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Overexpression of Cyclooxygenase-2 in NCI-H292 Human Alveolar Epithelial Carcinoma Cells: Roles of p38 MAPK, ERK-1/2, and PI3K/PKB Signaling Proteins

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ABSTRACT

Evidence suggests overexpression of COX-2 and its role in many human cancers, including lung. However, the regulatory mechanism underlying COX-2 overexpression in lung cancer is not fully understood. We herein investigated whether COX-2 is overexpressed in human airway cancer cell lines, including A549 (lung), Hep-2 (bronchial), and NCI-H292 (alveolar). When grown in cell culture medium containing 10% FBS (serum), of note, there was strong and transient induction of COX-2 protein and mRNA in NCI-H292 cells, but little or low COX-2 expression is seen in A549 or Hep-2 cells. Interestingly, strong and sustained activities of ERK-1/2, JNK-1/2, p38 MAPK, and PKB were also shown in NCI-H292 cells grown in presence of serum. Profoundly, results of pharmacological inhibition studies demonstrated that the serum-dependent COX-2 up-regulation in NCI-H292 cells is attributed to not only the p38 MAPK-, PI3K/PKB-, and ERK-1/2-mediated COX-2 transcriptional up-regulation but also the p38 MAPK- and ERK-1/2-mediated post-transcriptional COX-2 mRNA stabilization. Of further note, it was shown that the ERK-1/2 and PI3K/PKB (but not COX-2, p38 MAPK, and JNK-1/2) activities are necessary for growth of NCI-H292 cells. These findings collectively demonstrate for the first time that COX-2 expression is transiently up-regulated by serum addition in NCI-H292 cells and the serum-induced COX-2 expression is closely linked to the p38 MAPK-, ERK-1/2-, and PI3K/PKB-mediated COX-2 transcriptional and post-transcriptional up-regulation. J. Cell. Biochem. 112: 3015–3024, 2011.

KEY WORDS: COX-2; NCI-H292 CELLS; p38 MAPK; PKB; ERK-1/2

Cyclooxygenase (COX), also known as prostaglandin H synthase, is the rate-limiting enzyme in the biosynthesis of PGs from arachidonic acid [Smith et al., 2000]. Under physiological conditions, PGs involve in normal inflammatory responses, bone development, wound healing, and reproductive function [Smith and DeWitt, 1996]. However, if excessive, those PGs play causative roles in many inflammatory and neoplastic diseases [Vane et al., 1998].

Abbreviations: Act D, actimonycin D; CHX, cycloheximide; COX, cyclooxygenase; COX-2, cyclooxygenase-2; ERK-1/2, extracellular signal-regulated protein kinase-1/2; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK-1/2, c-Jun N-terminal protein kinase-1/2; MAPKs, mitogen-activated protein kinases; PGs, prostaglandins; PGE_2, prostaglandin E_2; PKB, protein kinase B; p38 MAPK, p38 mitogen-activated protein kinase.

Suhaeng Sung and Yukyung Park are equally contributed to this work.

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COX is expressed in two isoforms: COX-1 and COX-2 [Hla et al., 1993; Smith and DeWitt, 1996; Vane et al., 1998]. COX-1 is constitutively expressed in most cell types and the COX1-derived PGs involves in the above-mentioned physiological functions. On the other hand, COX-2 is inducible and its expression is largely increased in cells following the exposure of interleukin-1β, lipopolysaccharide, growth factors, hypoxia, or ultraviolet [Hla et al., 1993; Smith and DeWitt, 1996; Vane et al., 1998; Chen et al., 2001; Cho et al., 2005]. COX-2 overexpression is also frequently observed in many tumors, including colon and lung, and its overexpression correlates with the malignant changes in human cancers [Dubois et al., 1998; Hawk et al., 2002]. Interestingly, the detection of a markedly higher COX-2 expression in human lung adenocarcinomas but a lower expression of COX-2 in squamous cell carcinomas or small cell lung cancers has been reported [Petkova et al., 2004; Liu et al., 2006; Tong et al., 2006]. Importantly, clinical evidence that administration of COX-2 inhibitors reduces tumor in the patients with lung cancer [Czembirek et al., 2009; Mutter et al., 2009] suggests a role of COX-2 expression (and activity) in lung cancer.

At present, the regulatory mechanism by which COX-2 is overexpressed in lung cancer remains unclear. Primarily, COX-2 expression is regulated at the transcriptional level. Transcriptional induction of COX-2 gene is largely dependent of activities of multiple transcription factors, including NF-κB, NF-IL6, and AP-1, that specifically bind *cis*-acting elements within the promoter of COX-2 gene [Inoue et al., 1995; Newton et al., 1997]. COX-2 mRNA stabilization at the post-transcriptional level is also necessary to achieve maximal COX-2 expression [Ristimaki et al., 1994; Srivastava et al., 1994; Jang et al., 2003]. In addition, COX-2 expression (and/or activity) is further regulated at the translational levels such as the protein stability (turnover) and/or *N*-glycosylation status [Otto et al., 1993; Mbonye et al., 2006, 2008; Jang et al., 2007; Kang et al., 2007]. Many recent studies have also demonstrated involvement of signal transduction pathways, including ERK-1/2, p38 MAPK, JNK-1/2, and PI3K/PKB, in up-regulating COX-2 expression at the transcriptional, post-transcriptional, and/or translational stages [Jang et al., 2000, 2004, 2005; Chen et al., 2001; Hunot et al., 2004]. Interestingly, the activity of many signaling proteins in human tumor cells and their roles in tumor cell growth and/or oncogenic gene expression have been introduced [Xu et al., 2004; Cao et al., 2007; Yang et al., 2010]. However, little is known as to the relationship between activity of signaling proteins in lung cancer cells and/or their roles in COX-2 gene expression and lung cancer cell growth.

In this study, we investigated whether COX-2 is overexpressed in human airway cancer cell lines, including A549 (lung), Hep-2 (bronchial), or NCI-H292 (alveolar), and if any, delineated the molecular and signaling mechanisms associated.

MATERIALS AND METHODS

MATERIALS

Antibodies of phospho-ERK-1/2 (p-ERK-1/2), ERK-1/2, phospho-JNK-1/2 (p-JNK-1/2), JNK-1/2, phospho-p38 MAPK (p-p38 MAPK), p38 MAPK, phospho-PKB (p-PKB), and PKB were purchased from Cell Signaling Tech. (Beverly, MA). Anti-COX-2 polyclonal antibody was purchased from Cayman Chemicals (Ann Arbor, MI). Anti-rabbit or mouse secondary horseradish peroxidase antibodies were bought from Amersham Biosciences (Amersham, UK). SB203580 (SB, a p38 MAPK inhibitor), LY294002 (LY, a PI3K inhibitor), and PD98059 (PD, an ERK-1/2 inhibitor) were purchased from Biomol (Plymouth, PA). SP600125 (SP, a JNK-1/2 inhibitor) and NS-398 (a COX-2 enzymatic inhibitor) were obtained from Calbiochem (La Jolla, CA). Anti-actin antibody, actinomycin D (Act D), and cycloheximide (CHX) were purchased from Sigma–Aldrich (St. Louis, MO).

CELL LINES AND CULTURE

Three human airway epithelial carcinoma cell types with different origin were used in this study, including NCI-H292 (alveolar, ATCC CRL-1848), A549 (lung, ATCC CCL-185), and Hep-2 (bronchial, ATCC CCL-23). H292 or A549 cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Hep-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. The passage number of each cell line used in present study ranged from 100 to 110th.

CELL TREATMENTS

Typically, 3×10^5/ml cells were seeded the day before treatments without or with serum and/or inhibitors in each well of six-well plates in 2 ml volume for sample preparation of Western blot or RT-PCR. Briefly, to see the degree of COX-2 expression in NCI-H292, A549, or Hep-2 cells, the cells were grown in culture media containing 10% FBS (serum) for 8 h. For time course experiments, cells were grown in culture media without or with serum for 0, 2, 4, 8, or 24 h. For pharmacological inhibition studies, cells were pretreated with SB (25 μM), PD (50 μM), SP (25 μM), or LY (25 μM) for 1 h. The culture media were then removed from cells and cells were grown in fresh culture media containing serum in absence or presence of each inhibitor for additional 8 h. After treatments at the indicated time, whole cell lysates or total mRNA were prepared or extracted from the conditioned cells, and further analyzed by Western blot or RT-PCR.

WESTERN BLOT ANALYSIS

Aforementioned, following treatments at the indicated times or conditions, cells were washed with ice-cold phosphate-buffered saline (PBS) supplemented with 1 mM Na_3VO_4 and 1 mM NaF and lysed in a modified RIPA buffer [50 mM Tris–Cl (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, 1 mM EGTA, proteinase inhibitor cocktail (1×)]. 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) using bovine serum albumin as the standard. Equal amounts of protein (40 μg/lane) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and

transferred onto a nitrocellulose membrane (Millpore Co., Bedford, MA). 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 of p-ERK-1/2 (1:2,000), ERK-1/2 (1:2,000), p-JNK-1/2 (1:2,000), JNK-1/2 (1:2,000), p-p38 MAPK (1:2,000), p38 MAPK (1:2,000), p-PKB (1:2,000), PKB (1:2,000), COX-2 (1:2,000), and β-actin (1:10,000). The membrane was subsequently incubated with appropriate secondary antibodies coupled to horseradish peroxidase and developed in the enzyme-linked chemiluminescence (ECL) Western detection reagents (Amersham Pharmacia Biotech, Oakville, ON).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

After treatments at the indicated times or conditions, which were in detail described in cell treatment section above, total cellular RNA was isolated from the conditioned cells using TriZol-reagent (Invitrogen, Carlsbad, CA) according to manual instructions provided by the manufacturer. Five micrograms of total RNA were reverse transcribed using 8 μl of M-MLV RT 5× 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 5× 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 anti-sense primer of COX-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR products were analyzed on 1.2% agarose gel. The primer sequences used by PCR were as follows: COX-2 forward, 5'-CCG CGT CAG TAT CAA CTG CG-3', reverse 5'-CAA TCA TCA GGC ACA GGA GG-3'; GAPDH forward, 5'-CGT CTT CAC CAC CAT GGA GA-3', reverse 5'-CGG CCA TCA CGC CAC ACT TT-3'. The PCR conditions applied were: COX-2, 25 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s; GAPDH, 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, respectively.

REAL-TIME PCR

After treatments with each pharmacological inhibitor, total cellular RNA was isolated from the conditioned cells using TriZol-reagent (Invitrogen) according to manual instructions provided by the manufacturer. Five micrograms of total RNA were reverse transcribed using 8 μl of M-MLV RT 5× 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), and 1.5 μl of 50 pM oligo dT (Bioneer, Chungbuk, Korea) in 40 μl volume. The RT reactions were performed at 42°C for 60 min. Real-time PCR was performed with a LightCycler 2.0 Instrument and LightCycler FastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. PCR amplification mixtures (20 μl) contained 25 ng template cDNA, 3 mmol $MgCl_2$, 0.5 pmol primer, and 2 μl LightCycler FastStart DNA Master SYBR Green I. For detection of the COX-2 mRNA, a combination of a forward primer, 5'-CCG CGT CAG TAT CAA CTG CG-3', reverse 5'-CAA TCA TCA GGC ACA GGA GG-3'; GAPDH forward, 5'-CGT CTT CAC CAC CAT GGA GA-3', reverse 5'-CGG CCA TCA CGC CAC ACT TT-3', was used. The following LightCycler conditions were used: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for annealing at 56°C for 5 s, elongation at 72°C for 5 s and cooling at 40°C for 30 s. *Ct* (fit point method) and *Cp* (second derivative method) values were determined by the LightCycler® 480 software version 1.2 and exported into an MS Excel data sheet (Microsoft) for analysis. In order to normalize the differences in the amount of total RNA used in each reaction, we performed the amplification of GAPDH RNA as internal control.

CELL PROLIFERATION ASSAY

NCI-H292 cells were seeded in 96-well plates at 1×10^4 cells/plate in 100 μl volume. After overnight incubation, cells were treated without or with NS-398 (40 μM), SB (25 μM), LY (25 μM), or PD (50 μM) for 8, 24, and 48 h, respectively. At each time, cells were treated with 20 μl of the mixture of phenazine methosulfate in 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) and phenazine ethosulfate and then incubated at 37°C in 5% CO_2 for 1 h. Color change by formazan product was measured at the absorbance of 490 nm.

CELL COUNT ANALYSIS

NCI-H292 cells were seeded in 24-well plates at 1×10^5 cells/plate in 500 μl volume. After overnight incubation, cells were treated without or with NS-398 (40 μM), SB (25 μM), LY (25 μM), or PD (50 μM) for 8, 24, and 48 h, respectively. At each time, cells were detached from each well plate using 0.25% trypsin EDTA. An aliquot of cell suspension was exposed to trypan blue and spotted on Burker hemocytometer (Marienfeld GmbH, Marienfeld, Germany). The number of surviving cells, which were not stained with the dye, was counted under microscope.

ANALYSIS OF COX-2 MRNA STABILITY

NCI-H292 cells were seeded in each well of six-well plates at 3×10^5/plate in 2 ml volume. After overnight incubation, cells were primarily grown in culture media containing serum for 4 h to highly induce COX-2 transcripts and then treated without or with SB (25 μM), LY (25 μM), or PD (50 μM) in presence of Act D (2.5 μM) for 0, 1, 2, 4, or 8 h. At each time, total RNA was isolated and subjected to COX-2 or GAPDH RT-PCR to determine the amounts of each mRNA remained in the cells.

ANALYSIS OF COX-2 PROTEIN STABILITY

NCI-H292 cells were seeded in each well of six-well plates at 3×10^5/plate in 2 ml volume. After overnight incubation, cells were primarily grown in culture media containing serum for 4 h to highly induce COX-2 proteins and then treated without or with SB (25 μM), LY (25 μM), or PD (50 μM) in presence of CHX (10 μg/ml), a translation inhibitor, for 0, 2, 4, 8, or 24 h. At each time, whole cell lysates were prepared and subjected to immunoblot analysis for COX-2 or actin to determine the amounts of each protein remained in the cells.

STATISTICAL ANALYSES

Results are expressed as mean ± SE. The significance of difference was determined by one-way ANOVA. Differences were considered significant when P value was <0.05.

RESULTS

STRONG EXPRESSION OF COX-2 PROTEIN AND mRNA IN NCI-H292 CELLS

We initially investigated whether COX-2 is expressed in several human airway cancer cells with different origin. For this, A549 (lung), Hep-2 (bronchial), or NCI-H292 (laryngeal) cells plated overnight were grown in fresh media containing 10% FBS (serum) for 8 h. Whole cell lysates and total RNA were prepared and analyzed by Western blot and RT-PCR, respectively. Profoundly, while COX-2 protein expression was not seen in A549 or Hep-2 cells, there was an increased expression of COX-2 protein in NCI-H292 cells (Fig. 1A). RT-PCR experiments were then carried out to see expression of COX-2 transcripts in A549, Hep-2, or NCI-H292 cells. Similar to the pattern of COX-2 protein expression, while COX-2 mRNA expression was not detected in A549 or Hep-2 cells, there was an increased expression of COX-2 mRNA in NCI-H292 cells (Fig. 1B). Results of real-time PCR also showed the serum-induced expression of COX-2 mRNA in NCI-H292 cells (data not shown). It was thus suggested that increased COX-2 protein expression by serum addition in NCI-H292 cells was due to increased COX-2 transcripts. Control actin protein or GAPDH mRNA expression was constant in A549, Hep-2, or NCI-H292 cells. We next investigated whether cell–plastic interactions contribute to high expression of COX-2 in NCI-H292 cells. For this, NCI-H292, A549, or Hep-2 cells were grown under the same experimental conditions except in the suspension status. Notably, when cultured in the suspension conditions (using cell culture dishes without coating materials), COX-2 was still highly expressed in NCI-H292 cells, but there was no COX-2 expression in A549 or Hep2 cells (Fig. 1C), indicating that COX-2 overexpression in NCI-H292 cells is not due to cell–plastic interaction. Since the cell–plastic interaction is essential for cell viability for adherent cells, we also checked whether NCI-H292 cells grown in the suspension conditions remain alive. Results of cell count assays showed no difference in the survival of NCI-H292 cells between adherent and suspension conditions (Fig. 1D). These results collectively demonstrate an increased expression of COX-2 at the protein and mRNA levels in NCI-H292 cells.

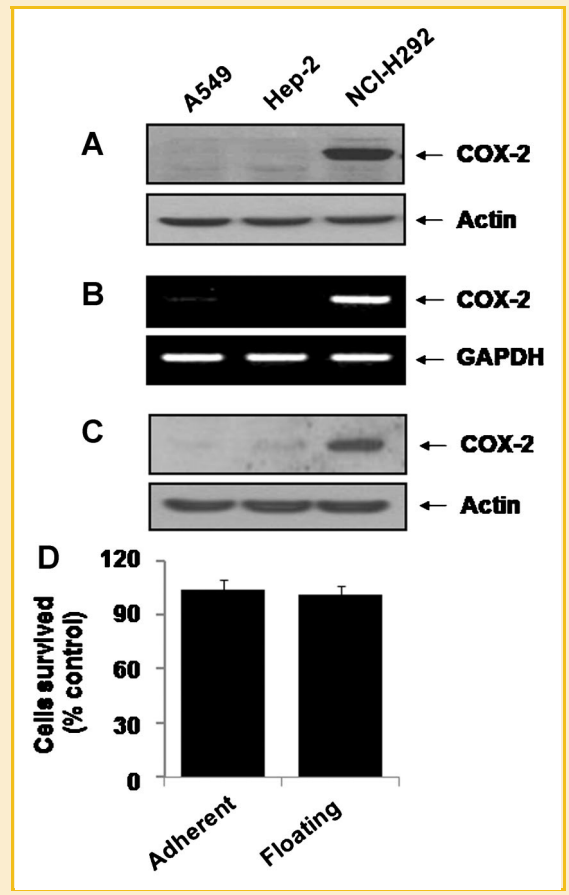

Fig. 1. Strong up-regulation of COX-2 protein and mRNA in NCI-H292 cells. A,B: A549, Hep-2, or NCI-H292 cells (6×10^5 cells/2 ml, respectively) seeded the day before treatments were grown in fresh culture media containing 10% FBS for 8 h. Whole cell lysates or total RNA were prepared, and analyzed by Western blot (A) or RT-PCR (B). C: A549, Hep-2, or NCI-H292 cells were grown in the same culture conditions as (A) except in cell suspension status using specific cell culture dishes with no coating materials. Whole cell lysates were prepared, and analyzed by Western blot. D: NCI-H292 cells were grown for 8 h in the same culture conditions as in (A) (adherent in culture dish) or in (C) (floating in culture dish). The number of cells survived was then determined by cell count analysis. Data are mean ± SE of three independent experiments. The picture in (A, B, or C) is a representative of three independent experiments.

COX-2 SERUM-DEPENDENT AND TRANSIENT COX-2 UP-REGULATION IN NCI-H292 CELLS

Kinetic studies were next performed to see the time of COX-2 protein and mRNA expression in NCI-H292 cells. For this, NCI-H292 cells plated the day before were grown in fresh culture media without or with serum for 0, 2, 4, 8, 24, or 48 h. At each time, whole cell lysates or total RNA were prepared and analyzed by Western blot or RT-PCR. As shown in Figure 2A, at time 0 h (lane 1), there was substantial expression of COX-2 at the protein and mRNA levels in NCI-H292 cells. However, in absence of serum, cellular levels of COX-2 protein were sharply declined over time (lanes 2–6). In presence of serum, compared with COX-2 protein levels at 0 h (lane 1), there was no significant change in expression levels of COX-2 protein at 2 h (lane 7). However, there was much induction of COX-2 protein in NCI-H292 cells at 4 or 8 h (lane 8 or 9), followed drastic reduction at 24 or 48 h (lane 10 or 11). As shown in Figure 2B, in absence of serum, there was a time-dependent decline of COX-2 mRNA levels (lanes 2–6). Notably, in presence of serum, compared with COX-2 mRNA levels at 0 h (lane 1), there was much induction of COX-2 mRNA at 2 or 4 h (lane 7 or 8), followed decline thereafter (lanes 9–11). The semi-quantitative analysis of Figure 2A,B is shown in Figure 2C,D, respectively. We next carried out additional time course experiments to see whether COX-2 is also induced by serum in other types of lung cancer cells A549 or Hep-2. As shown in Figure 2E, low levels of COX-2 protein were expressed in A549 at time 0 h (lane 1). Rather, cellular levels of COX-2 protein

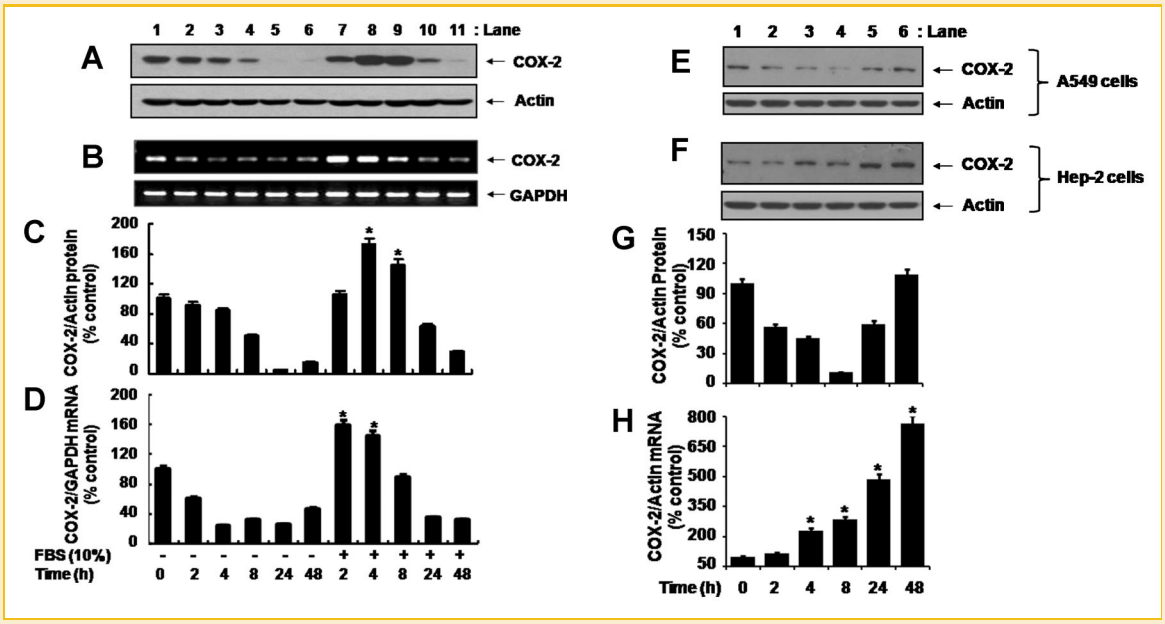

Fig. 2. Time course experiments for COX-2 expression in NCI-H292, A549, or Hep-2 cells grown in absence or presence of serum. A,B: NCI-H292 cells (6×10^5 cells/2 ml) seeded the day before treatments were grown in fresh culture media without or with 10% FBS for the indicated times. At each time, whole cell lysates or total RNA were prepared, and analyzed by Western blot (A) or RT-PCR (B). (C) and (D) are the semi-quantitative analysis of (A) and (B), respectively. Data are mean ± SE of three independent experiments. The graphs (C) and (D), respectively, show COX-2 protein and mRNA levels normalized to actin protein and mRNA levels as percentage of the value at the time 0. $^*P < 0.05$ compared to the value obtained at the same time point (h) in absence of FBS. E,F: A549 (E) or Hep-2 cells (F) at the density of 6×10^5 cells/2 ml seeded the day before treatments were grown in presence of 10% FBS for the indicated times. Whole cell lysates were prepared, and analyzed by Western blot. (G) and (H) are the semi-quantitative analysis of (E) and (F), respectively. Data are mean ± SE of three independent experiments. The graph (E) or (F) shows COX-2 protein levels normalized to actin protein as percentage of the value at the time 0. $^*P < 0.05$ compared to the value obtained at the time 0.

in A549 cells were decreased at 2, 4, or 8 h (lanes 2–4) but returned to basal level at 24 or 48 h (lane 5 or 6). On the other hand, as shown in Figure 2F, in Hep-2 cells, COX-2 protein levels were slightly increased at 4 or 8 h (lane 3 or 4) and there was much induction of COX-2 protein at 24 or 48 h (lane 5 or 6). The semi-quantitative analysis of Figure 2E,F is shown in Figure 2G,H, respectively.

INCREASED ACTIVITIES OF ERK-1/2, JNK-1/2, p38 MAPK, AND PKB BY SERUM IN NCI-H292 CELLS

Considering data of Figures 1 and 2 in present study, it was evident that NCI-H292 cells might have a unique system to up-regulate COX-2 expression in response to serum exposure. Given that COX-2 overexpression is largely dependent of activities of many intracellular signaling components, including the family of MAPKs and PKB [Jang et al., 2000, 2005; Chen et al., 2001; Hunot et al., 2004], kinetic studies were next utilized to see any change of activities of MAPKs and PKB in NCI-H292 cells upon the exposure of serum. In this study, activation of signaling protein was assessed by the amounts of phosphorylated forms of each protein in response to serum addition. As shown in Figure 3 (lanes 1–6), data of Western blot with antibodies recognizing total protein expression of ERK-1/2, p38 MAPK, JNK-1/2, or PKB showed that ERK-1/2, p38 MAPK, JNK-1/2, and PKB were endogenously expressed in NCI-H292 cells and their total expression levels were not changed by addition of serum for the indicated times (0–48 h). Profoundly, results of Western blot with respective phospho-specific antibody demonstrated a time-dependent activation of ERK-1/2, p38 MAPK, JNK-1/

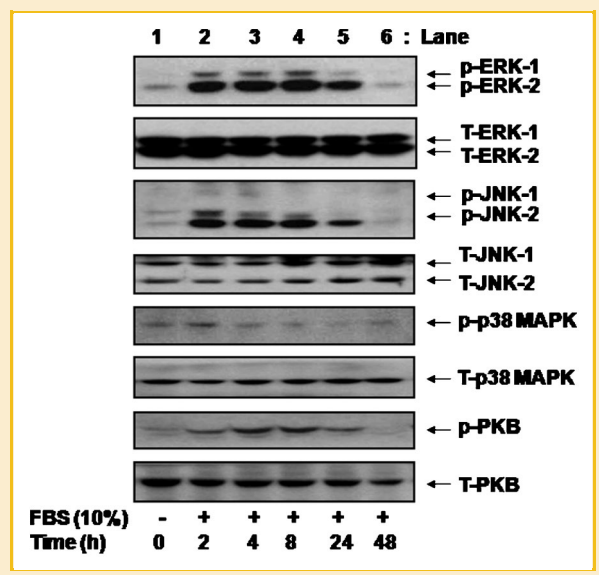

Fig. 3. Time course experiments for the expression and activity of MAPKs or PKB in NCI-H292 cells. NCI-H292 cells (6×10^5 cells/2 ml) seeded the day before treatments were grown in fresh culture media containing 10% FBS for the indicated times. At each time, whole cell lysates were prepared, and analyzed by Western blot. Each picture is a representative of three independent experiments. p-ERK-1/2, phospho-ERK-1/2; p-JNK-1/2 phospho-JNK-1/2; p-p38 MAPK, phospho-p38 MAPK; p-PKB, phospho-PKB; T-ERK-1/2, total ERK-1/2; T-JNK-1/2, total JNK-1/2; T-p38 MAPK, total p38 MAPK; T-PKB, total PKB.

2, and PKB by serum addition in NCI-H292 cells. Especially, there were increased and sustained activities of ERK-1/2, p38 MAPK, JNK-1/2, and PKB by serum treatment at 2, 4, and/or 8 h (lanes 2–4) in which high COX-2 protein and/or mRNA expression was induced in NCI-H292 cells. However, activities of ERK-1/2, p38 MAPK, JNK-1/2, and PKB were gradually or sharply declined at 24 or 48 h (lane 5 or 6), suggesting that activation of these signaling proteins by serum seemed to be also transient. Notably, results of additional kinetic analyses using serum addition for short periods (5–30 min) demonstrated that there was an increased phosphorylation ERK-1/2, but not p38 MAPK and JNK-1/2 in NCI-H292 cells exposed to serum for such short times (data not shown), suggesting an early and sustained activation of ERK-1/2 but a late and sustained activation of p38 MAPK, JNK-1/2, and PKB in response to serum exposure.

DOWN-REGULATION OF COX-2 PROTEIN AND mRNA EXPRESSIONS IN NCI-H292 CELLS BY PHARMACOLOGICAL INHIBITOR OF p38 MAPK, PI3K/PKB, OR ERK-1/2

We next determined whether activities of ERK-1/2, p38 MAPK, JNK-1/2, and/or PKB are necessary for COX-2 up-regulation by serum in NCI-H292 cells. For this, NCI-H292 cells plated the day before were treated without or with SB203580 (SB, a p38 MAPK inhibitor), SP600125 (SP, a JNK-1/2 inhibitor), LY294002 (LY, a PI3K/PKB inhibitor), or PD98059 (PD, an ERK-1/2 inhibitor), followed measurement of COX-2 protein and mRNA levels in the conditioned cells by Western blot and RT-PCR, respectively. As shown in Figure 4A,B, treatment with SB (lane 2) or SP (lane 4) strongly inhibited expression of COX-2 at the protein and mRNA levels. Moreover, treatment with PD (lane 5) also had an effect to substantially reduce COX-2 expression at the protein and mRNA levels. However, treatment with SP600125 (lane 3) did not influence COX-2 protein and mRNA expressions. The semi-quantitative analysis of Figure 4A,B is shown in Figure 4C,D, respectively. Data of real-time PCR further showed strong repression of the serum-induced COX-2 mRNA expression by SB, LY, or PD (Fig. 4E).

p38 MAPK- AND ERK-1/2-MEDIATED POST-TRANSCRIPTIONAL COX-2 mRNA STABILIZATION IN NCI-H292 CELLS

Aforementioned, COX-2 up-regulation is also attributed to increased COX-2 mRNA and/or protein stability. Act D and CHX chase experiments were next carried out to see the stability of COX-2 mRNA and protein in NCI-H292 cells in response to serum exposure, respectively. As shown in Figure 5A, results of Act D chase assays revealed that when transcription was blocked, COX-2 mRNA was fairly stable by 8 h in NCI-H292 cells (lanes 2–5), suggesting post-transcriptional stabilization of COX-2 mRNA in NCI-H292 cells

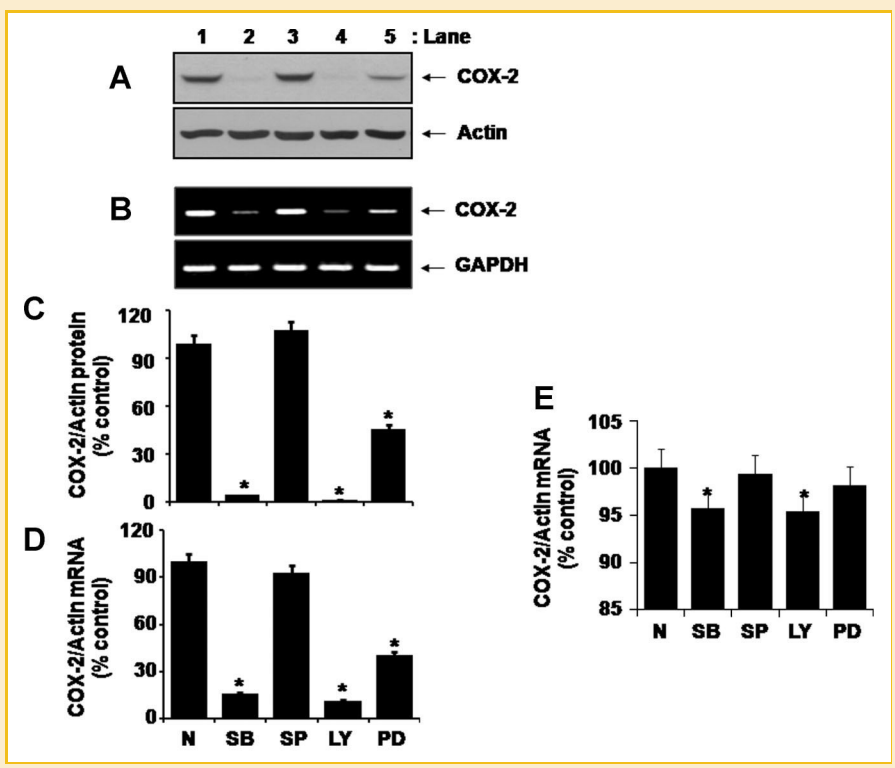

Fig. 4. Effects of treatment with pharmacological inhibitors on COX-2 expression in NCI-H292 cells. A,B: NCI-H292 cells (6×10^5 cells/2 ml) seeded the day before treatments were pretreated for 1 h with a p38 MAPK inhibitor SB203580 (SB, 25 μM), a JNK-1/2 inhibitor SP600125 (SP, 25 μM), a PI3K/PKB inhibitor LY294002 (LY, 25 μM), or an ERK-1/2 inhibitor PD98059 (PD, 50 μM). The culture media were then removed from cells and cells were grown in fresh media containing 10% FBS in absence or presence of each inhibitor for additional 8 h. Whole cell lysates or total RNA were then prepared and analyzed by Western blot (A) or RT-PCR (B). (C) and (D) are the semi-quantitative analysis of (A) and (B), respectively. Data are mean ± SE of three independent experiments. The graphs (C) and (D), respectively, show COX-2 protein and mRNA levels normalized to actin protein and mRNA levels as percentage of the value of control. $^*P < 0.05$ compared to the value of control. E: An aliquot of total RNA used for (B) was analyzed by real-time PCR. Data are mean ± SE of three independent experiments. The graph shows COX-2 mRNA levels normalized to actin mRNA levels as percentage of the value of control. $^*P < 0.05$ compared to the value of control.

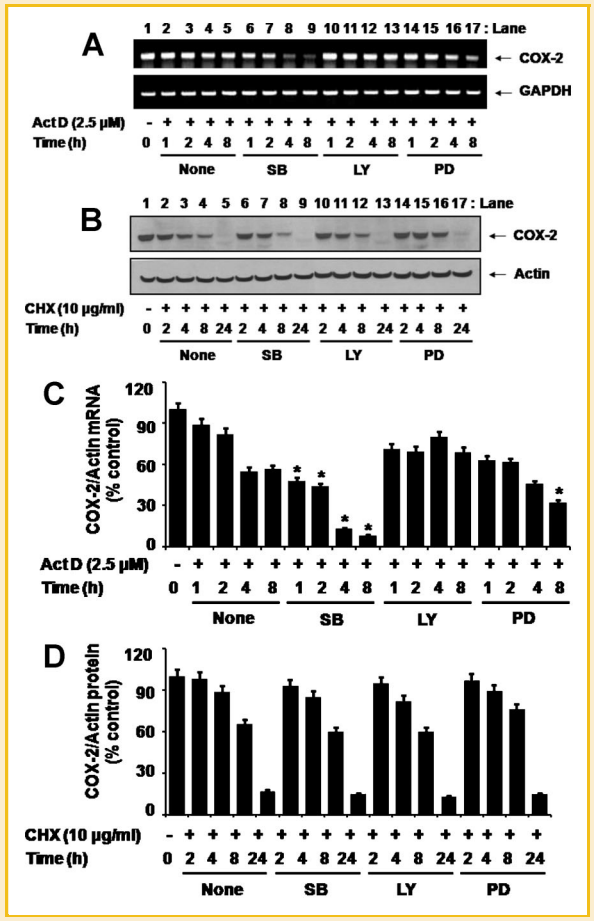

Fig. 5. Effects of treatment with pharmacological inhibitors on COX-2 mRNA or protein stability in NCI-H292 cells. A: NCI-H292 cells (6×10^5 cells/2 ml) seeded the day before treatments were primarily grown in fresh media containing 10% FBS for 4 h to induce high expression levels of COX-2 mRNA. Cells were exposed to fresh media containing 10% FBS and a transcriptional inhibitor actinomycin D (Act D) in absence or presence of SB203580 (SB, 25 μM), LY294002 (LY, 25 μM), or PD98059 (PD, 50 μM) for additional 1, 2, 4, or 8 h. At each time, total RNA was isolated and used for RT-PCR to measure the amounts of COX-2 or GAPDH mRNA remained in the cells, compared with those of COX-2 or GAPDH mRNA at 0 h (lane 1). B: NCI-H292 cells (6×10^5 cells/2 ml) seeded the day before treatments were primarily grown in fresh media containing 10% FBS for 4 h to induce high expression levels of COX-2 protein. Cells were then exposed to fresh media containing 10% FBS and a translational blocker cycloheximide (CHX) in absence or presence of SB, LY, or PD (50 μM) for additional 2, 4, 8, or 24 h. At each time, whole cell lysates were isolated and used for Western blotting to measure the amounts of COX-2 or actin protein remained in the cells, compared with those of COX-2 or actin protein at 0 h (lane 1). (C) and (D) are the semi-quantitative analysis of (A) and (B), respectively. Data are mean ± SE of three independent experiments. The graphs (C) and (D), respectively, show COX-2 mRNA and protein levels normalized to actin mRNA and protein levels as percentage of the value at 0 h. $^*P < 0.05$ compared to the value obtained at the same time point (h) in absence of any drug.

upon the exposure of serum. Profoundly, treatment with the p38 MAPK inhibitor (SB) led to a time-dependent strong reduction of COX-2 mRNA levels (lanes 6–9). Furthermore, albeit weak, there was also decline of COX-2 mRNA levels by at 4 or 8 h treatment with the ERK-1/2 inhibitor (PD; lane 16 or 17). However, treatment with the PI3K/PKB inhibitor (LY) did not affect the COX-2 mRNA stability (lanes 10–13). As shown in Figure 5B, data of CHX chase experiments showed that when translation was blocked, there was a time-dependent sharp decrease of COX-2 protein levels (lanes 2–5), suggesting that COX-2 protein is rapidly degraded in the conditioned cells. Treatment with SB (lanes 6–9), LY (lanes 10–13), or PD (lanes 14–17), however, did not modulate the intrinsic COX-2 protein turnover in NCI-H292 cells. The semi-quantitative analysis of Figure 5A,B is shown in Figure 5C,D, respectively.

ROLES OF INCREASED EXPRESSION AND/OR ACTIVITY OF COX-2, PI3K/PKB, ERK-1/2, AND/OR p38 MAPK IN GROWTH OF NCI-H292 CELLS

We next determined the relationship between increased expression and/or activity of COX-2, PKB, ERK-1/2, or p38 MAPK and growth of NCI-H292 cells. For this, NCI-H292 cells were treated without or with NS-398 (a COX-2 enzymatic inhibitor), SB, LY, or PD for 0, 8, 24, or 48 h, followed measurement of the cell viability and number by MTS and cell count analysis, respectively. As shown in Figure 6A,B, while treatment with SB for the times tested did not influence the cell viability and number, LY or PD treatment strongly

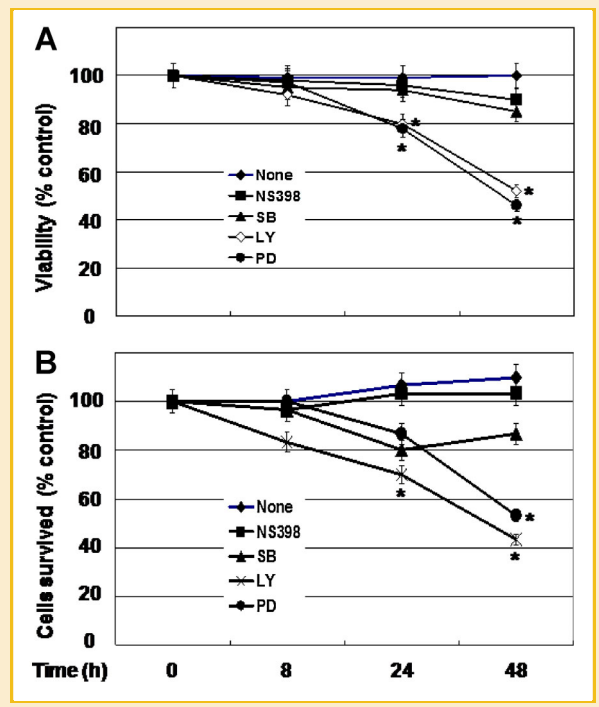

Fig. 6. Effects of NS-398, SB203580, LY294002, or PD98059 on growth of NCI-H292 cells. A,B: NCI-H292 cells (1×10^4 cells/100 μl/well/96-well plate) seeded the day before treatments were exposed to fresh media containing 10% FBS in absence or presence of a COX-2 enzymatic inhibitor NS-398 (40 μM), a p38 MAPK inhibitor SB203580 (SB, 25 μM), a JNK-1/2 inhibitor SP600125 (SP, 25 μM), a PI3K/PKB inhibitor LY294002 (LY, 25 μM), or an ERK-1/2 inhibitor PD98059 (PD, 50 μM) for 0, 8, 24, or 48 h, followed measurement of the cell viability by MTS assay (A) or of the number of cells by cell count analysis (B). Data are mean ± SE of three independent experiments. The viability and survival was normalized as percentage of the value at 0 h. $^*P < 0.05$ compared to the value obtained at the same time point (h) in absence of any drug.

reduced the cell viability and number in a time-dependent fashion. Treatment with NS-398 at the times applied had little effect on the cell viability and number. To confirm the activity of COX-2, ELISA assay was carried out to measure the amounts of PGE_2, a major COX-2 metabolite in culture media obtained from NCI-H292 cells grown in presence of serum for 8 h in which high COX-2 protein expression is induced. Unfortunately, we were unable to see the levels of PGE_2 since the PGE_2 levels were far below the standard curve in the assay, which might be due to extremely low PGE_2 or no PGE_2 in the sample. We assume that because of its intrinsic instability (short half-life) PGE_2 may be degraded during cell culture condition and/or sample collection before ELISA assay.

DISCUSSION

Evidence suggests deregulated COX-2 expression and its role in lung cancer [Petkova et al., 2004; Mutter et al., 2009]. It is thus a worthy goal to delineate molecular, cellular, and signaling mechanisms underlined aberrant COX-2 expression in lung cancer. We herein report for the first time a serum-dependent COX-2 transcriptional and post-transcriptional up-regulation in NCI-H292 human alveolar epithelial carcinoma cells via activations of multiple signaling proteins, including p38 MAPK, ERK-1/2, and PI3K/PKB.

Through initial experiments using various human airway cancer cell lines, we demonstrate that COX-2 protein expression is transiently up-regulated by serum addition in NCI-H292 cells (Figs. 1A and 2A). Notably, the present findings that albeit weak, COX-2 protein is also induced by serum addition in A549 or Hep-2 cells (Fig. 2E) suggest that up-regulation of COX-2 protein by serum addition is observed in different human lung cancer cell types but with different kinetics and magnitudes.

COX-2 expression is regulated at multiple steps, including transcription, post-transcription, and translation [Herschman et al., 1997]. Thus, the present findings showing high COX-2 mRNA steady-state levels (mostly at 2 or 4 h) in NCI-H292 cells upon the exposure of serum (Figs. 1B and 2B) indicate that up-regulation of COX-2 protein by serum in NCI-H292 cells is primarily due to COX-2 transcriptional up-regulation. There are numerous studies implicating roles of PI3K/PKB, ERK-1/2, JNK-1/2, and/or p38 MAPK in COX-2 transcriptional up-regulation [Guan et al., 1998; Ridley et al., 1998; Dean et al., 1999; Sheng et al., 2000]. Once activated, some of these signaling proteins results in a multitude of cytoplasmic and nuclear changes, which contribute to alterations in gene expression [Cohen, 1997]. Accordingly, the constitutive activity of PI3K/PKB and MAPKs in many types of cancer cells has been reported [Stauffer et al., 2005; Yang et al., 2010]. In this study, we have shown that activities of PKB, ERK-1/2, JNK-1/2, and p38 MAPK are increased by serum addition in NCI-H292 cells (Fig. 3). Importantly, the present findings that treatment with p38 MAPK inhibitor (SB), PI3K/PKB inhibitor (LY), or ERK-1/2 inhibitor (PD) strongly blocks expression of COX-2 protein and mRNA induced by serum in NCI-H292 cells (Fig. 4) imply significance of increased activities of p38 MAPK, PI3K/PKB, and ERK-1/2 in the serum-induced COX-2 transcriptional up-regulation in the cells. However, given that JNK-1/2 inhibitor (SP) does not affect COX-2 expression (Fig. 4) and also growth (Fig. 6A,B) in NCI-H292 cells grown in presence of serum, it is evident that activities of JNK-1/2 do not facilitate the serum-mediated COX-2 up-regulation and growth in NCI-H292 cells.

COX-2 mRNA is labile and its half-life is about 1.5 h in cells. However, recent studies demonstrate post-transcriptional COX-2 mRNA stabilization in many types of tumor cells, including breast and colon [Jang et al., 2000; Sheng et al., 2000]. There is now compelling evidence that post-transcriptional COX-2 mRNA stabilization is greatly influenced by signaling proteins (p38 MAPK, ERK-1/2, PI3K/PKB) [Ridley et al., 1998; Dean et al., 1999; Jang et al., 2000, 2004; Zhang et al., 2000], RNA-binding proteins (HuR, TTP) [Sengupta et al., 2003; Young et al., 2009], and AU-rich elements in COX-2 mRNA 3′-UTR [Srivastava et al., 1994; Cok and Morrison, 2001]. Remarkably, analysis of Act D chase experiments in present study shows that COX-2 mRNA is stable by 8 h in NCI-H292 cells (Fig. 5A), suggesting post-transcriptional COX-2 mRNA stabilization. Importantly, the present findings that treatment with SB or PD, but not LY, accelerates degradation of COX-2 mRNA in NCI-H292 cells indicate roles of p38 MAPK and ERK-1/2 in post-transcriptional COX-2 mRNA stabilization in the cells. There are numerous studies illustrating that COX-2 expression is further affected by the protein turnover [Otto et al., 1993; Mbonye et al., 2006, 2008; Jang et al., 2007; Kang et al., 2007]. In this study, analysis of CHX chase experiments with pharmacological inhibitors shows that COX-2 protein is rapidly degraded in NCI-H292 cells and treatment with SB, PD, or LY does not accelerate the protein turnover (Fig. 5B), which may imply that the serum-induced COX-2 up-regulation in NCI-H292 cells is largely attributed to sustained COX-2 mRNA stability rather than COX-2 protein stability levels.

COX-2 and PGE_2, a major metabolite facilitate growth of tumor tissues and cells, which is evidenced by recent in vivo and in vitro studies with suppressive effects of administration of COX-2 inhibitors alone or combinatorial treatment with other anti-cancer therapies on tumor mass and cancer cell growth [Eberhart et al., 1994; Talmadge et al., 2007; Kurihara et al., 2009; Zhang et al., 2009]. However, the COX-2-independent tumor cell growth also has been implicated [Park et al., 2010]. In this study, we have shown that treatment with NS-398 (a specific COX-2 inhibitor) for the times applied does not influence the viability and number of NCI-H292 cells (Fig. 6A,B), addressing that the growth of NCI-H292 cells is COX-2-independent. A positive role of ERK-1/2 in growth of tumor cells has been addressed [Balmanno and Cook, 2009]. The PI3K/PKB- or p38 MAPK-dependent growth of tumor cells also has been reported [Xu et al., 2004; Yang et al., 2010]. Thus, the present findings that treatment with PD or LY, but not SB, decreases the viability and survival of NCI-H292 cells (Fig. 6A,B) suggest that activities of ERK-1/2 and PKB are necessary for growth of NCI-H292 cells (Fig. 6A,B). Considering the roles of PI3K/PKB and ERK-1/2 in both COX-2 expression and growth in NCI-H-292 cells herein, it is further suggestive that PI3K/PKB or ERK-1/2 pharmacological inhibitor in single and/or combinatorial regimens along with established anti-cancer drugs may be potentially useful against human alveolar epithelial carcinoma cells even where COX-2 is overexpressed. Established cancer cell lines are poor indicators of the tumor biology. Thus, pathophysiological relevance of the present findings remains unclear at present. However, considering

the role of COX-2 in lung cancer but lack of the regulatory mechanisms underlined lung tumor-associated COX-2 overexpression, the present study has a significance to address novel ERK-1/2, p38 MAPK, and PKB-mediated COX-2 transcriptional and post-transcriptional up-regulation induced by serum in NCI-H292 cells, which may contribute to better understanding of molecular, cellular, and signaling mechanisms involved in aberrant COX-2 expression in lung cancer.

In conclusion, we demonstrate that (1) COX-2 is transiently up-regulated upon the exposure of serum in NCI-H292 cells, (2) the serum-induced COX-2 up-regulation (transcriptional and post-transcriptional) is largely dependent of activities of PI3K/PKB, p38 MAPK, and ERK-1/2 signaling proteins, and (3) there is an necessity of the ERK-1/2 and PI3K/PKB (but not COX-2, p38 MAPK, and JKN-1/2) activities in growth of NCI-H292 cells.

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