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Psoralidin, a dual inhibitor of COX-2 and 5-LOX, regulates ionizing radiation (IR)-induced pulmonary inflammation

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ABSTRACT

Radiotherapy is the most significant non-surgical cure for the elimination of tumor, however it is restricted by two major problems: radioresistance and normal tissue damage. Efficiency improvement on radiotherapy is demanded to achieve cancer treatment. We focused on radiation-induced normal cell damage, and are concerned about inflammation reported to act as a main limiting factor in the radiotherapy. Psoralidin, a coumestan derivative isolated from the seed of *Psoralea corylifolia*, has been studied for anti-cancer and anti-bacterial properties. However, little is known regarding its effects on IR-induced pulmonary inflammation. The aim of this study is to investigate mechanisms of IR-induced inflammation and to examine therapeutic mechanisms of psoralidin in human normal lung fibroblasts and mice. Here, we demonstrated that IR-induced ROS activated cyclooxygenases-2 (COX-2) and 5-lipoxygenase (5-LOX) pathway in HFL-1 and MRC-5 cells. Psoralidin inhibited the IR-induced COX-2 expression and PGE_2 production through regulation of PI3K/Akt and NF-κB pathway. Also, psoralidin blocked IR-induced LTB_4 production, and it was due to direct interaction of psoralidin and 5-lipoxygenase activating protein (FLAP) in 5-LOX pathway. IR-induced fibroblast migration was notably attenuated in the presence of psoralidin. Moreover, *in vivo* results from mouse lung indicate that psoralidin suppresses IR-induced expression of pro-inflammatory cytokines (TNF-α, TGF-β, IL-6 and IL-1 α/β) and ICAM-1. Taken together, our findings reveal a regulatory mechanism of IR-induced pulmonary inflammation in human normal lung fibroblast and mice, and suggest that psoralidin may be useful as a potential lead compound for development of a better radiopreventive agent against radiation-induced normal tissue injury.

1. Introduction

Lung cancer is a leading cause of cancer death throughout the world [1]. Only a small number of patients (15–25%) diagnosed with early stage non-small-cell lung cancer (NSCLC) are treated with surgery, while the majority of NSCLC patients cannot undergo surgery. Accordingly, radiotherapy have been considered a preferred modality for these inoperable NSCLC patients [2]. Radiotherapy depends on two fundamental principles: killing cancer cells, but not normal cells [3]. Unfortunately, application of radiotherapy inevitably leads to normal tissue irradiation. The exact mechanisms of radiation-induced normal tissue injury are not fully established, however, accumulating studies indicate that the process starts with energy deposition and production of reactive oxygen species (ROS), followed by molecular modification, activation of transcription factors and cross-talk of signal transduction pathways [4].

Inflammation is reported to act as a main limiting factor in the radiotherapy process [5]. Also, an association between inflammation and cancer development or progression has been well demonstrated [6–8]. Many researchers have paid attention to importance of inflammation, and have demonstrated that anti-inflammatory drug administration offers advantages in cancer

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; DCFH-DA, 2′,7′-dichlorodihydrofluorescin diacetate; FLAP, 5-lipoxygenase activating protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM, intercellular adhesion molecule; IκBα, inhibitors of NF-κB α; IL, interleukin; IR, ionizing radiation; ITC, isothermal titration calorimetry; JNK, c-Jun N-terminal kinase; LOX, lipoxygenase; LT, leukotriene; MAPK, mitogen activated protein kinase; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; PG, prostaglandin; PI3K, phosphoinositide3-kinase; ROS, reactive oxygen species; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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patients [9]. Inflammation is a complex biological response to harmful stimuli, and is characterized by influx of inflammatory cells, change in cytokine milieu, production of pro-inflammatory mediators and fibroblast recruitment [4]. There is abundant evidence to prove that cyclooxygenases (COXs) and 5-lipoxygenase (5-LOX) play a pivotal role in inflammation and organ dysfunction. Prostaglandins (PGs) and leukotrienes (LTs) are specialized lipids derived from oxygenation of arachidonic acid (AA) by COXs and 5-LOX activity, respectively [10–12]. In addition, membrane-embedded 5-lipoxygenase activating protein (FLAP) selectively transfers AA to 5-LOX, and enhances LTs synthesis [13]. Pharmacological studies have been developed for anti-inflammatory drugs targeting COXs, 5-LOXs and FLAP.

Common non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2 isoforms. COX-1 and COX-2 differ in their expression patterns and biological functions. COX-2 is usually absent in most normal tissues, and is induced rapidly in response to pathological stimuli. COX-1, in contrast, is constitutively expressed throughout the body, and is responsible for platelet aggregation, normal tissue integrity and homeostatic physiological functions. Although NSAIDs have remarkable anti-inflammatory effects, continuous use of the NSAIDs has been restricted by severe side effects in gastrointestinal (GI) tract and kidney. Considering the COX-1 inhibition as a major possibility of GI toxicity, COX-2 specific inhibitors have been developed [14–16]. However, as some physiological functions of COX-2 are reported, doubt about safety of the COX-2 specific inhibitor have raised [17]. On the other hand, 5-LOX has a crucial role in AA metabolism of the inflammatory process. Recent studies have indicated that COXs inhibition could cause up-regulation of the 5-LOX pathway: PGs (synthesized by COXs) and LTs (synthesized by 5-LOX) are complementally related to each other [17]. As a result, a "dual inhibitor" of COX-2 and 5-LOX has been proposed, indicating that it could have synergistic and safer anti-inflammatory effects [18].

Chemoprevention by phytochemicals provides new paradigm for various disease management [19]. Psoralidin (Fig. 1A) is coumestan derivative isolated from seed of *Psoralea corylifolia*, which is broadly used in traditional medicine for bleeding, enuresis, pollakiuria, vitiligo, and psoriasis [20]. Psoralidin has demonstrated a variety of biological activities such as anti-cancer, anti-oxidant, anti-bacterial, anti-depressant activities and regulation of insulin signaling [21–23].

Here, we investigated IR-induced pulmonary inflammation mechanism and anti-inflammatory role of psoralidin in human normal lung fibroblasts and mice. Also, we provided the first evidence for anti-inflammatory property of psoralidin as a dual inhibitor of COX-2 and 5-LOX. These results suggest that psoralidin would have possibility of preventing and minimizing radiation-induced normal tissue injury, and could be potential radio-preventive agent.

2. Materials and methods

2.1. Reagents

Cell culture media, FBS, glutamine, penicillin, streptomycin and Trizol® were acquired from Gibco (Grand Island, NY). Psoralidin and antibodies against COX-2, 5-LOX, FLAP, Akt, p-Akt (Ser473, Thr308), β-actin, and Lamin A/C were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phospho-inhibitors of NF-κB α (p-IκBα) and NF-κB (p65) were acquired from Cell Signaling Technology (Beverly, MA). Inhibitor X (Akt inhibitor),

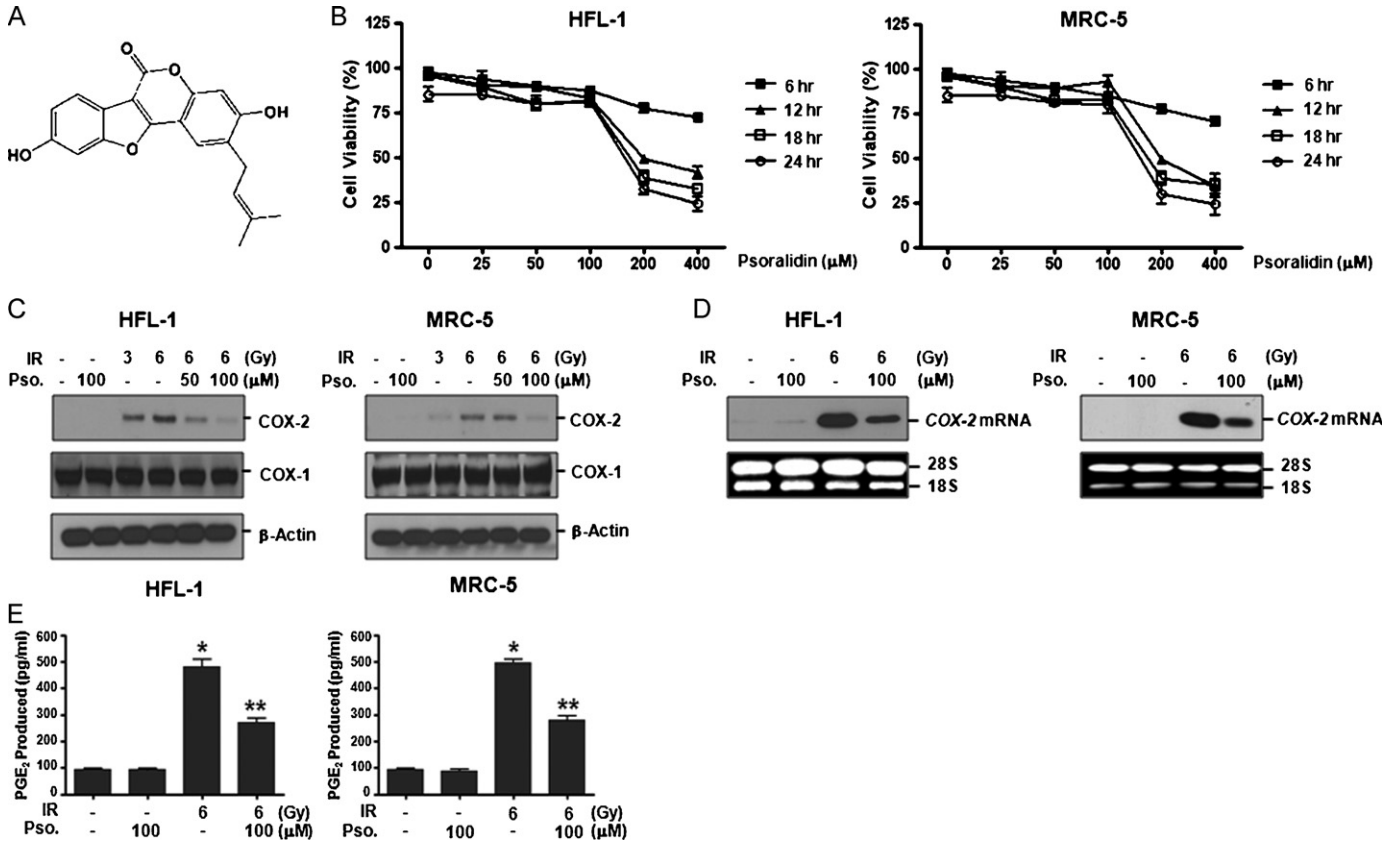

Fig. 1. Psoralidin inhibits COX-2 in IR-irradiated normal lung fibroblasts. (A) Chemical structure of psoralidin. (B) Cytotoxicity of psoralidin in HFL-1 and MRC-5 cells was assessed by MTT assay. (C) The inhibitory effect of psoralidin in IR-induced COX-2 protein expression was detected by immunoblotting. (D) The inhibitory effect of psoralidin in IR-induced *COX-2* mRNA expression was detected by northern blotting. (E) The inhibitory effect of psoralidin in IR-induced COX-2 activity was detected by enzyme-immunoassay. $^{*}P < 0.05$; irradiated cells vs. control cells, $^{**}P < 0.05$; psoralidin-treated irradiated cells vs. psoralidin-untreated irradiated cells.

LY294002 (PI3K inhibitor), SP600125 (c-Jun N-terminal kinase (JNK) inhibitor), PD98059 (extracellular signal-regulated kinase1/2 (ERK1/2) inhibitor), and SB203580 (p38 inhibitor) were acquired from Calbiochem (San Diego, CA). Methylthiazolyldiphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA), pyrrolidine dithiocarbamate (PDTC, NF-κB inhibitor), procaterol (inhibitor of lung fibroblast migration), MK-886 (FLAP inhibitor), and sepharose 4B were acquired from Sigma (St. Louis, MO).

2.2. Cell culture, psoralidin treatment, and irradiation

Human normal lung fibroblasts (HFL-1 and MRC-5) were cultured in F-12K medium and DMEM, respectively, and supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 μg/ml), and 10% FBS. Cells were treated with psoralidin (50 and 100 μM) dissolved in DMSO for 2 h, and exposed to IR using the Gamma Cell 40 Exactor (Nordion International, Inc., Kanata Ontario, Canada) at a dose rate of 0.81 Gy/min. Irradiated cells were incubated for an additional 4 h.

2.3. MTT assay

MTT assay was performed as described previously [24]. HFL-1 and MRC-5 cells were seeded and cultured in 24-well plates with or without psoralidin for the indicated time period. Media were removed, and 0.05% MTT solution was added, followed by incubation at 37 °C for 2 h. Then MTT solution was replaced with DMSO and incubated for 10 min. After incubation, the solution was aliquoted into 96-well plates in duplicate, and absorbance was measured at 570 nm.

2.4. Western blot analysis

Cell lysates (5×10^6 cells) were prepared using RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 25 mM NaF, 1 mM DTT, 20 mM EGTA, 1 mM Na_3VO_4, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), and 5 U/ml aprotinin). Cell fractionation was performed as described previously [25]. Proteins (40 μg) were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, blocked, and probed with specific primary antibodies. Following the addition of peroxidase-conjugated secondary antibody, the proteins were visualized using an ECL detection system (GE Healthcare, Little Chalfont, Buckinghamshire, England). To ensure equal protein loading, the blots were stripped and reprobed with β-actin or Lamin A/C antibody.

2.5. Northern blot analysis

Following the desired treatments (3×10^6 cells), total cellular RNA was isolated from cell monolayers using Trizol®, electrophoresed on a formaldehyde-containing 1.2% agarose gel, and transferred to nylon membranes. Following UV-cross linking, membranes were prehybridized for 30 min in an ExpressHyb Hybridization solution (Clontech, Palo Alto, CA) and hybridized for 1 h at 65 °C with radiolabeled DNA probes for human *COX-2* cDNA. The membranes were washed twice for 30 min at room temperature in 1 × SSC and 0.1% SDS solution. Then washed membranes were subjected to autoradiography. *COX-2* probe was labeled with the [α-^{32}P] CTP using a random priming kit (GE Healthcare, Little Chalfont, Buckinghamshire, England).

2.6. Measurement of PGE_2 and LTB_4 production

Cells (6×10^5) were plated in 6-well plates and grown to 80% confluence. Following the desired treatments, the amounts of PGE_2 or LTB_4 released into the media were measured using the enzyme-immunoassay kit (GE Healthcare, Little Chalfont, Buckinghamshire, England), according to the manufacturer's instructions.

2.7. Measurement of intracellular ROS

After psoralidin treatment and irradiation, cells (5×10^5) were seeded in black 96-well plates, washed with PBS, and then incubated with DCFH-DA (20 μM) in PBS at 37 °C for 2 h. DCF fluorescence was measured at an emission wavelength (530 nm) and an excitation wavelength (485 nm) using a FLX 800 microplate fluorescence reader (BioTek Instruments Inc., Winooski, VT). The background was from cell-free conditions. Results were expressed as percentage of control (not irradiated fibroblast cells) fluorescence intensity.

2.8. Transient transfection

Cells (5×10^5) were plated in 6-well plates and incubated for 4 h for stabilization. The cells were transiently transfected with pCL-3 empty vector (2.5 μg) or pGL-3-Luc (2.5 μg), containing the NF-κB promoter region ligated to luciferase and/or 1 μg of pCDNA3.1/hisB/lacZ (coding galactosidase for normalization of luciferase activity) using Lipofectin (Gibco, Grand Island, NY) according to the manufacturer's instructions. Dominant negative IκB mutant plasmid (pcDNA3.1/IκBα-SR, 2 μg) was co-transfected into the cells. Also, cells were transiently transfected with pCMV6 vector (2 μg) containing CA-Akt1 (Myr-Akt1-HA, Upstate Biotechnology, Lake Placid, NY) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA).

2.9. Luciferase reporter gene assay

Following overnight transfection, the medium was changed, treated with psoralidin for 2 h and irradiated. After 4 h, cells were washed twice with cold PBS and lysed with reporter lysis buffer (Promega, Madison, WI). After vortex-mixing and centrifugation at 12,000 × *g* for 1 min at 4 °C, the cell extract (20 μl) and luciferase assay reagent (100 μl) were mixed. Then luciferase activity is detected by a luminometer (AutoLumat LB 953, Berthold Technologies, Bad Widbad, Germany).

2.10. Preparation of psoralidin-sepharose 4B and pull-down assay

Preparation of psoralidin-sepharose 4B using psoralidin (100 mg) and sepharose 4B freeze-dried powder (500 mg) was performed as previously described [26]. For the *ex vivo* and *in vitro* pull-down assay, cellular supernatant fraction of irradiated cells (500 μg) or purified FLAP (10 μg) was incubated with psoralidin-sepharose 4B (or sepharose 4B alone as a control) beads (100 μl, 50% slurry) in reaction buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP-40, 2 μg/ml BSA, 0.02 mM PMSF, and 1 μg protease inhibitor mixture). After incubation with gentle rocking overnight at 4 °C, the beads were washed five times with reaction buffer not containing BSA, and proteins bound to the beads were analyzed by immunoblotting. For the *in vivo* pull-down assay, BALB/c mice received intraperitoneal application of 500 μl DMSO alone or psoralidin (5 mg/kg) in 500 μl DMSO 30 min before and 1 h after IR irradiation (20 Gy). After 24 h, one lung from each mouse was excised and placed on ice for the preparation of lung lysates. Any fat was removed, and the skin was placed in liquid nitrogen and immediately pulverized with a mortar and pestle. The pulverized lung was blended on ice with a homogenizer (IKA T10 basic, IKA Laboratory Equipment, Staufen, Germany), and lung lysates were centrifuged at 12,000 rpm for 20 min. After the protein content was determined using the Bio-Rad protein assay

kit, 500 μg of the lung lysates was incubated with psoralidin-sepharose 4B (or sepharose 4B alone as a control) beads (100 μl, 50% slurry) in reaction buffer as previously described for the *ex vivo* and *in vitro* pull-down assay. Beads were incubated and washed. Proteins bound to the beads were analyzed by immunoblotting as described above.

2.11. FLAP preparation and isothermal titration calorimetry (ITC) assay

Recombinant human FLAP was prepared according to a previously described protocol [27]. The gene encoding full-length human FLAP was cloned into the pET28a vector, which includes a C-terminal 6His-tag. Recombinant protein was expressed in *E. coli* BL21 (DE3) cells by induction (at OD600 ≈ 0.8) with 1 mM IPTG for 6 h at 30 °C. Cells were harvested, and lysed in Tris (20 mM, pH 7.4), 50 mM NaCl, 10% glycerol and 1 mM DTT. FLAP was extracted from purified membranes for 90 min at room temperature in lysis buffer by addition of 2% (*w*/*v*) dodecylmaltoside, centrifuged at 40,000 × *g* for 30 min, applied to Ni-NTA resin, and eluted with 300 mM imidazole at 4 °C. The protein was further purified by ion exchange and gel filtration chromatography. The direct interaction between FLAP and psoralidin was measured using a MicroCal VP-ITC instrument at 25 °C. Recombinant human FLAP, 5-LOX (Cayman Chemical, Ann Arbor, MI) or COX-2 (Sigma, St. Louis, MO) was equilibrated with Tris buffer (20 mM, pH 7.5) containing 150 mM NaCl. Protein was added to the calorimetric reaction cell at a concentration of 0.01 mM, and titrated with 0.1 mM psoralidin in the same buffer. Enzyme and ligand solutions were degassed prior to use. Each titration experiment was performed with 29 injections of 10 μl at 300^{-s} equilibration intervals. Heat of dilution for an individual ligand was determined by titration of the ligand into the same buffer without protein, and used in correction of the protein titration. Data were fit to a single-site binding model by non-linear least-square regression using the Origin software package. The fit of the data yields the binding affinity (K_d), enthalpy change (ΔH), entropy change (ΔS), and binding stoichiometry for the titration.

2.12. Cell migration assay

HFL-1 and MRC-5 cells (1×10^5) were plated in DMEM/1% FBS in the upper chamber of 5 μm pore (24-well) transwells (Costar, High Wycombe, England), and allowed to adhere for 4 h. Cells were treated with psoralidin (0, 50 and 100 μM) and procaterol (10 μM, lung fibroblast migration inhibitor) 2 h before irradiation (0 and 6 Gy). The cells were incubated for 24 h, rinsed with PBS, and fixed in 100% methanol. Cells remaining at the top of the polycarbonate membrane were removed. Cells migrated through pores to the lower surface, were stained with ethanol-based crystal violet solution. Membranes were mounted on microslides, and cells were counted. The fold increase (relative to not irradiated fibroblast cells) of migrating cells is shown based on the mean of the cell number in ten randomly selected fields from a single representative experiment.

2.13. Animal study

Male BALB/c strain mice (eight weeks, average weight 22–25 g) were obtained from Central Lab Animals Inc. (Seoul, South Korea), and divided into eight groups for specific experiments, as indicated ($n = 5$ in each group). To anesthetize the animals during irradiation, mice were intraperitoneally injected with a mixture of ketamine (80 mg/kg) and xylazine (16 mg/kg). Mice were irradiated once in the thorax area with 20 Gy of radiation using a Gamma Cell 40 Exactor (Nordion International, Inc., Kanata Ontario, Canada) at a dose rate of 0.81 Gy/min. Control mice were subjected to sham-irradiation. On the day of the experiments, psoralidin (5 mg/kg) were administrated in a home cage in a quiet room, so that stress to the animals would be minimized. All animal use procedures were approved by the Institutional Animal Care and Use Committee of Pusan National University, and performed in accordance with the provisions of the NIH Guide for the Care and Use of Laboratory Animals.

2.14. Real time reverse transcription-polymerase chain reaction (real time RT-PCR)

Mice ($n = 5$ in each group) were sacrificed at 12 h and 1 week after irradiation (20 Gy), and the lungs were dissected. Lung lysate was prepared as described above (see Section 2.10) and total cellular RNA was isolated using Trizol® reagents for real time RT-PCR. Biotin-labeled probes (Ambion, Austin, TX) were hybridized to the target mRNA. The samples were mixed with loading buffer and separated by electrophoresis on a 9% sequencing gel. The intensity of each gene was normalized against with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. RNA (2 μg) was subjected to reverse transcription with a random primer using the superscript II kit (Invitrogen, Carlsbad, CA) to obtain the first cDNA strand. Primers (Table 1) for TNF-α, TGF-β, IL-6, IL-1 α/β, and intercellular adhesion molecule (ICAM-1) were designed using Primer Express® 4.0 (Applied Biosystems, Foster, CA) for PCR analysis. To allow for loading differences, a GAPDH primer was used as a control. Optimized PCR was performed using an iCycler iQ multicolor real time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Significant PCR fluorescence signals were normalized to a PCR fluorescence signal obtained from the mean value for not irradiated control mice.

2.15. Statistical analysis

All numeric data are presented as mean ± SD from independent experiments, and were analyzed using a one-way analysis of variance on ranked data, followed by a Tukey's honestly significant difference test in Prism 4 (GraphPad Software, San Diego, CA). A p value < 0.05 was considered statistically significant.

All results described in this paper were confirmed by three independent experiments.

Table 1
Primer pairs of the specific mRNA for cytokines and cell adhesion molecule.

	Primer forward 5′3′	Primer reverse 5′3′
TNF-α	5′-CGAGTGACAAGCCTGTAGCC-3′	5′-GGTTGACTTTCTCCTGGTATGAG-3′
TGF-β	5′-CCACTATTGCTTCAGCTCCAC-3′	5′-TCAGCTGCACTTGCAGGAGCGCAC-3′
IL-6	5′-ATGTTCTCTGGGAAATCGTG-3′	5′-GAAGGACTCTGGCTTTGTCTT-3′
IL-1α	5′-TGCAAGCTATGAGCCACTTC-3′	5′-GCATTCCTGGTGGATGACTC-3′
IL-1β	5′-GAGAACCAAGCAACGACAAAATACC-3′	5′-GCATTAGAAACAGTCCAGCCCATAC-3′
ICAM-1	5′-CACCCCAAGGACCCCAAGGAGAT-3′	5′-CGACGCCGCTCAGAAGAACCAC-3′

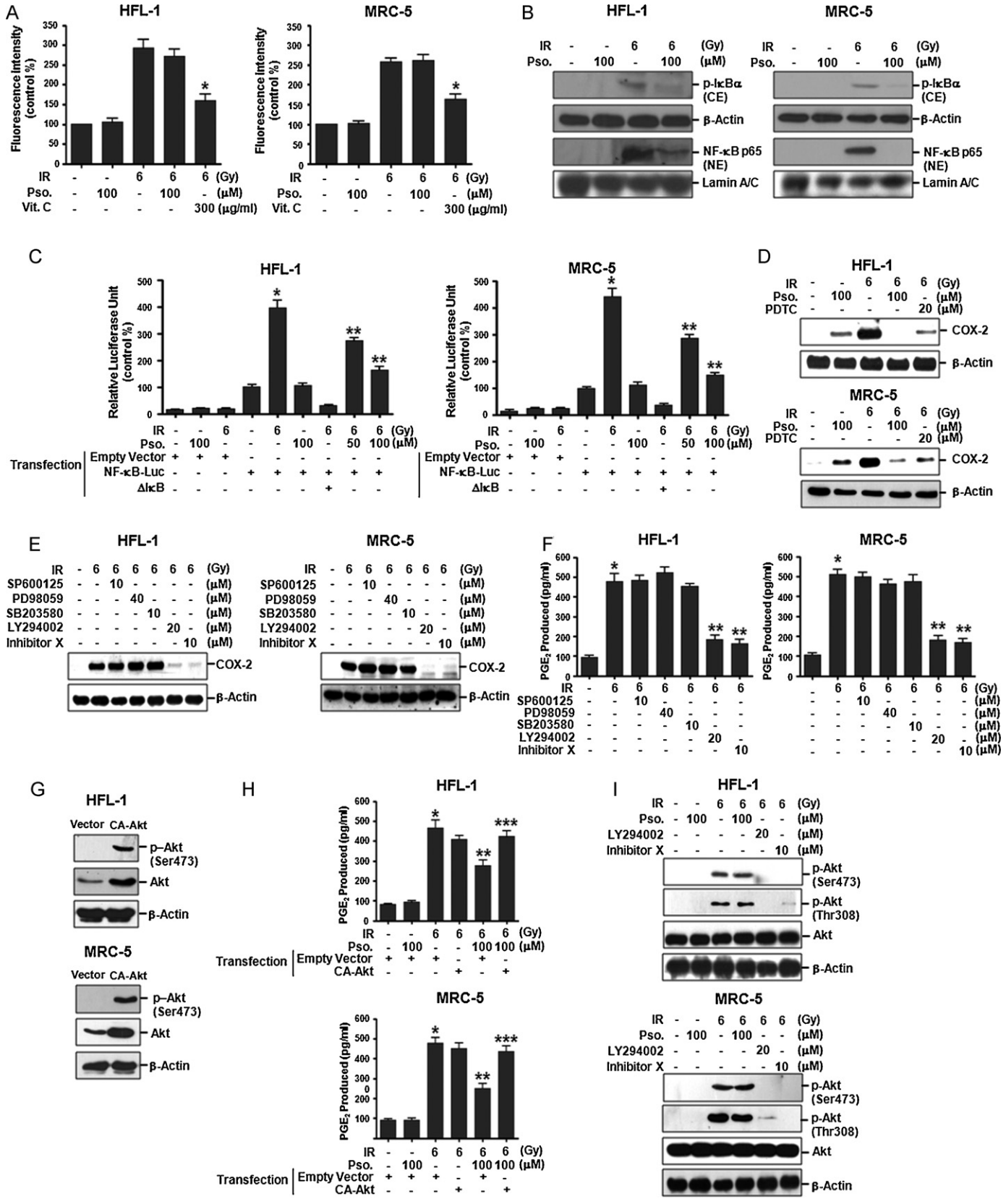

Fig. 2. The PI3K/Akt and NF-κB pathway is pivotal for COX-2 inhibition by psoralidin in IR-irradiated normal lung fibroblasts. (A) Involvement of psoralidin in IR-induced intracellular ROS generation was analyzed through measurement of the intensity of DCF fluorescence. The Vitamin C (Vit.C, 300 μg/ml) as a positive control, was added 30 min before irradiation. $^{*}P < 0.05$; Vit.C-treated irradiated cells vs. Vit.C-untreated irradiated cells. (B) The inhibitory effects of psoralidin in NF-κB (p65) nuclear translocation and IκBα phosphorylation were assayed by immunoblotting. (C) The inhibitory effect of psoralidin in NF-κB transcriptional activity was measured by luciferase assay. $^{*}P < 0.05$; irradiated cells vs. control cells, $^{**}P < 0.05$; psoralidin-treated irradiated cells vs. psoralidin-untreated irradiated cells. (D) Involvement of NF-κB in IR-induced COX-2 expression was detected by immunoblotting. (E) Involvement of PI3K/Akt and MAPKs in IR-induced COX-2 expression was detected by immunoblotting.

3. Results

3.1. COX-2 inhibitory effects of psoralidin in IR-irradiated normal lung fibroblasts

To determine concentration of psoralidin that can be used without affecting cell viability, HFL-1 and MRC-5 cells were treated with psoralidin at different concentrations or DMSO vehicle control (Fig. 1B). Psoralidin did not affect cell viability up to 100 μM, and non-cytotoxic concentration of psoralidin used in this study was determined: 50 and 100 μM. To investigate that COX-1, 2 could be regulated by psoralidin, cells were treated with psoralidin under IR irradiation. Exposure of 3 or 6 Gy (doses belonging to the radiation range typically administered in radiation biology experiment on cells [3]) dramatically induced COX-2 expression, and a marked decrease in COX-2 expression was noted after treatment with psoralidin. On the other hand, COX-1 is constitutively expressed, and psoralidin did not cause any significant changes in COX-1 expression (Fig. 1C). Furthermore, we assured that IR-induced *COX-2* mRNA expression and COX-2 activity (PGE_2 production) were reduced by treatment of psoralidin in both types of cells (Fig. 1D and E). These findings suggest that psoralidin in IR-irradiated normal lung fibroblasts could act as a specific inhibitor of COX-2, but not COX-1.

3.2. Possible involvement of the PI3K/Akt and NF-κB in the COX-2 inhibitory effects of psoralidin in IR-irradiated normal lung fibroblasts

Biological effects of radiation are induced by direct DNA damage and indirect cellular damage through ROS generation. Recent studies have demonstrated that IR-induced ROS acts as a pathogenic initiator through mechanisms such as DNA damage, lipid peroxidation and alteration of enzyme activity [4,28]. To determine whether COX-2 inhibition by psoralidin might associate with the IR-induced ROS generation, intracellular ROS level was determined by measurement of the intensity of DCF fluorescence. DCF fluorescence in irradiated HFL-1 and MRC-5 cells was significantly increased. However, pre-incubation with psoralidin did not significantly reduce the IR-induced increase of ROS generation (Fig. 2A). These data suggest that inhibition of COX-2 by psoralidin could occur in downstream of ROS production and associate with ROS-mediated signaling, indicating slight effect of psoralidin as a ROS scavenger.

NF-κB has known to be the most critical regulator of pro-inflammatory gene expression [29–31]. In this research, we demonstrated that IR-induced NF-κB (p65) nuclear translocation and IκBα phosphorylation are prevented by psoralidin treatment in both types of cells (Fig. 2B). Furthermore, NF-κB transcriptional activity was demonstrated through a luciferase reporter gene assay. IR-increased NF-κB-luciferase activity was approximately 4-fold higher compared to control (not irradiated cells), and psoralidin significantly inhibited IR-induced NF-κB-luciferase activity. When pcDNA3.1/IκBα-SR (ΔIκB) was transfected, the result showed basal luciferase activity (Fig. 2C). To determine whether NF-κB could subsequently regulate COX-2 expression, cells were treated with PDTC (20 μM, NF-κB inhibitor) (Fig. 2D). These results suggest possible involvement of NF-κB in inhibition of COX-2 expression by psoralidin in IR-irradiated normal lung fibroblasts.

Mitogen activated protein kinases (MAPKs) and PI3K/Akt have been implicated in regulation of NF-κB-mediated inflammatory processes in response to various stimuli [32–34]. To investigate regulators for the activation of NF-κB and COX-2 in IR-irradiated normal lung fibroblasts, inhibitors of protein kinases associated with inflammatory response were treated in the subsequent experiments. IR-induced COX-2 expression and activity were significantly diminished following addition of LY294002 (PI3K inhibitor) and inhibitor X (Akt inhibitor), but not SP600125 (JNK inhibitor), PD98059 (ERK1/2 inhibitor), and SB203580 (p38 inhibitor) (Fig. 2E and F). These results indicate that COX-2 expression and activity in IR-irradiated normal lung fibroblasts could be closely related to PI3K/Akt than MAPKs.

To further assess that Akt could regulate COX-2 activity, cells were transfected with CA-Akt (Fig. 2G). IR-induced COX-2 activity (PGE_2 production) was reduced by treatment of psoralidin (Fig. 1E), and the psoralidin-reduced PGE_2 production showed significant recovery by Akt overexpression (Fig. 2H). We hypothesize that psoralidin might directly modulate Akt kinase activity, however, increased Akt phosphorylation after irradiation did not change with psoralidin treatment (Fig. 2I). These results indicate that PI3K/Akt could act as an important mediator of IR-induced COX-2 expression and activation, and suggest that NF-κB activation through the PI3K/Akt pathway could be a target of COX-2 inhibition by psoralidin in IR-irradiated normal lung fibroblasts. However, additional study will be needed to prove exact details of how psoralidin inhibits the signaling pathway between PI3K/Akt and NF-κB suggested in this study.

3.3. 5-LOX inhibitory effect of psoralidin in IR-induced normal lung fibroblasts

To determine whether 5-LOX could be regulated by psoralidin in IR-irradiated normal lung fibroblasts, HFL-1 and MRC-5 cells were treated with psoralidin (50 and 100 μM) under IR irradiation. 5-LOX was constitutively expressed regardless of irradiation, and revealed no significant alteration in psoralidin treatment (Fig. 3A). However, interestingly, although there was no change in 5-LOX expression after irradiation, 5-LOX activity (LTB_4 production) was significantly increased by irradiation, and IR-induced LTB_4 production attenuated by psoralidin (Fig. 3B). In addition, we confirmed noticeable inhibitory effect of psoralidin on IR-induced 5-LOX activity through use of a zileuton (10 μM, 5-LOX inhibitor), a positive control. According to our experiments, we suggest that 5-LOX activity might be associated with indirect regulation of other partner(s) rather than its own expression in IR-irradiated normal lung fibroblasts.

3.4. Possible involvement of FLAP in the 5-LOX inhibitory effect of psoralidin in IR-irradiated normal lung fibroblasts

Once cells are activated, 5-LOX translocates to the nuclear envelope and interacts with FLAP, regardless of its cellular localization [35,36]. FLAP transfers AA to 5-LOX, thereby enabling 5-LOX to efficiently generate oxidized lipid products (e.g. LTB_4). Considering the biochemical relationships between 5-LOX and FLAP, we hypothesized that FLAP could be a direct target for psoralidin in regulation of 5-LOX activity under IR irradiation. Interestingly, FLAP expression significantly increased in IR dose-dependent manner, however, IR-induced FLAP expression was not

Each inhibitor was treated 30 min before irradiation. (F) The inhibitory effect of PI3K/Akt in IR-induced PGE_2 production was detected by enzyme-immunoassay. $^{*}P < 0.05$; irradiated cells vs. control cells, $^{**}P < 0.05$; LY294002- or inhibitor X-treated irradiated cells vs. LY294002- or inhibitor X-untreated irradiated cells. (G) HFL-1and MRC-5 cells were transfected with CA-Akt and confirmed by immunoblotting. (H) The inhibitory effect of psoralidin in IR-induced PGE_2 production was reversed by AKT overexpression. $^{*}P < 0.05$; irradiated cells vs. control cells, $^{**}P < 0.05$; psoralidin-treated irradiated cells vs. psoralidin-untreated irradiated cells, $^{***}P < 0.05$; psoralidin-treated irradiated cells with CA-Akt transfection vs. psoralidin-treated irradiated cells without CA-Akt transfection. (I) Inhibitory effects of psoralidin in IR-induced Akt activation were detected by immunoblotting.

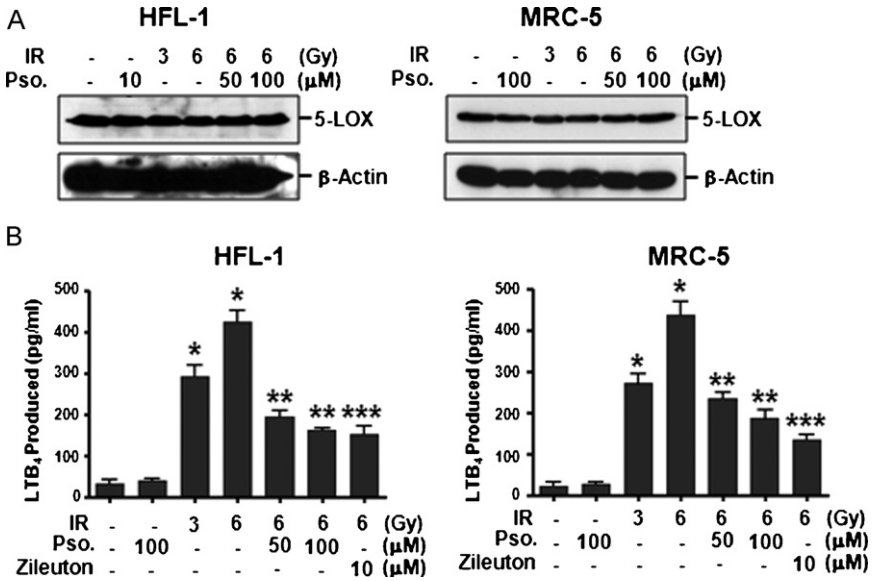

Fig. 3. Psoralidin inhibits 5-LOX pathway in IR-irradiated normal lung fibroblasts. (A) The effect of psoralidin in 5-LOX expression was detected by immunoblotting. (B) The inhibitory effect of psoralidin on IR-induced LTB_4 production was detected by enzyme-immunoassay. $^{*}P < 0.05$; irradiated cells vs. control cells, $^{**}P < 0.05$; psoralidin-treated irradiated cells vs. psoralidin-untreated irradiated cells, $^{***}P < 0.05$; zileuton-treated irradiated cells vs. zileuton-untreated irradiated cells.

changed by psoralidin treatments (Fig. 4A and B). To determine whether FLAP control the LTB_4 generation, HFL-1 and MRC-5 cells were treated with MK-886 (FLAP inhibitor) (Fig. 4C). Increased LTB_4 production by irradiation was reduced with MK-886 treatment in a concentration-dependent manner. These results suggest that FLAP could acts as an important regulator of IR-induced 5-LOX pathway.

Furthermore, we assessed pull-down assay, and the results indicated that FLAP can directly bind to psoralidin in *ex vivo* and *in vitro* experiment (Fig. 4D). ITC experiment was employed for investigation of the binding parameters between psoralidin and FLAP. Calorimetric data revealed that heat was released when psoralidin was associated with FLAP, indicating the significant enthalpic contributions to psoralidin-FLAP interaction (K_d = 21.0 μM, $\Delta H = -18.5 \pm 1.5$ kcal/mol, $\Delta S = -26.5$ cal/mol degree) (Fig. 4E). ITC data showed that psoralidin can bind to FLAP (but not 5-LOX and COX-2) with a stoichiometry of 1:1. According to these results, we suggest that psoralidin inhibits IR-induced 5-LOX activity by direct binding with the FALP, and could be a potent FLAP inhibitor for suppression of 5-LOX pathway.

3.5. Anti-migration effect of psoralidin in IR-irradiated normal lung fibroblasts

In some uncontrolled inflammatory processes, fibroblasts excessively migrate toward the area of injury. Fibroblast accumulation results in replacement of normal parenchymal elements and alteration of organ functions [37]. To investigate the effect of psoralidin in IR-induced fibroblast migration, transwell migration assay was assessed. We demonstrated that IR induces fibroblast migration in a dose-dependent manner, and psoralidin has a tendency to inhibit migration in IR-irradiated normal lung fibroblasts (Fig. 5). In addition, we confirmed noticeable anti-migration effect of psoralidin in IR-induced normal lung fibroblast through use of a procaterol (10 μM, inhibitor of lung fibroblast migration), a positive control (Fig. 5B).

3.6. Anti-inflammatory effect of psoralidin in IR-irradiated mice

Before examining the anti-inflammatory effect of proralidin, we confirmed that FLAP from lung extract of IR-irradiated mice can directly bind to psoralidin using *in vivo* pull down assay (Fig. 6A). Inflammation is mediated by the action of various pro-inflammatory cytokines [38]. Previous studies have indicated that radiotoxicity is associated with numerous mediators: tumor growth factors and pro-inflammatory cytokines such as IL-1α/-1β, IL-6, IL-8, IL-10, TNF-α, TGF-β and IFN-γ [39–41]. To investigate the anti-inflammatory effect of psoralidin in IR-irradiated lung of BALB/c mouse, we analyzed that psoralidin could regulate expression of pro-inflammatory cytokines that play an important role in inflammatory diseases. Mice were injected intraperitoneally with psoralidin (5 mg/kg) 30 min before and once a day after irradiation (20 Gy), and maintained for 12 h or 1 week. To determine mRNA expression of representative pro-inflammatory cytokines (TNF-α, TGF-β, IL-6, IL-1α/-1β) and ICAM-1 (adhesion molecule involved fibroblast migration), we quantified expression of these cytokines and adhesion molecule after radiation and/or psoralidin treatment by real time RT-PCR. Expression of TNF-α, TGF-β, IL-6, IL-1α/-1β and ICAM-1 showed basal level in unirradiated mice, and there were no significant changes after psoralidin treatment. However, interestingly, radiation-increased mRNAs (corresponding to TNF-α, TGF-β, IL-6, IL-1α/-1β, and ICAM-1) notably attenuated in psoralidin treatment at 12 h and 1 week after irradiation (Fig. 6B and C). At 12 h, psoralidin inhibited the radiation-increased mRNA level of TNF-α, TGF-β and ICAM-1 by half, and suppressed the radiation-increased mRNA level of IL-6 and IL-1α/-1β by one quarter. The inhibitory effects of psoralidin were more effective at 1 week after irradiation. These data suggest that psoralidin could effectively act *in vivo*, and would be a potential component regulating inflammation in both IR-irradiated normal lung fibroblast and mice, without toxicity.

4. Discussion

Radiotherapy is the most significant non-surgical cure for the elimination of tumor. About 50% of all cancer patients are given radiotherapy during the course of their treatment [42]. Efficiency of radiotherapy depends on (total) radiation dose, however, radiation-induced damage on normal cells surrounding the tumor, limits increase of the dose intensity [43]. Because preventing and minimizing of normal tissue damage are momentous in radiotherapy, it is essential to comprehend the damaging mechanism of

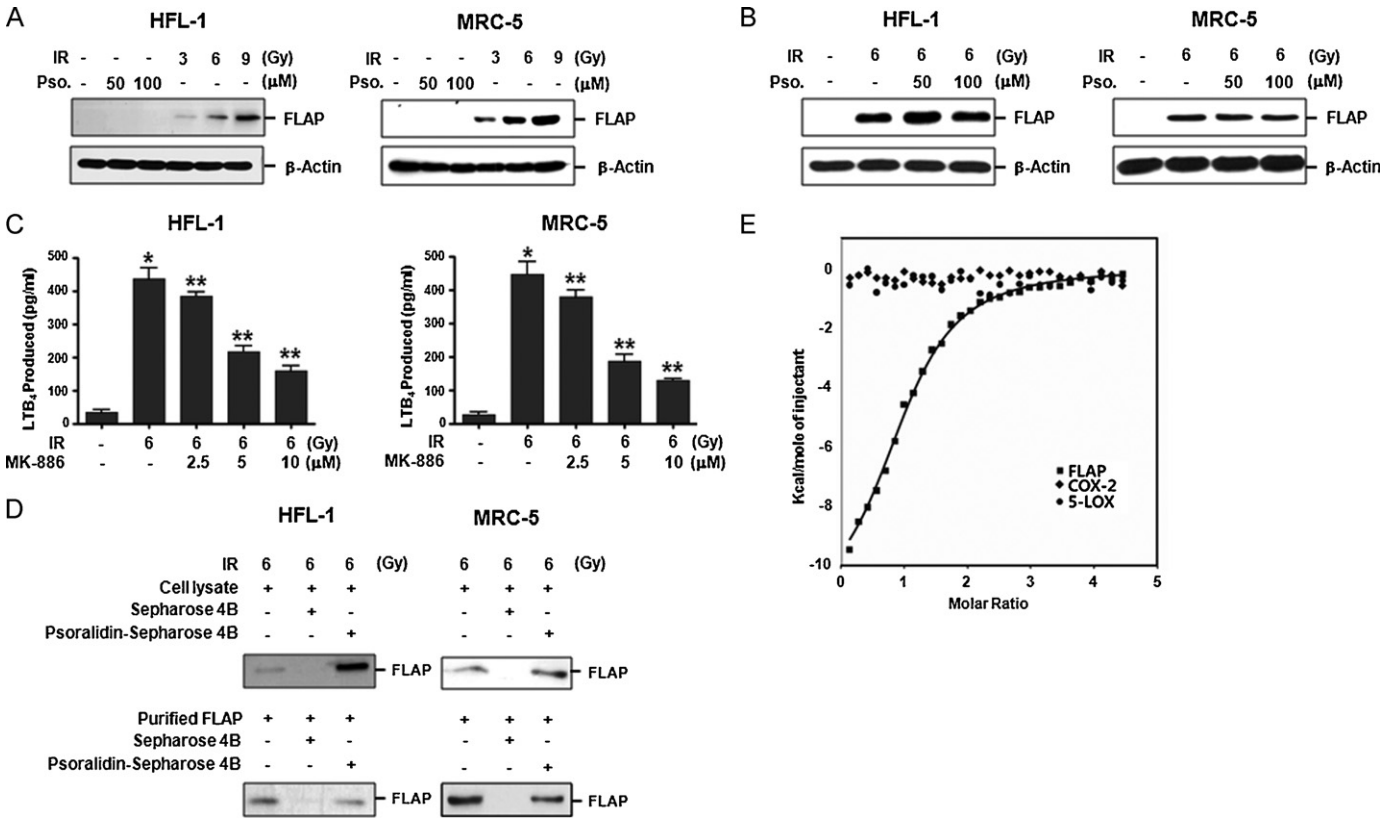

Fig. 4. FLAP is involved in inhibition of 5-LOX pathway by psoralidin in IR-irradiated normal lung fibroblasts. (A and B) The association between psoralidin and IR-induced FLAP expression was detected by immunoblotting. (C) Involvement of FLAP in IR-induced LTB_4 production was determined through MK-886 treatment by enzyme-immunoassay. $^{*}P < 0.05$; irradiated cells vs. control cells, $^{**}P < 0.05$; MK-886-treated irradiated cells vs. MK-886-untreated irradiated cells. (D) Direct binding of psoralidin to FLAP was assessed by pull-down assay. *Ex vivo* (top) and *in vitro* (bottom), binding of psoralidin to FLAP was confirmed by immunoblotting. (E) Characterization of the interaction between psoralidin and FLAP was investigated by ITC experiment. Heat of injection was experimentally determined during titration of psoralidin in the presence of FLAP (■), COX-2 (◆) or 5-LOX (●). Solid lines represent the least square fits of the data using a one-site binding model.

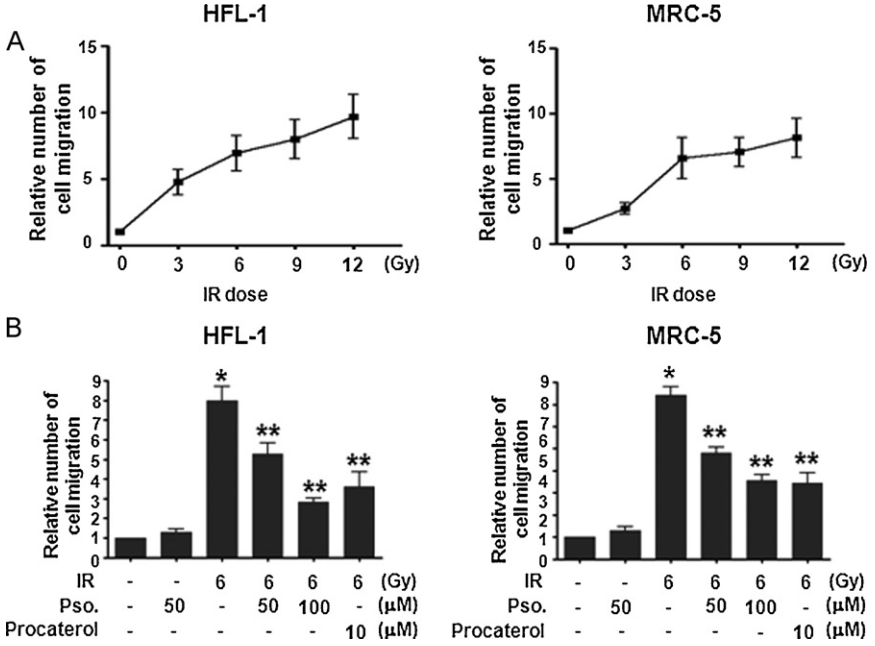

Fig. 5. Psoralidin effectively inhibits IR-increase fibroblast migration. (A) IR-induced fibroblast migration was detected by the Transwell migration assay. (B) The inhibitory effect of psoralidin in IR-induced fibroblasts migration was measured by the Transwell migration assay. The results are expressed as fold increase of migration compared with unirradiated control and based on the relative cell number in ten randomly selected fields from a single representative experiment. $^{*}P < 0.05$; irradiated cells vs. control cells, $^{**}P < 0.05$; psoralidin- or procaterol-treated irradiated cells vs. psoralidin- or procaterol-untreated irradiated cells.

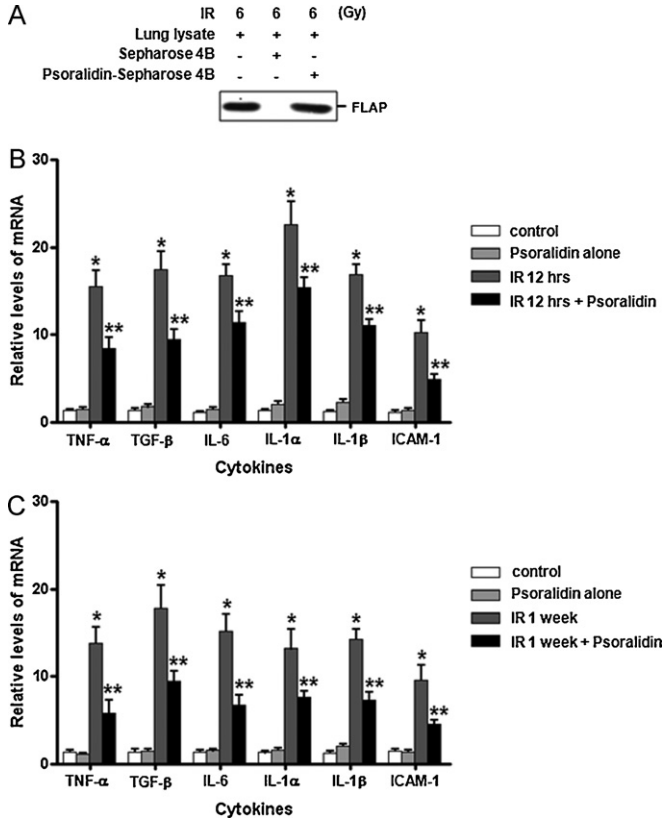

Fig. 6. Psoralidin inhibits gene expression of the pro-inflammatory cytokines and cell adhesion molecule in IR-irradiated mice. (A) The *in vivo* binding of psoralidin to FLAP in lung extracts of IR-irradiated mice was detected by pull-down assay and immunoblotting. (B and C) The inhibitory effects of psoralidin in IR-irradiated mice were determined through the alteration of the mRNA for TNF-α, TGF-β, IL-6, IL-1α/-1β and ICAM-1 by real time RT-PCR. $^{*}P < 0.05$; irradiated mice vs. control mice, $^{**}P < 0.05$; psoralidin-treated irradiated mice vs. psoralidin-untreated irradiated mice.

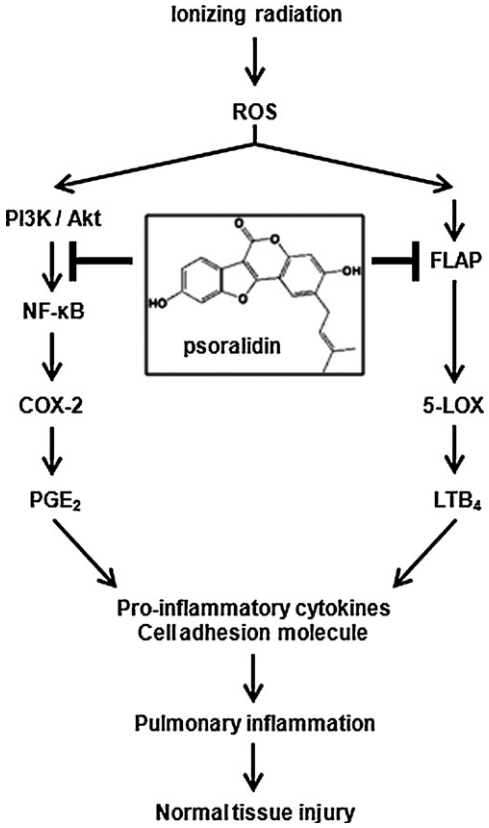

Fig. 7. Schematic diagram illustrating the IR-induced pulmonary inflammation pathway and dual inhibitory mechanism of psoralidin.

normal tissue exposed to radiation and to identify effective therapeutic agents. Based upon previous researches, we suggested that radiation-induced inflammation could have an important influence on normal cell damage. On the other hand, we are concerned various biological properties of natural products as therapeutic approach toward radiation-induced normal cell damage. According to our focus on lung cancer (as mentioned earlier in Section 1), we targeted human normal lung fibroblasts, which are highly abundant in the lung.

Fibroblasts were once believed to be comparatively less important as structure elements, producing only collagen and extracellular matrix (ECM) proteins. However, there are many reports that fibroblast are sentinel cells performing a pivotal role in production of pro-inflammatory mediators, cytokines and chemokines that regulate immunity and inflammation [44,45]. These mediators provoke epithelial and endothelial cells within the lung parenchyma, causing prolonged inflammation and tissue remodeling [46].

In this study, we sought to clarify inflammatory processes induced by IR and dual inhibitory mechanisms of psoralidin in human normal lung fibroblasts. Also, we identified anti-inflammatory property of psoralinin *in vivo*. A schematic model based on our results is presented in Fig. 7. Several important findings include the following: (i) In normal lung fibroblasts, IR-induced ROS activated the PI3K/Akt, NF-κB pathway, resulting in COX-2 mediated PGE_2 production, and the FLAP/5-LOX pathway, resulting in LTB_4 production. (ii) Psoralidin inhibited the COX-2 through suppression of NF-κB activation at a step after Akt phosphorylation, blocked the 5-LOX activity by direct binding with the FLAP, and decreased fibroblast migration in IR-irradiated normal lung fibroblasts. (iii) Furthermore, psoralidin inhibited the IR-induced gene expression of the pro-inflammatory cytokines *in vivo*.

Radiation responses can be distinguished by time appearing pathological symptoms: acute and late effects [47]. Acute effects including erythema, mucositis, nausea and diarrhea, are expressed during or within weeks of radiotherapy, and mainly associated with inflammation [48]. From months to years, late effects such as fibrosis and atrophy, are presented through tissue remodeling by excessive ECM synthesis and collagen deposition [49]. Recent progresses in molecular pathology and radiobiology have revealed that duration and severity of acute effects could increase the risk of late effects [50,51]. Abnormal continuance of inflammation could contribute to fibrosis, tumorigenesis and carcinogenesis [52]. Similarly, there are researches that not only cytokines production but also persistent cytokine expression during inflammation (acute stage) could initiate the process of epitherial–mesenchymal transition (EMT) leading to organ fibrosis (late stage) [53]. Interestingly, we identified that expression of pro-inflammatory cytokines (TNF-α, TGF-β, IL-6, IL-1α/-1β) and adhesion molecule (ICAM-1), persistently maintained from 12 h to 1 week in IR-irradiated mouse model. And psoralidin showed inhibitory effect in both time points (Fig. 6). According to these results, we assured that psoralidin could effectively act *in vivo*, and could have possibility for preventing radiation late effect by inhibition of the prolonged acute effect. Although parameters (TNF-α, TGF-β, IL-6, IL-1α/-1β and ICAM-1) representing IR-induced *in vivo* response are different to the parameters (COX-1, -2, 5-LOX, FLAP and migration) *in vitro*, because we focused on pro-inflammatory cytokines acting to make inflammatory disease worse and ICAM-1 involving fibroblasts migration in mouse model, we carefully suggest that *in vivo* results could cover for *in vitro* results, and

psoralidin could have anti-inflammatory effect *in vivo*. Further, to demonstrate exact mechanism of psoralidin *in vivo*, we will need to construction of appropriate mouse model to clarify IR-induced normal tissue effects and to demonstrate ability of psoralidin *in vivo*.

Previous studies of psoralidin were limited in cancer cell lines, and no report has described action mechanism of psoralidin under radiation. Although the anti-inflammatory effects of psoralidin on 5-LOX, 12-LOX, COX-1, and COX-2 have been briefly mentioned [54], until now, no report has described the mechanisms and effects of psoralidin on radiation-induced inflammation processes in normal lung fibroblasts. Psoralidin was reported as a potential cancer therapeutic agent in gastric (SNU-1, -16), colon (HT29), and breast (MCF-7) cancer cells [55,56]. Meaningful effects were not observed in lung (A549) and liver (HepG2) cancer cells [56]. These studies suggest that psoralidin could have cell type specific anti-cancer mechanism. Psoralidin is informed of possessing an anti-oxidant activity [57]. However, in our study, this effect was not remarkable, although psoralidin show slight anti-oxidant response in HFL-1 cells. This result suggested that inhibition of COX-2 by psoralidin could occur in downstream of ROS production and associate with ROS-mediated signaling, indicating slight effect of psoralidin as a ROS scavenger.

Something to notice in our research is that we provide the first evidence of the anti-inflammatory effect of psoralidin: dual inhibitory mechanism of COX-2 and 5-LOX, and association between psoralidin and FLAP. Physiological roles of COX-1 and pathological roles of COX-2 have generally been accepted [58], however, recent studies have shown that the relation between the two isoforms is not so straightforward. Indeed, COX-1 may contribute to inflammation processes whereas COX-2 is constitutively expressed in several organs and associated with several physiological functions. Recent pharmacological studies appear that only selective COX-2 inhibitors do not fully satisfy for new safer anti-inflammatory agents, and alternative medicine could be dual inhibitor of COX-2 and 5-LOX.

As a dual inhibitor, psoralidin could be applied for effective prevention of the side effects—normal tissue damage during the radiotherapy through balance of AA metabolism. Also, because COX-2 and 5-LOX are up-regulated in numerous cancers, development of drugs targeting both enzymes could be a helpful for cancer chemoprevention. We suggest that psoralidin may be useful as a potential lead compound for development of a better radiopreventive agent against radiation-induced normal tissue injury and effective adjuvant against radiotherapy.

Acknowledgments

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