receptor (GPCR)-mediated transactivation of receptor tyrosine kinases was mediated through the participation of intracellular component, c-Src, or extracellular regulators, metalloproteinases (33). To differentiate whether c-Src or metalloproteinases participated in ATPγS-stimulated expression of COX-2, PP1 (the inhibitor of c-Src) and GM-6001 (the inhibitor of metalloproteinases) were employed. Data showed that pretreatment with PP1 but not GM-6001 attenuated ATPγS-regulated COX-2 expression, suggesting that c-Src but not metalloproteinases may contribute to COX-2 induction (Fig. 3, A and B). Pretreatment with PP1 also inhibited ATPγS-enhanced COX-2 mRNA expression and promoter activity (Fig. 3, C and D). To confirm the character of c-Src, c-Src siRNA was used. Transfection with c-Src siRNA inhibited ATPγS-mediated COX-2 expression (Fig. 3E). We found that ATPγS stimulated c-Src phosphorylation, which was inhibited by pretreatment with PP1 (Fig. 3F). We next investigated the relationship between ROS and c-Src. As shown in Fig. 3F, pretreatment with edaravone, APO, or DPI markedly reduced ATPγS-induced c-Src activation. These data suggested that ATPγS induced ROS-dependent c-Src activation.

Participation of EGFR in ATPγS-stimulated COX-2 expression. To investigate whether the activation of receptor tyrosine kinases plays a key role in ATPγS-stimulated COX-2 expression, the inhibitors of EGFR (AG-1478) and PDGFR (AG-1296) were used. Data showed that pretreatment with AG-1478 but not AG-1296 inhibited ATPγS-stimulated COX-2 expression (Fig. 3A). ATPγS-induced COX-2 mRNA expression was inhibited by preincubation with AG-1478 (Fig. 3B). To further confirm that EGFR but not PDGFR is involved in ATPγS-stimulated COX-2 expression, siRNAs of EGFR and PDGFR were used. As shown in Fig. 3C, transfection with EGFR siRNA markedly inhibited ATPγS-induced COX-2 expression. In addition, ATPγS-stimulated COX-2 promoter activity was also inhibited by pretreatment with AG-1478 (Fig. 3D). These data revealed that EGFR may contribute to ATPγS-induced COX-2 expression. We next investigated whether ATPγS could stimulate EGFR activation. As shown in Fig. 3E, ATPγS stimulated EGFR phosphorylation, which was reduced by pretreatment with AG-1478. In
addition, ATPγS-induced EGFR phosphorylation was inhibited by pretreatment with PP1, edaravone, DPI, and APO (Fig. 4E). These data implied that ATPγS regulated EGFR phosphorylation through NADPH oxidase.

Akt activation contributes to ATPγS-mediated COX-2 expression. Several studies revealed that EGFR-dependent activation of Akt participated in various inflammatory processes. To examine whether Akt plays a key role in ATPγS-upregulated COX-2 expression, the inhibitor of PI3K, LY-294002, was used. We found that ATPγS-induced COX-2 expression was inhibited by pretreatment with LY-294002 (Fig. 5A). Pretreatment with LY-294002 also attenuated ATPγS-induced COX-2 mRNA expression and promoter activity (Fig. 5, B and C). On the other hand, ATPγS-induced COX-2 expression was inhibited by transfection with Akt siRNA (Fig. 5D). Furthermore, ATPγS stimulated Akt phosphorylation directly, which was reduced by pretreatment with LY-294002, edaravone, DPI, APO, PP1, and AG-1478 (Fig. 5, E and F). These data showed that ATPγS upregulated COX-2 expression through NADPH oxidases-dependent Akt activation.

Involvement of NF-κB in ATPγS-stimulated COX-2 expression. We investigated whether ATPγS could stimulate COX-2 expression through nuclear factor (NF)-κB. As shown in Fig. 6A, pretreatment with Bay11–7082 (an inhibitor of NF-κB) markedly inhibited ATPγS-induced COX-2 expression. ATPγS-induced COX-2 mRNA expression and promoter activity were also inhibited by pretreatment with Bay11–7082 (Fig. 6, B and C). Moreover, ATPγS stimulated NF-κB promoter activity, which was inhibited by pretreatment with edaravone, DPI, APO, PP1, AG-1478, and LY-294002 (Fig. 6, D and E). On the other hand, transfection with p65 siRNA significantly reduced p65 protein expression and ATPγS-induced COX-2 expression in A549 cells (Fig. 6F). Next, we showed that ATPγS induced p65 phosphorylation, which was reduced by pretreatment with edaravone, DPI, APO, PP1, AG-1478, and LY-294002 (Fig. 6G). In addition, ATPγS induced NF-κB p65 subunit translocation from the cytosol to the nucleus, which was inhibited by pretreatment with Bay11–7082, edaravone, APO, PP1, AG-1478, and LY-294002 (Fig. 6H). These data suggested that ATPγS induced COX-2 expression through NADPH oxidase/ROS/c-Src/EGFR/PI3K/Akt cascade-dependent NF-κB activation.

ATPγS stimulates COX-2 expression through p300. Activated NF-κB is found to complex with p300, which is a histone acetylator and functions to loosen the chromatin structure, thus facilitating the binding between transcription factors and gene promoters (26). Moreover, we found that pretreatment with GR-343 (the inhibitor of p300) inhibited ATPγS-induced COX-2 protein expression (Fig. 7A). ATPγS also induced COX-2 mRNA levels and promoter activity, which were reduced by pretreatment with GR-343 (Fig. 7, B and C). In addition, transfection with p300 siRNA reduced ATPγS-induced COX-2 expression (Fig. 7D), suggesting that p300 plays a key role in COX-2 induction in response to ATPγS. Moreover, several studies demonstrated that activated Akt has the ability to translocate into the nucleus, form complex with p300, and regulate the activities of p300 (19). Here, immunoprecipitation assay revealed that nuclear-translocated Akt complexed with p65 and p300 in a time-dependent manner with maximum response at 30 min after ATPγS stimulation (Fig. 7E). In addition, ATPγS stimulated Akt translocation (Fig. 7F). These data suggested that ATPγS led Akt nuclear localization and complexed with p65 and p300. The formation of Akt/p65/p300 complexes played an important role in regulating the acetylation of histone, which represented the loosening of chromatin structure and gene expression. As proved by Western blot analysis with specific anti-acetyl histone H4 Ab, ATPγS stimulated the acetylation of histone H4 in a time-dependent manner (Fig. 7G). Pretreatment with a ROS scavenger and the inhibitors of NADPH oxidase, c-Src, EGFR, and PI3K, significantly attenuated ATPγS-induced acetylation of histone H4 (Fig. 7G). These data suggested that ATPγS mediated COX-2 expression through NADPH oxidase/c-Src/EGFR/PI3K/Akt-dependent p300 activation and histone acetylation.

ATPγS induces PGE2 production. It has been demonstrated that the expression of COX-2 is mainly responsible for the production of prostanooid synthesis (32). Here, we found that ATPγS stimulated PGE2 release in a time-dependent manner (Fig. 8A), which was blocked by transfection with siRNAs of NOX2, p47phox, c-Src, Akt, EGFR, p65, and p300 (Fig. 8B). These results indicated that ATPγS induced PGE2 release through a NADPH oxidase-dependent signaling pathway.

DISCUSSION

Extracellular nucleotides releasing from stressed endothelial and epithelial cells or LPS-stimulated monocytes may act in an autocrine or paracrine fashion to mediate cell inflammation. Inflammation is the predominant feature of cancer, including NSCLC. It has been found that high expression levels of COX-2 exist in NSCLC that correlates to the diminished disease-free survival rate of NSCLC. However, whether ATP promoted A549 cells to express
COX-2 protein remains unknown. Here, mechanisms of ATPS/H9253S-stimulated COX-2 expression were investigated in A549 cells. We found that ATPS/H9253S caused COX-2/PGE2 expression. Moreover, ATPS/H9253S-induced COX-2 expression and PGE2 release were attenuated by pretreatment of a ROS scavenger (edaravone), the inhibitors of NADPH oxidase (DPI and APO), c-Src (PP1), EGFR (AG-1478), PI3K (LY-294002), NF-κB (Bay11–7082), and p300 (GR-343). Transfection with siRNA of NOX2, p47phox, c-Src, EGFR, Akt, p65, or p300 also reduced ATPS/H9253S-stimulated COX-2 expression. These data suggested that NADPH oxidase, c-Src, EGFR, PI3K/Akt, NF-κB, and p300 may contribute to ATPS-mediated COX-2 expression. Furthermore, ATPS led to the activation of NADPH oxidase and ROS production, which resulted in downstream phosphorylation of c-Src, EGFR, Akt, p65, and p300. Thus, increase of p300 activities impacted on enhanced acetylation of histone H4, which represented the freeing of DNA binding sites for transcription factor. In summary, ATPS-mediated COX-2 expression through upregulated activation of the NADPH oxidase/c-Src/EGFR/PI3K/Akt/NF-κB/p300 cascade.

The actions of ATP on physiology were multiple, including excitatory neurotransmission, tissue development, nociception, and platelet aggregation. However, roles of ATP in cancer were controversial. Several studies revealed that ATP may promote cytotoxicity of endometrial cells (21), colon cancer...
cells (18), esophageal cancer cells (29), and lung tumor growth (17). ATP therapy can inhibit body weight loss and cachexia (1). In addition, ATP may also facilitate proliferation of C6 glioma cells (40), breast cancer cells (41), and epithelial lung cancer (34). High expression levels of COX-2 are positively related to low survival rate and diminished disease-free survival rate of NSCLC (22). Thus, we thought that ATP may enhance the seriousness of NSCLC through promoting COX-2 expression.

ROS are found to participate in promoting lung carcinogenesis (3, 23). One of the main sources of ROS generation is NADPH oxidase, which is composed of two membrane-bound elements (gp91phox and p22phox), three cytosolic components (p67phox, p47phox, and p40phox), and a low-molecular-weight G protein (either rac 2 or rac 1) (4). Here, we proved that ATPyS stimulated membrane localization of p47phox and upregulated activities of NADPH oxidase, thus resulting in accumulation of ROS. To link the relationships between ROS and COX-2 expression in ATPyS-stimulated A549 cells, cells were pre-treated with or without AG-1478 (10 μM), MCI-186 (100 μM), DPI (10 μM), or APO (100 μM) for 1 h, and then stimulated with ATPyS (100 μM) for the indicated time or pretreated with AG-1478 (10 μM) or AG-1296 (10 μM) for 1 h, and then stimulated with ATPyS for 10 min. The phosphorylation (p) of EGFR was determined by Western blot. Data are expressed as means ± SE of three independent experiments. *P < 0.5 and **P < 0.01 compared with the cells exposed to ATPyS alone.

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Several studies demonstrated that EGFR and COX-2 have related signaling pathways that interact to regulate cellular responses (7, 10, 35). Thus, it is interesting in evaluating the correlation between EGFR and COX-2 in NSCLC. It was found that intracellular c-Src or extracellular metalloproteinases may participate in GPCR-mediated transactivation of EGFR (33). Thus, in ATPyS-stimulated A549 cells, the role...
ATP induces ROS-dependent COX-2 expression

A. Fold of basal COX-2

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B. COX-2

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C. COX-2 promoter activity

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D. Akt

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E. Time (min)

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F. Inhibitors

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Fig. 5. ATPγS induces COX-2 expression via the activation of protein kinase B (Akt). Cells were pretreated with LY-294002 for 1 h and then treated with ATPγS for 6 h (A) or 2 h (B). COX-2 protein (A) and mRNA (B) expression was detected by Western blot and RT-PCR, respectively. C: cells were transiently transfected with COX-2-luc reporter gene together with β-galactosidase plasmid, pretreated with LY-294002 for 1 h, and then stimulated with ATPγS for 4 h. COX-2 luciferase activity was determined. D: cells were transfected with scrambled siRNA or Akt siRNA and then stimulated with ATPγS for 6 h. Akt and COX-2 protein expression was detected by Western blot. Cells were pretreated with or without LY-294002 (30 µM) for 1 h and then stimulated with ATPγS (100 µM) for the indicated time (E) or pretreated with MCI-186 (100 µM), DPI (10 µM), APO (100 µM), PP1 (10 µM), or AG-1478 (10 µM) for 1 h (F) and then stimulated with ATPγS for 10 min. The phosphorylation of Akt was determined by Western blot. Data are expressed as means ± SE of three independent experiments. *P < 0.5 and #P < 0.01 compared with the cells exposed to ATPγS alone.

of GPCR in EGFR activation was investigated. Furthermore, activation of EGFR may transport its downstream signaling through activation of PI3K/Akt to regulate cell function (8). Here, we showed that ATPγS stimulated phosphorylation of c-Src, EGFR, and Akt via ROS. Pretreatment with the inhibitors of c-Src, EGFR, and PI3K or transfection with siRNA of c-Src, EGFR, or Akt attenuated ATPγS-induced COX-2 expression. These results demonstrated that, in ATPγS-stimulated A549 cells, the expression of COX-2 was regulated by ROS-dependent activation of the c-Src/EGFR/PI3K/Akt pathway. These results were consistent with those obtained on colon cancer cells indicating that activation of EGFR by transforming growth factor-α stimulates COX-2 production and PGE2 release (10) and, in NIH3T3 cells, H2O2 activates Akt via an EGFR/PI3K-dependent manner (42).

NF-κB is composed of five members: RelA (p65), RelB, Rel (cRel), NFκB1 (p50 and its precursor p105), and NFκB2 (p52 and its precursor p100) in mammals (5). Recent studies demonstrate that NSCLC exhibits dysregulated antiapoptotic pathways involving the transcription factor NF-κB (12). Based on sequence analysis, NF-κB has been identified...
Fig. 7. ATP$_\gamma$S induces COX-2 expression via the activation of p300. Cells were pretreated with GR343 for 1 h and then treated with ATP$_\gamma$S for 6 h (A) or 2 h (B). COX-2 protein (A) and mRNA (B) expression was detected by Western blot and RT-PCR, respectively. C: cells were transiently transfected with COX-2-luc reporter gene together with β-galactosidase plasmid, pretreated with GR-343 for 1 h, and then stimulated with ATP$_\gamma$S for 4 h. COX-2 luciferase activity was determined. D: cells were transfected with scrambled siRNA or p300 siRNA and then stimulated with ATP$_\gamma$S for 6 h. p300 and COX-2 protein expression was detected by Western blot. E: cells were transfected with scrambled siRNA or p300 siRNA and then stimulated with ATP$_\gamma$S for 6 h. COX-2 protein expression was detected by Western blot. F: cells were transfected with scrambled siRNA or p300 siRNA and then stimulated with ATP$_\gamma$S for 6 h. COX-2 protein expression was detected by Western blot. G: cells were transfected with scrambled siRNA or p300 siRNA and then stimulated with ATP$_\gamma$S for 6 h. COX-2 protein expression was detected by Western blot. 

*P < 0.5 and #P < 0.01 compared with the cells exposed to ATP$_\gamma$S alone [A, C, and G (below)]. *P < 0.01 compared with the vehicle treatment (G, above).
as transcriptional regulatory elements on the COX-2 gene (20). Here, we found that ATP<sub>H9253</sub>S led to nuclear localization and phosphorylation of p65 together with enhancement of NF-κB promoter activity. ATP<sub>H9253</sub>S-mediated COX-2 expression and PGE<sub>2</sub> release were abrogated by pretreatment with the NF-κB inhibitor or transfection with p65 siRNA. Moreover, we found that ATP<sub>H9253</sub>S-mediated NF-κB activation was inhibited by the inhibitors of NADPH oxidase, ROS, c-Src, EGFR, and PI3K. These data suggested that ATP<sub>H9253</sub>S-induced COX-2 expression and PGE<sub>2</sub> release were mediated via NADPH oxidase/c-Src/EGFR/PI3K-dependent NF-κB activation. These results were consistent with those obtained on TNF-α-treated A549 cells showing that expression of COX-2 is mediated by Akt-activated NF-κB (13).

p300 has been implicated in transcriptional responses to disparate extracellular and intracellular signals and has the activity of histone acetyltransferase to regulate chromatin structure and cellular program determination (16). Moreover, our data showed that pretreatment with GR343 or transfection with p300 siRNA inhibited ATP<sub>H9253</sub>S-induced COX-2 expression and PGE<sub>2</sub> release. Furthermore, we proved that activated Akt translocated into nucleus and complexed with p65 and p300 in response to ATP<sub>H9253</sub>S. Thus, ATP<sub>H9253</sub>S enhanced p300 activities, resulting in an increase of histone H4 acetylation. These results were similar to the effect of human papillomavirus E6 and E7 oncoproteins on cervical cancer cells, indicating that expression of COX-2 is also regulated by p300 protein (36).

In conclusion, we showed that ATP<sub>H9253</sub>S stimulated NADPH oxidase activation, resulting in ROS generation, which then activated the downstream c-Src/EGFR/PI3K/Akt/NF-κB/p300 cascade to regulate the expression of COX-2 and synthesis of PGE<sub>2</sub> in A549 cells. Based on observations from the literature and our findings, Fig. 8C depicts a model for the signaling mechanisms underlying ATP<sub>H9253</sub>S-induced COX-2 expression in A549 cells. These findings imply that ATP<sub>H9253</sub>S might play a key role in pathological processes of NSCLC. Targeting COX-2 and their upstream signaling components should yield useful therapeutic targets for treatment of NSCLC.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


