BAI, a 3-aminoindazole derivative, inhibits interleukin-1β-induced expression of cyclooxygenase-2 in A549 human airway cells

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Abstract. Cyclooxygenase (COX)-2 and its products, including PGE_2, are key inflammatory mediators. In this study, we have assessed the pharmacological characteristics of BAI, a 3-aminoindazole derivative and a novel cyclin-dependent kinase (CDK) inhibitor, for regulation of COX-2 expression induced by interleukin (IL)-1β in A549 human airway cells. Treatment with BAI strongly inhibited IL-1β-induced expression of COX-2 at both the protein and mRNA levels. Results of luciferase experiments also revealed that BAI treatment reduced IL-1β-induced COX-2 promoter activity. Distinctly, treatment with BAI did not affect IL-1β-induced phosphorylation of extracellular signal-regulated protein kinase-1/2 (ERK-1/2), p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal protein kinase-1/2 (JNK-1/2) and proteolysis of IκB-α, an inhibitor of nuclear factor (NF)-κB, but inhibited IL-1β-induced phosphorylation of histone H1, a target for phosphorylation by CDKs. siRNA transfection experiments demonstrated that knockdown of CDK2 and CDK4 led to a slight reduction of IL-1β-induced histone H1 phosphorylation but had no effect on IL-1β-induced COX-2 expression. Interestingly, additional cell culture experiments showed the ability of BAI to repress the PMA-induced COX-2 expression in A549 cells and serum-dependent COX-2 expression in NCI-H292 cells, a human laryngeal cell line. Collectively, these results demonstrate firstly that BAI downregulates IL-1β-induced COX-2 expression through transcriptional repression, which appears to be independent of CDK2, CDK4, MAPKs and NF-κB, in A549 cells. It is suggested that BAI may be a potential candidate for treatment of the airway inflammatory diseases where COX-2 overexpression is problematic.

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Introduction

Cyclooxygenase (COX), also referred to as prostaglandin (PG) H synthase, is the rate-limiting enzyme in the biosynthesis of PGs and related eicosanoids from arachidonic acid metabolism (1). Physiologically, PGs are involved in the inflammatory response, bone development, wound healing, and reproductive system. However, excessive PGs play a pathogenic role in inflammatory and neoplastic diseases (2,3).

In eukaryotic cells, COX is expressed in two isoforms, COX-1 and COX-2. While COX-1 is constitutively expressed in most cells and the COX-1-derived PGs are involved in the maintenance of physiological functions, COX-2 is inducible by pro-inflammatory cytokines, tumor promoters, mitogens, and growth factors in many cell types, including monocytes, fibroblasts, and endothelial cells (1-5). Importantly, evidence that non-steroidal anti-inflammatory drugs or compounds that inhibit COX-2 lessen major inflammatory symptoms including fever and pain strongly suggests the role of COX-2 in inflammation (6). Expression of COX-2 gene is controlled at the transcriptional, post-transcriptional, and/or translational level. For example, transcription of COX-2 is induced in cells following the exposure of exogenous stimuli, which leads to activation of intracellular signaling pathways that in turn modulate the activity of transcription factors and hence stimulate the COX-2 promoter (7). Multiple cis-acting elements within the COX-2 promoter, such as cyclic AMP responsive element, activator protein-1, nuclear factor (NF)-interleukin (IL)-6, and NF-κB, are found to be critical for the COX-2 transcriptional induction (8,9). In addition, post-transcriptional COX-2 RNA nuclear export and COX-2 mRNA stabilization have shown to be necessary for maximal COX-2 induction (10-13). Regulation of cellular COX-2 protein levels by protein degradation has also been reported (14,15). COX-2 protein N-glycosylation at a co-translational step is also suggested to be critical for the protein stability, localization, and activity (15-19). There are also many reports indicating that activities of many intracellular signaling proteins, including extracellular signal-regulated protein kinase-1/2 (ERK-1/2), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal

protein kinase-1/2 (JNK-1/2), and protein kinase B (PKB) are necessary for the transcriptional, post-transcriptional, and/or translational upregulation of COX-2 expression (20-24).

In a previous study, we synthesized a chemical compound, 2-([1,1'-biphenyl]-4-yl)-N-(5-(1,1-dioxo-1λ^6-isothiazolidin-2-yl)-1H-indazol-3-yl)acetamide (BAI), one of the 3-aminoindazole derivatives, as a novel cyclin-dependent kinase (CDK) inhibitor (25) and reported that BAI inhibits proliferation and induces apoptosis via activation of caspases in head and neck cancer cells (26), suggesting its anticancer activity. To gain insight into the BAI's anti-inflammatory potential, in this study, we evaluated the effect of BAI on IL-1β-mediated induction of COX-2 expression in A549 human airway cells and determined the possible molecular and cellular mechanisms involved.

Materials and methods

Materials. RPMI-1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Welgene (Daegu, Korea). Antibodies of phospho-ERK-1/2 (p-ERK-1/2), total ERK-1/2 (T-ERK-1/2), phospho-JNK-1/2 (p-JNK-1/2), total JNK-1/2 (T-JNK-1/2), phospho-p38 MAPK (p-p38 MAPK), total p38 MAPK (T-p38 MAPK), phospho-PKB (p-PKB), and total PKB (T-PKB) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-COX-2 rabbit polyclonal antibody was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Anti-rabbit or mouse secondary horseradish peroxidase antibodies were bought from Amersham Biosciences (Amersham, UK). RNAzol-B reagent was purchased from Tel-Test (Houston, TX, USA). Lipofectamine Plus reagents were purchased from Invitrogen (Carlsbad, CA, USA). Enzyme-linked chemiluminescence (ECL) Western detection reagents were purchased from Thermo Scientific (Waltham, MA, USA). An antibody for phospho-histone H1 (p-histone H1) and nitrocellulose membranes were acquired from Millipore (Rockford, IL, USA). IL-1β was purchased from R&D Systems (Minneapolis, MN, USA). Antibodies of CDK2 and CDK4 and siRNAs of scrambled, CDK2, and CDK4 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). Other reagents, including PMA, were purchased from Sigma (St. Louis, MO, USA).

Cell culture. Human airway cells, A549 (lung) or NCI-H292 (laryngeal), were maintained at 37˚C in a humidified condition of 95% air and 5% CO_2 in RPMI supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Preparation of whole cell lysates. After treatment at the indicated times or conditions, A549 cells or NCI-H292 cells were washed with ice-cold phosphate-buffered saline (PBS) supplemented with 1 mM Na_3VO_4 and 1 mM NaF, and lysed with a modified RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA and 1 mM EGTA, proteinase inhibitor cocktail (1X)]. After centrifugation at 12,000 rpm for 20 min at 4˚C, the supernatant was collected and the protein concentration was determined with Bradford reagent (Bio-Rad, Mississauga, ON, Canada) using bovine serum albumin as the standard.

Western blot analysis. Equal amounts of protein (40 μg/lane) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. The membrane was washed with Tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl) containing 0.05% Tween-20 (TBST) and blocked in TBST supplemented with 5% non-fat dried milk. The membrane was further incubated with primary antibodies against COX-2 (1:2,000), p-histone H1 (1:2,000), CDK2 (1:2,000), CDK4 (1:2,000), p-JNK-1/2 (1:2,000), T-JNK-1/2 (1:2,000), p-ERK-1/2 (1:2,000), T-ERK-1/2 (1:2,000), p-p38 MAPK (1:2,000), T-p38 MAPK (1:2,000), IκB-α (1:2,000) or actin (1:10,000). The membrane was subsequently incubated with the appropriate secondary antibodies coupled to horseradish peroxidase and developed by ECL Western detection reagents.

Reverse transcription-polymerase chain reaction (RT-PCR). After treatments at the indicated times or conditions, total cellular RNA was isolated from the conditioned cells using RNAzol-B reagent according to the manufacturer's instructions. Five micrograms of total-RNA were reverse transcribed using 8 μl of M-MLV RT 5X buffer, 3 μl of 10 mM dNTPs, 0.45 μl of 10,000 U RNAse inhibitor, 0.3 μl of 50,000 U M-MLV reverse transcriptase (Promega, Madison, WI), and 1.5 μl of 50 pM oligo(dT) (Bioneer, Chungbuk, Korea) in 40 μl volume. Single stranded cDNA was then amplified by PCR using 4 μl of 5X green Go Taq Flexi buffer, 0.4 μl of 10 mM dNTPs, 0.1 μl of 500 U Taq polymerase, 1.2 μl of 25 mM $MgCl_2$ (Promega), and 0.4 μl of each 20 pM of specific sense and antisense primer of COX-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR products were analyzed on 1.2% agarose gels. The primer sequences used by PCR were as follows: COX-2, forward, 5'-CCG CGT CAG TAT CAA CTG CG-3' and reverse 5'-CAA TCA TCA GGC ACA GGA GG-3'; GAPDH, forward, 5'-CGT CTT CAC CAC CAT GGA GA-3' and reverse 5'-CGG CCA TCA CGC CAC ACT TT-3'. The PCR conditions applied were: COX-2, 25 cycles of denaturation at 94˚C for 30 sec, annealing at 59˚C for 30 sec, and extension at 72˚C for 30 sec; GAPDH, 25 cycles of denaturation at 94˚C for 30 sec, annealing at 56˚C for 30 sec, and extension at 72˚C for 30 sec, respectively. GAPDH was used as an internal control to evaluate the relative expression of COX-2.

Analysis of COX-2 mRNA stability. A549 cells were initially grown in the absence or presence of IL-1β for 6 h to highly induce cellular levels of COX-2 mRNA. Cells were then treated with IL-1β alone or IL-1β plus BAI for 10, 30, 45 or 60 min in the presence of ActD, a transcription inhibitor, to block ongoing transcription. Total-RNA was isolated and subjected to COX-2 or GAPDH RT-PCR at each time point to determine the mRNA amounts of each gene that remained in the cells.

Analysis of COX-2 protein stability. A549 cells were initially grown in the absence or presence of IL-1β for 6 h to highly induce cellular levels of COX-2 protein. Cells were then treated with IL-1β alone or IL-1β plus BAI for 2, 4 or 8 h in the absence or presence of CHX, a translation inhibitor, to block ongoing

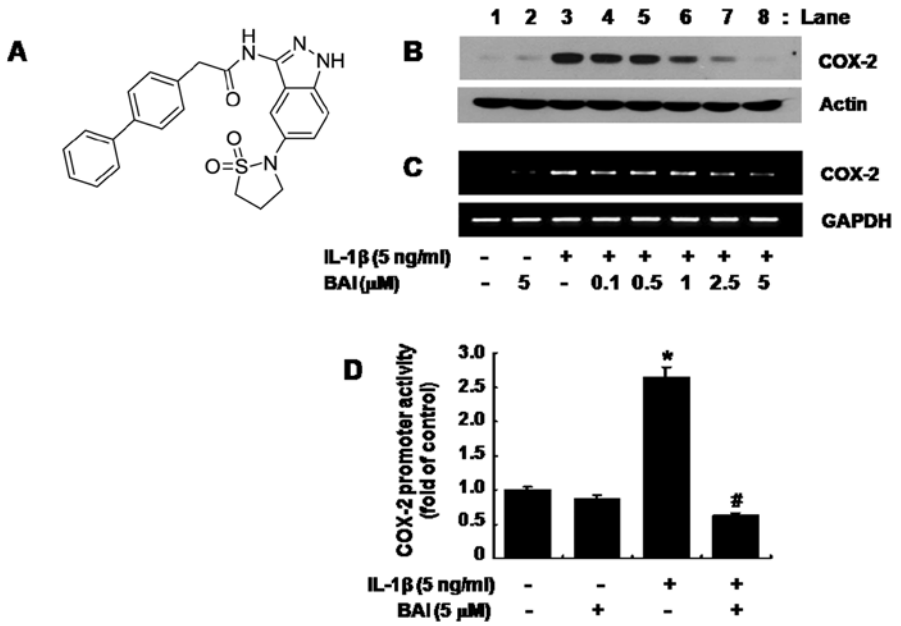

Figure 1. The structure of BAI and the effect of BAI on COX-2 protein and mRNA expressions and promoter activity in IL-1β-treated A549 cells. (A) The chemical structure of BAI. (B and C) A549 cells were pretreated without or with the indicated doses of BAI for 1 h and treated without or with IL-1β in the absence or presence of BAI for an additional 6 h. Whole cell lysates and total-RNA were prepared and subjected to immunoblot analysis (B) and to RT-PCR (C), respectively. Data in (B) or (C) are representative from three independent experiments. (D) A549 cells were co-transfected with 1 μg of the human COX-2 promoter-containing luciferase DNA along with 20 ng of control pRL-TK DNA for 24 h. Transfected cells were pretreated without or with BAI for 1 h and treated without or with IL-1β in the absence or presence of BAI for additional 6 h. Whole cell lysates were prepared, and used for the reporter gene activity. Data are mean ± SE of three independent experiments. *P<0.05 compared to the values of control (no IL-1β); $^{\#}$P<0.05 compared to the values of IL-1β without BAI.

translation. At each time, whole cell lysate was prepared and subjected to immunoblot analysis for COX-2 or actin to determine the amounts of each protein remaining in the cells.

Luciferase sssay. A549 cells grown in 24-well plates were transfected for 24 h with a luciferase DNA construct containing a COX-2 promoter. Cells were treated with or without IL-1β in the absence or presence of BAI for additional 6 h. Cell lysates were prepared and assayed with the luciferase assay system (Promega) using a TD 20/20 lumino-meter (Turner Designs Instruments).

siRNA transfection. A549 cells were plated at $2x10^6$ cells/well in 6-well plates. After overnight incubation, cells were transfected with control siRNA (80 nM) or siRNAs of CDK2 (40 nM) and CDK4 (40 nM) using Lipofectamine Plus reagents. At 48 h post-transfection, the transfected cells were treated without or with IL-1β in the absence or presence of BAI for additional 6 h. Cell lysates were then prepared and analyzed by Western blotting to determine expression of CDK2, CDK4, COX-2 or actin protein.

Statistical analysis. Results are expressed as means ± standard error (SE). The significance of the differences was determined by one-way ANOVA. Differences were considered significant at P-value <0.05.

Results

BAI inhibits COX-2 protein and mRNA expressions and promoter activity in IL-1β-treated A549 cells. The chemical structure of BAI is shown in Fig. 1A. Initially, the effect of different concentrations of BAI on expression of COX-2 protein induced by IL-1β in A549 cells was investigated. As shown in Fig. 1B, compared with control (lane 1), the exposure of IL-1β for 6 h into A549 cells largely increased the expression of COX-2 protein (lane 3). However, treatment with BAI suppressed IL-1β-induced COX-2 protein expression in a concentration-dependent manner (lanes 4-8). In particular, treatment with BAI at 5 μM almost completely repressed expression of COX-2 protein by IL-1β (lane 8). Expression of control actin protein was not affected in A549 cells treated without or with IL-1β for 6 h in the absence or presence of BAI at the doses tested (Fig. 1B, lanes 1-8).

RT-PCR experiments were next performed to evaluate the effect of BAI on the expression of COX-2 mRNA induced by IL-1β. As shown in Fig. 1C, compared with control (lane 1), the exposure of IL-1β for 6 h into A549 cells largely increased the expression of COX-2 mRNA (lane 3). However, BAI treatment also inhibited IL-1β-induced COX-2 mRNA expression in a concentration-dependent fashion (lanes 4-8). Expression of control GAPDH mRNA remained constant in A549 cells treated without or with IL-1β for 6 h in the absence or presence of BAI at the doses tested (Fig. 1C, lanes 1-8). Due to strongest inhibitory effect on IL-1β-induced COX-2 protein and mRNA expression levels, the 5 μM dose of BAI was chosen for further studies. Luciferase experiments were next utilized to determine whether BAI-mediated downregulation of COX-2 mRNA was due to inhibition of COX-2 promoter activity. As shown in Fig. 1D, compared with control (column 1), treatment with IL-1β for 6 h stimulated COX-2 promoter-driven luciferase expression in A549 cells (column 3). However, BAI

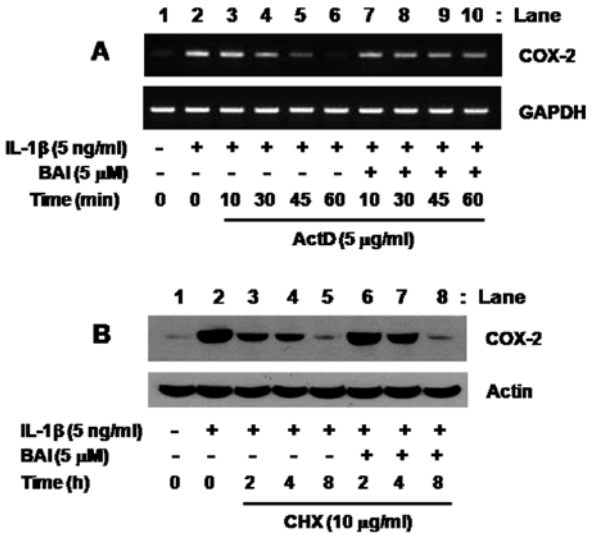

Figure 2. The effect of BAI on COX-2 protein or mRNA stability in IL-1β-treated A549 cells. (A) A549 cells were initially treated without or with IL-1β for 6 h to highly induce COX-2 mRNA and then exposed to IL-1β without or with BAI in the absence or presence of actinomycin D (ActD), a transcriptional inhibitor, for the indicated times. At each time point, total-RNA was isolated and used for COX-2 or GAPDH RT-PCR to measure the amounts of COX-2 mRNA that remained in the cells at the respective time. (B) A549 cells were initially treated without or with IL-1β for 6 h to highly induce COX-2 protein and then exposed to IL-1β without or with BAI in the absence or presence of cycloheximide (CHX), a translational inhibitor, for the indicated times. At each time, whole cell lysates were prepared and analyzed by immunoblot analysis using antibodies for COX-2 or actin to measure the amounts of COX-2 protein that remained in the cells at the respective time. The data are representative from three independent experiments.

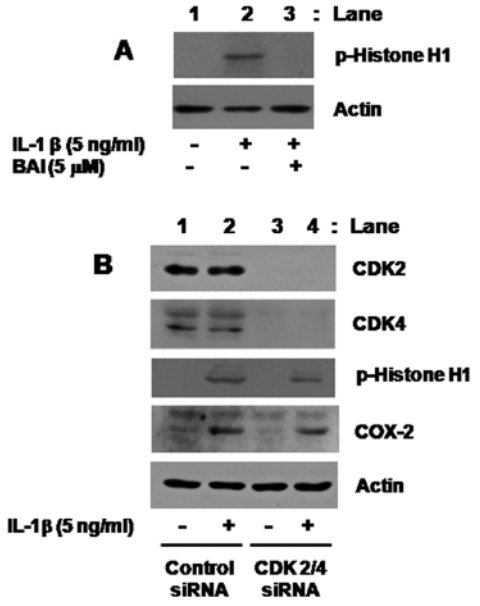

Figure 3. The effect of BAI or CDK2 and CDK4 knockdowns on IL-1β-induced histone H1 phosphorylation and/or COX-2 expression in A549 cells. (A) A549 cells were pretreated without or with BAI for 1 h, and treated with IL-1β alone or IL-1β plus BAI for additional 6 h. Whole cell lysates were prepared and analyzed by immunoblot analysis for measuring the amounts of p-histone H1 or actin. (B) A549 cells were transfected with control siRNAs (80 nM) or the mixture of CDK2 (40 nM) and CDK4 (40 nM) siRNAs. At 48 h post-transfection, cells were treated without or with IL-1β for an additional 6 h. Whole cell lysates were prepared and analyzed by Western blotting for measuring the amounts of CDK2, CDK4, COX-2, p-histone H1 or actin. p-Histone H1, phosphorylated histone H1. The picture in (A) or (B) is a representative from three independent experiments.

treatment strongly repressed IL-1β-induced COX-2 promoter-driven luciferase expression (column 4). Data of MTS and cell survival analyses indicated no cytotoxicity of BAI at 5 μM in A549 cells (data not shown).

BAI does not influence COX-2 mRNA and protein stability in IL-1β-treated A549 cells. In addition to the transcriptional regulation, COX-2 expression was also affected at the stability of COX-2 mRNA and/or protein. The effect of BAI on the stability of COX-2 mRNA was next determined by ActD chase experiments. As shown in Fig. 2A, when ongoing transcription was blocked in the presence of ActD, the amounts of COX-2 mRNA induced by IL-1β were decreased within 1 h (lanes 2-6), suggesting the intrinsic instability of COX-2 mRNA. BAI treatment, however, did not further accelerate COX-2 mRNA degradation (lanes 7-10); rather BAI treatment at the time of 45 and 60 min seemed to enhance COX-2 mRNA stability. Expression of GAPDH mRNA remained constant in A549 cells treated without or with IL-1β in the absence or presence of BAI at the doses and times tested (Fig. 1A, lanes 1-10). The effect of BAI on the stability of COX-2 protein was further analyzed by CHX chase experiments. As shown in Fig. 2B when ongoing translation was blocked in the presence of CHX, the amounts of COX-2 protein induced by IL-1β were decreased in a time-dependent manner (lanes 2-5), suggesting that COX-2 protein is also unstable and is degraded within 8 h. However, BAI treatment also did not accelerate COX-2 protein degradation (lanes 6-8). Expression of actin protein was not affected in A549 cells treated without or with IL-1β in the absence or presence of BAI at the doses and times tested (Fig. 2B, lanes 1-8).

BAI-mediated downregulation of COX-2 expression in IL-1β-treated A549 cells is independent of CDK2 and CDK4. Considering BAI as a novel CDK inhibitor, we hypothesized that BAI might exert its inhibitory effect on COX-2 expression in IL-1β-treated A549 cells via modulation of CDKs. CDKs are involved in the cell cycle in part by phosphorylating histone H1, a CDK downstream effector (27). This promptly led us to investigate the effect of BAI on phosphorylation of histone H1 in IL-1β-treated A549 cells. As shown in Fig. 3A compared with control (lane 1), IL-1β treatment strongly induced phosphorylation of histone H1 in A549 cells (lane 2). However, IL-1β-induced phosphorylation of histone H1 in A549 cells was not shown by treatment with BAI (lane 3). Expression of actin protein remained constant in A549 cells treated without or with IL-1β in the absence or presence of BAI (Fig. 3A, lanes 1-3). To directly see whether activities of CDKs, herein CDK2 and CDK4 are necessary for IL-1β-induced histone H1 phosphorylation and COX-2 expression in A549 cells, CDK2 and CDK4 siRNA transfection experiments were next carried out. As shown in Fig. 3B there was a substantial expression of endogenous CDK2 and CDK4 in scrambled siRNA-transfected A549 cells (lane 1 and 2). On the other hand, CDK2 and CDK4 expressions were not seen in CDK2 and CDK4 siRNA-transfected A549 cells (lane 3 and 4), indicating the high transfection efficiency. As shown in Fig. 3B, compared with IL-1β-induced

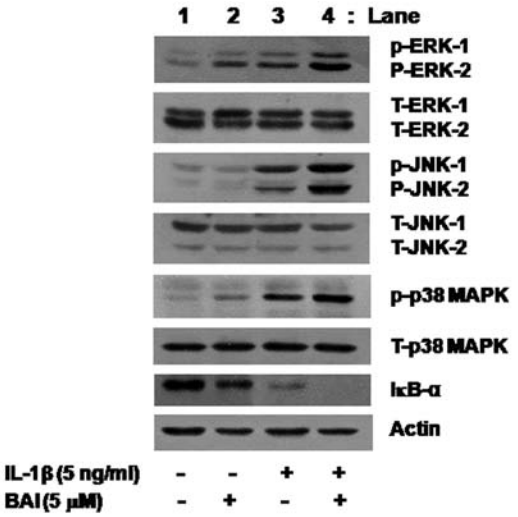

Figure 4. The effect of BAI on the phosphorylation of MAPKs and proteolysis of IκB-α in IL-1β-treated A549 cells. A549 cells were pretreated without or with BAI for 1 h and then treated without or with IL-1β in the absence or presence of BAI for additional 0.5 h. Whole cell lysates were prepared and analyzed by immunoblot analysis for measuring the amounts of p-ERK-1/2, ERK-1/2, p-JNK-1/2, JNK-1/2, p-p38 MAPK or p38 MAPK (A) and IκB-α or actin (B). p-ERK-1/2, phosphorylated ERK-1/2; p-JNK-1/2, phosphorylated JNK-1/2; p-p38 MAPK, phosphorylated p38 MAPK; T-ERK-1/2, total ERK-1/2; T-JNK-1/2, total JNK-1/2; T-p38 MAPK, total p38 MAPK. The results shown are representative of three independent experiments.

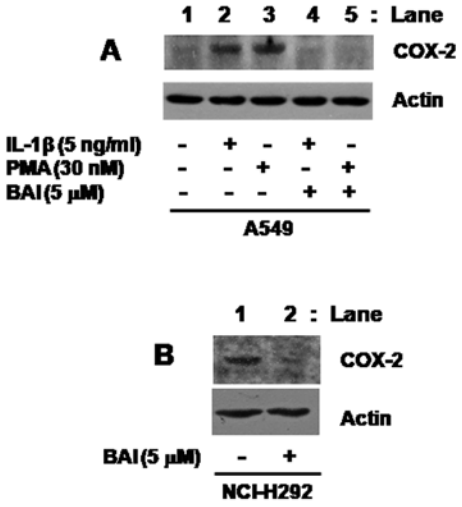

Figure 5. The effect of BAI on the PMA- or serum-induced COX-2 expression. (A) A549 cells were treated without or with IL-1β or PMA, other COX-2 inducer, in the absence or presence of BAI for 6 h. Whole cell lysates were prepared and subjected to immunoblot analysis. (B) NCI-H292 human laryngeal cells were grown in cell culture media containing 10% FBS in the presence or absence of BAI for 6 h. Whole cell lysates were prepared and subjected to immunoblot analysis. The data shown are representative of three independent experiments.

phosphorylation level of histone H1 in scrambled siRNA-transfected A549 cells (lane 2), there was less phosphorylation of histone H1 by IL-1β in CDK2 and CDK4-depleted A549 cells (lane 4). Of note, as shown in Fig. 3B, there was no difference in IL-1β-induced COX-2 expression in scrambled or CDK2 and CDK4 siRNA-transfected A549 cells (lane 2 and 4). Expression of actin protein remained constant in scrambled or CDK2 and CDK4 siRNA-transfected A549 cells treated without or with IL-1β (Fig. 3B, lanes 1-4).

BAI does not interfere with IL-1β signaling to induce phosphorylation of MAPKs and proteolysis of IκB-α in A549 cells. COX-2 expression is also influenced by activities of a variety of proteins, such as the family of MAPKs (ERK-1/2, JNK-1/2 and p38 MAPK) and NF-κB transcription factor. Therefore, we next investigated whether BAI treatment affects activation of MAPKs and NF-κB in IL-1β-treated A549 cells. In this study, activation of the family of MAPKs and NF-κB, respectively, was assessed by measuring the phosphorylation level of each member of the MAPKs and proteolysis of IκB-α (an inhibitor of NF-κB activation) in A549 cells treated without or with IL-1β in the absence or presence of BAI. As shown in Fig. 4, compared with the control (lane 1), the exposure of IL-1β into A549 cells increased not only the phosphorylation of ERK-1/2, JNK-1/2, and p38 MAPK but also proteolysis of IκB-α (lane 3). Western blot analysis applying an antibody, that recognizes the total expression levels of ERK-1/2, JNK-1/2, or p38 MAPK, into the stripped immunoblot used for detecting phosphorylated forms of ERK-1/2, JNK-1/2, or p38 MAPK demonstrated that IL-1β treatment did not affect total expression level of ERK-1/2, JNK-1/2, and p38 MAPK, suggesting the ability of IL-1β to increase phosphorylation level of pre-existed ERK-1/2, JNK-1/2, and p38 MAPK in A549 cells without *de novo* protein synthesis of these proteins. Notably, BAI treatment did not interfere with IL-1β-induced phosphorylation of ERK-1/2, JNK-1/2 and p38 MAPK and proteolysis of IκB-α in A549 cells (lane 4); rather BAI treatment slightly augmented phosphorylation of ERK-1/2, JNK-1/2 and p38 MAPK in A549 cells treated without or with IL-1β (lane 2 or 4). As shown in Fig. 4, expression of actin remained constant in A549 cells treated without or with IL-1β in the absence or presence of BAI (lanes 1-4).

BAI is also capable of inhibiting phorbol-12-myristate-13-acetate (PMA)-induced COX-2 in A549 cells and serum-dependent expression of COX-2 in NCI-H292 cells. The specificity of the BAI effects were assessed by examining its effect on the expression of COX-2 induced by PMA, another COX-2 inducer (28), in A549 cells. As shown in Fig. 5A, compared with the control (lane 1), the exposure of IL-1β or PMA into A549 cells stimulated the expression of COX-2 (lane 2 or 3). Apparently, BAI treatment that had a strong repressive effect on IL-1β-induced expression of COX-2 (lane 4) also largely suppressed PMA-induced COX-2 expression in A549 cells (lane 5). Expression of actin remained constant in A549 cells treated without or with IL-1β or PMA in the absence or presence of BAI (Fig. 5A, lanes 1-5). In a recent study, we demonstrated the serum-dependent induction of COX-2 expression in NCI-H292 cells, a human laryngeal cell line (29). As shown in Fig. 5B, high COX-2 expression was detected in NCI-H292 cells grown in normal culture media containing only FBS (lane 1). However, BAI treatment strongly inhibited the serum-mediated induction of COX-2 expression in NCI-H292 cells (lane 2). Expression of actin remained constant in NCI-H292 cells treated without or with BAI (Fig. 5B, lane 1 and 2).

Discussion

Chemicals that inhibit COX-2 have the potential to be clinically useful against inflammatory diseases. In this study, we have assessed pharmacological characteristics of BAI, one of 3-aminoindazole derivatives for regulation of COX-2 expression induced by IL-1β in A549 human airway cells.

The inflammatory cytokine IL-1β is involved in inflammatory processes by inducing its receptor-mediated expression of many effector proteins, including COX-2. Overexpression of COX-2 is linked to airway inflammatory disorders. COX-2 expression is regulated at the transcriptional, post-transcriptional, and translational level. Thus, the present findings that BAI downregulates COX-2 protein and mRNA expressions (Fig. 1A and B) and promoter activity (Fig. 1C), but does not affect the stability of COX-2 mRNA and protein (Fig. 2A and B) in IL-1β-treated A549 cells strongly indicate that BAI targets COX-2 at the transcriptional level in IL-1β-treated A549 cells. Considering COX-2 transcriptional upregulation by IL-1β is largely associated with activities of a variety of intracellular signaling components and transcription factors (7-9,12,21,22,24), we hypothesized that BAI-mediated COX-2 transcriptional repression might be due to the ability of BAI to interfere with the IL-1β signal to trigger activation of signaling proteins and/or transcription factors responsible for COX-2 transcriptional induction in IL-1β-treated A549 cells.

It has been demonstrated that IL-1β signals to activate multiple intracellular signaling proteins, including ERK-1/2, p38 MAPK, and JNK-1/2, and transcription factors, such as NF-κB, and their activations are critical for IL-1β-mediated induction of COX-2 expression in A549 cells (9,12,19,22). In resting cells, NF-κB resides in the cytoplasm by interacting with IκB-α, a NF-κB inhibitory protein. However, when cells are exposed to inflammatory cytokines, IκB-α is rapidly (within 1 h) phosphorylated, ubiquitinated, and degraded by the 26S proteasomal complex (30), thereby enabling NF-κB to localize into the nucleus where NF-κB participates in the transcriptional regulation of many target genes, including COX-2. In the present study, we have shown that BAI does not inhibit the IL-1β signal to induce the phosphorylation of ERK-1/2, p38 MAPK, and JNK-1/2, and proteolysis of IκB-α in A549 cells (Fig. 4), suggesting the independence of MAPKs and NF-κB pathways in BAI-mediated COX-2 transcriptional repression in IL-1β-treated A549 cells.

The transcriptional regulation of genes is primarily influenced by the structure of chromatin. Chromatin structure is affected by chromatin components or chromatin modifiers, including histone H1 and high-mobility group proteins. Among those, histone H1 is a linker protein involved in the higher-order and compact chromatin structure by interacting with nucleosomes (31) and has been reported to be post-translationally modified, by phosphorylation, acetylation, and methylation (32,33). Of note, there are several reports to indicate that phosphorylation of histone H1 plays an important role in regulating specific gene expression, as demonstrated by that loss of histone H1 causes both upregulation and downregulation of specific gene expression (34,35). Of further interest, there are previous studies demonstrating that phosphorylated histone H1 is enriched in transcriptionally active chromatin (36), histone H1 phosphorylation affects chromatin condensation (37,38), and phosphorylation of histone H1 leads to destabilization of the chromatin structure, allowing for access to the chromatin template of factors required for gene expression (39).

In this study, we have shown the ability of BAI to repress phosphorylation of histone H1 induced by IL-1β in A549 cells (Fig. 3A), which may, albeit weakly, suggest that inhibition of histone H1 phosphorylation by BAI may cause transcriptionally inactive chromatin and inaccessibility of transcriptional machineries or factors responsible for COX-2 transcriptional stimulation in IL-1β-treated A549 cells. At present, however, precise mechanisms of how BAI induces inhibitions of histone H1 phosphorylation and COX-2 transcriptional inactivation in response to IL-1β signal remain unclear.

There are previous reports to show phosphorylation of histone H1 by CDKs, including CDK2 (40-42). There is further evidence of CDK2-dependent COX-2 expression in the IL-1β-treated H358 human non-small cell lung carcinoma cell line under serum starvation conditions, as determined by CDK2 siRNA transfection or treatment with BMS-387082, a potent CDK2 inhibitor (42). However, the present findings that siRNA-mediated knockdown of CDK2 and CDK4 does not greatly affect IL-1β-mediated induction of histone H1 phosphorylation and COX-2 expression in A549 cells (Fig. 3B) suggest no involvement of these CDKs in IL-1β-induced histone H1 phosphorylation and COX-2 expression in A549 cells. As a CDK inhibitor, BAI is not likely to exert its repressive effects on IL-1β-induced histone H1 phosphorylation and COX-2 expression in A549 cells through modulation of the expression and/or activities of CDK2 and CDK4. BAI was originally synthesized as a CDK2 inhibitor with the IC_{50} concentration of 14 nM, based on the investigation of the X-ray structure of CDK2 in association with the docking study with the ATP binding pocket of the protein kinase (25). It therefore appears that the 5 μM concentration of BAI, which inhibits IL-1β-induced COX-2 expression in A549 cells, is much far above the dose to inhibit CDK2 activity and thus BAI at such a high concentration may inhibit not only CDK2 and other CDKs but also many cellular protein kinases with similar structure and/or the ATP binding pocket of CDK2, which may contribute to the BAI's repressive effect on COX-2 transcription in response to the IL-1β signal. This notion may be strengthened by a recent study demonstrating inhibition of JNK-2 by the aminoindazoles (43), whose structure is similar to BAI herein.

The present study additionally provides experimental evidence that BAI-mediated repression of COX-2 expression is not limited to the IL-1β signaling in A549 cells, which is deduced from that BAI inhibits the PMA-induced COX-2 expression in A549 cells (Fig. 5A) and the serum-dependent expression of COX-2 in the NCI-H292 human laryngeal cell line (Fig. 5B).

In conclusion, we demonstrate for the first time the ability of BAI to inhibit the COX-2 expression by transcriptional repression in IL-1β-treated A549 cells. BAI-mediated COX-2 transcriptional repression appears to be independent of MAPKs, NF-κB, CDK2 and CDK4. Considering COX-2 as a key inflammatory mediator, it is suggested that BAI (or its derivatives) may be potential candidates for the treatment of inflammatory diseases in which overexpression of COX-2 is problematic.

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