Lipoxin (LX) A_4 modulates radiation induced lung injury (RILI)

Hyunjung Kim

Department of Medicine

The Graduate School, Yonsei University

Lipoxin (LX) A_4 modulates radiation induced lung injury (RILI)

Hyunjung Kim

Department of Medicine

The Graduate School, Yonsei University

Lipoxin (LX) A_4 modulates radiation induced lung injury (RILI)

Directed by Professor Jaeho Cho

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy in Medical Science

Hyunjung Kim

December 2021

This certifies that the Doctoral Dissertation of Hyunjung Kim is approved.

Thesis Supervisor: Chang Geol Lee

Thesis Committee Member#1: Yun-Sil Lee

Thesis Committee Member#2: Moo Suk Park

Thesis Committee Member#3: Yoon-Jin Lee

Thesis Committee Member#4: Jaeho Cho

The Graduate School
Yonsei University

December 2021

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Professor Jaeho Cho, who gave me an opportunity and hope at every moment of giving up, and my parents, who supported me under any conditions.

In addition, I am profoundly thankful to Professor Moo Suk Park, Yun-Sil Lee, Yoon-Jin Lee, and Chang Geol Lee, who led the academic discussion and gave keen advice on this study.

<TABLE OF CONTENTS>

LIST OF FIGURES

Lipoxin (LX) A4 modulates radiation induced lung injury (RILI)

Hyunjung Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Jaeho cho)

Radiation-induced lung injury (RILI) is an unavoidable side effect in external beam radiotherapy on the thorax, which lasts for an extended period can cause irreversible radiation-induced fibrosis, compromising the respiratory ability of patients, so that it substantially affects the life quality. Despite the incidence and its seriousness, no fundamental treatment method has been developed and only symptomatic treatments are applied. Lipoxin, a substance proven to have anti-inflammatory effects, is a lipid mediator that is mostly secreted in the body and induced by drugs such as aspirin that can be easily obtained. We investigated the effects of lipoxin A_4(LXA_4) on RILI using a small-animal model following focal-ablative lung irradiation (IR). LXA_4 significantly inhibited immune-cell recruitment and reduced IR-induced expression of pro-inflammatory cytokines and fibrotic proteins in the irradiated lung sites. Micro-CT revealed that LXA_4 reduced IR-induced increases in lung consolidation volume. The FlexiVent^{TM} assays showed that LXA_4 reversed IR-induced lung function damage. In conclusion, Lipoxin shows its potential as a modulator and drug for the treatment of RILI.

Key words: radiation induced injury; lung; Lipoxin; LXA_4

Lipoxin (LX) A4 modulates radiation induced lung injury (RILI)

Hyunjung Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Jaeho Cho)

I. INTRODUCTION

External beam radiotherapy (EBRT) is an anti-cancer treatment that was advantageous as a non-invasive local treatment. It was applied either as monotherapy or combined therapy with surgery, chemotherapy, and immunotherapy[1]. According to the principle of radiotherapy, "As Low as Reasonably Achievable (ALARA)", radiotherapy has been developed to have the least side effects in normal tissue while radiating on tumors using 3-dimensional conformal radiotherapy (3D-CRT), Intensity-modulated radiation therapy (IMRT), or stereotactic ablative radiotherapy (SABR). However, 60-70% of patients who received radiotherapy have various side effects from minor to major problems[2]. Especially, radiation-induced lung fibrosis related to respiration is not only interfered with the quality of life but also has no fundamental treatments and effective prevention methods. Therefore, we should study to overcome radiation-induced lung fibrosis.

During the EBRT on the thoracic area, the radiation has been passing through normal lung tissues to reach a tumor. The normal lung tissues that surrounded the tumor occur the inflammation responses. About 70-80% of lung cancer patients with radiotherapy were showed radiological pneumonitis, and 20-30% of them occurred clinical symptoms. Radiation-induced pneumonitis is bound up with the

time duration of side effects. Radiation-induced pneumonitis leads to chronic courses; it can result in radiation-induced lung fibrosis that was an irreversible stage. And the chances of the cure the tumor with radiotherapy are decreased due to the chronic and severe side symptoms.

The Ethyol™ (Amifostine), an antioxidant that has been developed for the prevention of radiation injury in normal tissues, was approved the effects for the prevention and regulation of radiation side effects. But it is notorious for its toxicity and high prices[3,4]. Recently, it has been reported that drugs such as angiotensin-converting enzyme (ACE) inhibitors were helpful for the reduction of radiation pneumonitis symptoms [5,6]; however, there has been no verification of large-scale prospective studies. There were the drugs used for side effects of radiotherapy in-clinic include prednisone, glucocorticosteroid for reduction of inflammation; colchicine to inhibit the synthesis of collagen; penicillamine; antibiotic therapy; and immunosuppressant; however, most of them are symptomatic drugs and their secondary side effects are significant. So that they have limitations in fundamental treatment.[7] Therefore, it is necessary to develop radiation-induced lung fibrosis modulator which has fewer side effects, is more affordable, and enables to treat it fundamentally.

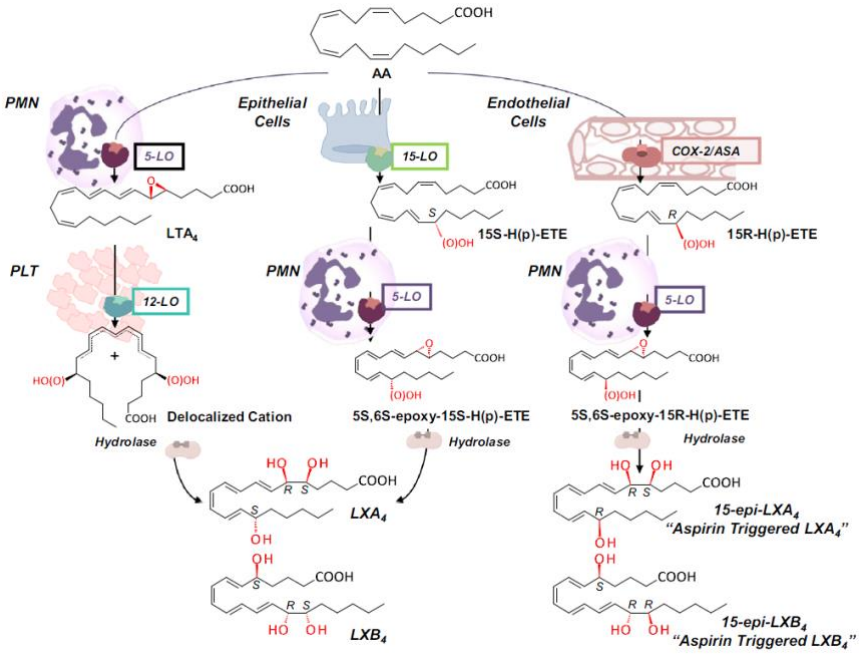

Figure 1. Biogenesis of Lipoxin A_4 derived from Arachidonic acid. Adapted from "Lipoxins and aspirin-triggered lipoxins in resolution of inflammation", by M. Romano, 2015, European journal of pharmacology, 760, p. 50, copyright 2015 by the Elsevier B.V.

Aspirin, an analgesic and antipyretic drug that has been used for a long time, is cheap and can be easily obtained, and its mechanism was well known (Figure 1). It inactivates Cyclooxygenase (COX)-1, through which it inhibits production of Prostaglandin and Thromboxane A_2 that are substances to cause inflammation. In addition, 15R-hyrodperoxyeicosatetraenoic acids (15R-HETE) are formed on membranes of epithelial or endothelial cells by the function of acetylated COX-2 by aspirin, which is converted to 15(R)-epi-lipoxin A_4. Lipoxin (LX), as a lipid mediator, functions anti-inflammatory roles by inhibiting the recruitment of inflammatory cells, suppressing the secretion of cytokines, and reducing vascular permeability.[8,9] Although LX is mostly produced endogenously in the early stage

of inflammation, it is also produced by external stimulation such as aspirin as described before ATL (Aspirin Triggered Lipoxin). Released LX binds to lipoxin A_4 receptor/formyl peptide receptor 2 (ALX/FPR2) receptor on cellular membranes[10] for cellular functions (Figure 2), and this receptor is mostly expressed in endothelial cells or myeloid lineages cells, regulating innate immunity[11] and adaptive immunity[12], which has a great inflammation reduction effect[13-15] as well as an anti-cancer effect.[16,17]

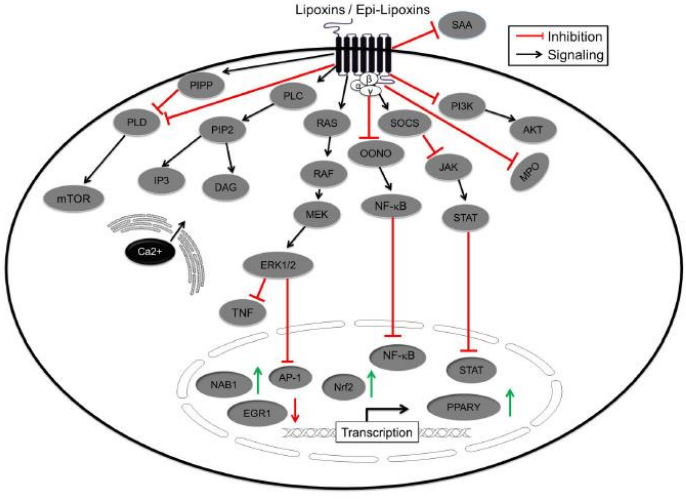

Figure 2. Effects of lipoxins on cytoplasmic signaling cascades and transcription factors. Adapted from "Lipoxins: nature's way to resolve inflammation", by Chandrasekharan and Sharma-Walia, 2015, Journal of inflammation research, 8, p. 184, copyright 2015 by the Chandrasekharan and Sharma-Walia.

It has been proven that LX increases in broad ranges of patients or animals with respiratory inflammation diseases, and LX and ATL have anti-inflammatory functions in respiratory inflammation disease through in vivo and in vitro studies.[18-20] In addition, it has been reported that LX decreased in the respiratory tract under chronic inflammation setting in patients with cystic fibrosis or

asthma[21,22], which means LX is involved only in the early inflammatory responses. The longer the duration of the inflammatory response, the lower the endogenous production of lipoxin. In other words, Whereas the absence or reduction of LX in the inflammatory responses can lead to chronic inflammation status. Therefore, the LX shows the potential as a mediator to regulate inflammation or fibrosis.

Nevertheless, LX has not been used clinically, because LX failed to last long due to the activities of monocytes or macrophages[23]; however, a recently developed stable analog complemented its sustaining power.[24,25] Thus, commercialized stable LX enables to study of anti-fibrotic, anti-inflammatory effects of LX on the lung.

In the present study, we evaluated the effects of LXA_4 on the reduction of radiation-induced lung injury (RILI) in various aspects including histology, molecular biology, alteration of cytokine expression, and functional test of the lung using an animal model.

II. MATERIALS AND METHODS

1. Materials

A. Lipoxin A_4 (LXA_4) and WRW4

LXA_4, a long-lasting exogenous lipoxin analog, and WRW4 which is an FPR2 receptor antagonist were purchased from Charles River Korea (Orient Bio, Seongnam, South Korea).

B. Mice

Male C57BL/6 mice (age, 6 weeks; weight, 20–25 g) were purchased from Charles River Korea (Orient Bio, Seongnam, South Korea) and allowed to acclimatize (n = 5 per cage) for a week before irradiation (IR). All protocols involving the use of mice were approved by the Animal Care and Use Committees of Yonsei University Medical School, Seoul, South Korea (YUHS-IACUC; 2016-0199), and were performed under the relevant guidelines.

C. MLE12 cell line

The mouse lung epithelial cell line, MLE12, obtained from the American Type Culture Collection, was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. The cells were seeded (density, 1.0×10^6 cells) in a 60-mm plate. After 24 h, the cells were washed and maintained in serum-free medium before the experiments.

2. Methods

A. Irradiation

A single dose of 75 Gy IR was delivered to the left lung using an image-guided small-animal irradiator (X-RAD 320; Precision, North Branford, CT, USA)

equipped with a collimator comprising 3.5 cm-thick copper and an imaging subsystem comprising a fluorescent screen coupled to a charge-coupled-device camera. In all experiments, 3-mm collimators were used to mimic clinical SBRT conditions with a small IR volume in lung tissues. The mice were randomly divided into the following four groups (n = 3–5 per group): (1) control group; (2) IR group exposed to a single dose of 75 Gy delivered to the left lung in a single fraction; (3) IR + LXA_4 group treated intravenously with LXA_4 (0.4, 1.2, or 2 μg/mouse) with IR; (4) IR+LXA_4+WRW4 group treated intravenously with WRW4 (10 μg/mouse) before the injection of LXA_4. The mice were euthanized by CO2 inhalation, and their lung tissues were collected for analysis. LXA_4 levels are measured in serum samples using an LXA_4 kit (Cusabio Biotech, Wuhan, China) according to the manufacturer's protocol.

B. Preparation and histological evaluation of lung tissue sections

Tissues from the left lung of the irradiated mice were fixed in 4% paraformaldehyde for 24 h, dehydrated and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E), Masson's trichrome (MT), and TGF-β, α-SMA, Twist, IL-6 antibodies, CD4, and CD163 (Abcam, Cambridge, MA, USA).

C. Immunohistochemistry

Lung tissue sections were stained with H&E and MT staining. To detect TGF-β, α-SMA, Twist, IL-6, CD4, and CD163, tissue sections were probed with primary antibodies (4°C overnight), followed by incubation with the avidin-biotin-peroxidase complex (ABC Kit, Vector Laboratories, CA, USA), and were developed using 3, 3′-diaminobenzidine tetrachloride (DAB; Zymed Laboratories, CA, USA).

For histological inflammation and fibrosis evaluation[26], lung tissue sections were

stained with TGF-β1 staining. Randomly selected fields of each slide were scored for area and intensity of positively stained (brown) cytoplasm and cell membrane. Inflammation scores were assigned as follows: 0 = no appreciable staining (negative); 1 = barely detectable staining (weak); 2 = readily appreciable brown staining (moderate); and 3 = dark brown staining (strong positivity). The total score was calculated by adding the intensity scores from five independent views in each sample, resulting in a final score of 0 to 15. For statistical analysis, scores 3–15 and 0–2 was defined as indicating positive and negative expression, respectively.

D. Lipoxygenase (LOX) activity assay

LOX activity in conditioned medium was measured using a LOX activity assay (AAT Bioquest, SunnyVale, CA) according to the manufacturer's instructions.

E. Colony formation assay

MLE12 cells were cultured in 35-mm dishes using DMEM supplemented with 10% FBS in the background of IR (3 Gy and 6 Gy) using an X-rad320 (Precision, North Branford, CT, USA). After 2 weeks—with or without LXA_4 treatment—the cells were fixed, stained with 0.5% crystal violet, and quantified for colony number.

F. Micro-CT analysis

Micro-computed tomography (CT) images were acquired using a volumetric CT scanner (NFRPolaris- G90MVC: NanoFocusRay, Iksan, South Korea) at 50 kVp, 180 μA, and 150 mGy (number of views, 700; frame rate, 142 ms). Images were reconstructed (image size, 1232 × 1120 pixels; number of slices, 512) by volumetric cone-beam reconstruction (Feldkamp-Davis-Kress method) in in-line/off-line modes. Volumetric analysis was performed using the Image J

software. To minimize inter-specimen variations in measurement, identical level settings were used for analysis of all images.

G. Functional lung assessment

Lung function in irradiated mice was evaluated by FlexiVent system (FlexiVent®; SCIREQ©, Montreal, QC, Canada), which measures flow-volume relationships in the respiratory system, including forced oscillation, to discriminate between airway and lung tissue variables. Evaluations were performed according to manufacturer's instructions. Briefly, after anesthetization, mice were connected to computer-controlled small-animal ventilator and quasi-sinusoidally ventilated with tidal volume of 10 mL/kg at frequency of 150 breaths per minute. Measurement commenced when a stable ventilation pattern, without obvious spontaneous ventilator effort, was observed at ventilation pressure tracing. All perturbations were performed sequentially until three acceptable measurements (coefficient of determination >0.95) were recorded for each subject, from which an average was calculated.

H. Analysis of Bronchoalveolar lavage (BAL) fluid

After sacrifice, a 1-mL syringe containing 1 mL PBS was inserted into the exposed trachea of mice. The PBS was injected and aspirated back into the syringe to collect BAL fluid. This procedure was repeated three times. The fluid was centrifuged at 1300 rpm for 10 min. Cell pellets were resuspended in 1 mL PBS and collected on glass slides by cytocentrifugation. Total live-cell count was determined using a haemocytometer. In addition, the cell counts of macrophages, neutrophils, eosinophils, and lymphocytes were determined by evaluating BAL fluid cytospin smears stained with the Diff-Quick stain (Life Technologies, Auckland, New Zealand); 500 cells were counted per slide. The supernatants of BAL fluid were stored at −80 °C for further analysis.

I. Quantitative real-time polymerase chain reaction (qRT- PCR)

Total RNA was isolated from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and cDNA was synthesized using a Quantifect Reverse Transcription Kit (Qiagen, Hilden, Germany). qRT- PCR was performed using SYBR Premix Ex Taq (Takara, Hercules, CA, USA), primers, RNase-free H2O, and cDNA (final reaction volume, 20 μL) under the following cycling conditions: 95 °C (10 min); and 50 cycles of 95°C, (20 sec), 55°C (30 sec), and 72°C (20 sec). All experiments were performed in triplicate, and the results were normalized to glyceraldehyde 3- phosphate dehydrogenase expression. mRNA expression was calculated using the delta Ct method.

J. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences between the means of two groups were evaluated using Student's t-tests. Differences between the means of multiple groups were evaluated by a one-way analysis of variance. The threshold for significance was $p < 0.05$, and all values were expressed as the mean ± SEM.

III. RESULTS

1. The natural course of the lung irradiation and the expression of FPR2 receptors

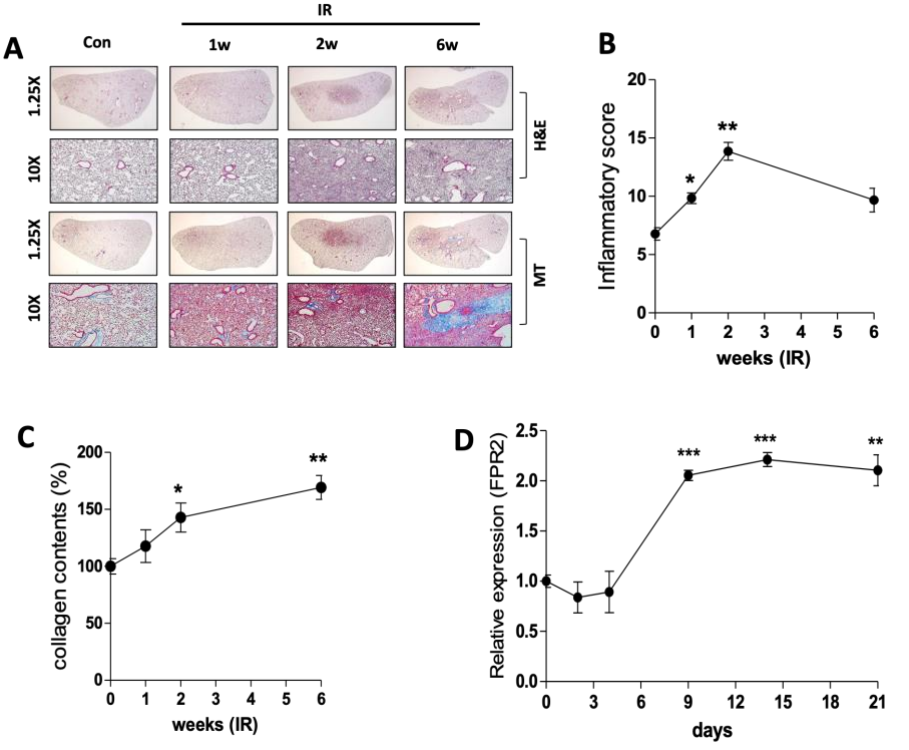

Figure 3. The natural course of the lung irradiation and the expression of FPR2 receptors. control, irradiation (IR) after 1wk;1w, IR after 2 weeks; 2w, IR after 6 weeks; 6w. **A**. Hematoxylin and eosin (H&E) stained lung sections. Masson's trichrome (MT) stained lung section. Magnification, 1.25x, 10x. **B**. Quantification of inflammatory foci. **C**. Quantification of collagen content after IR. **D**. the level of FPR2 receptor expression after IR. Data are expressed as the mean ± S.E.M. (n=3, *p<0.05, **p<0.01, ***p < 0.001 versus control).

To investigate the natural course of lung irradiation, we checked the inflammatory scores and collagen depositions in the 1, 2, 6 weeks. As shown in

Fig. 3, the inflammatory scores presented the maximum at 2 weeks, and the collagen depositions presented gradually increased. We found that the FPR2 receptor expression level reached the maximum level after 2 weeks.

2. Radiation reduces the endogenous Lipoxin (LXA_4) product

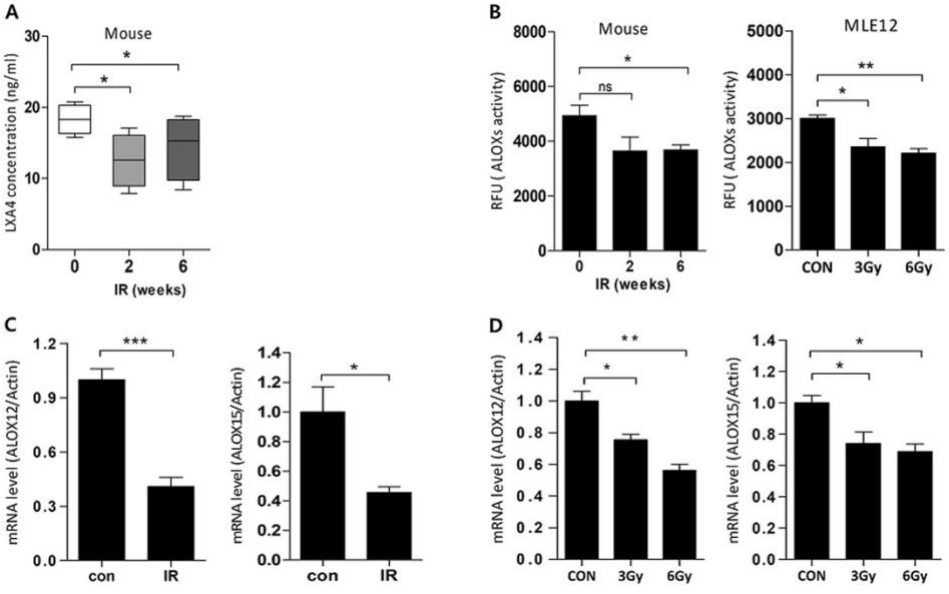

Figure 4. Radiation reduces the endogenous Lipoxin production

A. Endogenous LipoxinA_4(LXA_4) concentration after irradiation. 0, 2, and 6 weeks examinates by ELISA. **B**. Arachidonate lipoxygenase activity in vivo (mice) after irradiation (IR), and in vitro (MLE 12 cell) with 3 and 6 Gy. **C**. Arachidonate 12-lipoxygenase (ALOX12) and Arachidonate 15-lipoxygenase (ALOX15) level after IR in vivo. **D**. ALOX12 and ALOX15 level after IR in vitro. Data are expressed as the mean ± S.E.M. (n=3, *p<0.05, **p<0.01, ***p < 0.001 versus control).

To investigate the effect of the LXA_4 in RILI, the endogenous LXA_4 level was examined by ELISA. As shown in Fig. 4A, endogenous LXA_4 levels were significantly lower 2 weeks (12.56 ± 1.885 ng/ mL) and 6 weeks (14.46 ± 2.248 ng/mL) after IR than those in the control group (18.33 ± 1.037 ng/mL) in mouse blood by ELISA. The IR also reduced the lipoxygenase activity in the mouse

model and MLE12 cells (Fig. 4B) and downregulated Alox12 (12-lipoxygenase) and Alox15 (15- lipoxygenase), lipoxygenase variants which involved in arachidonic acid metabolism in irradiated sites (Fig. 4C) and MLE12 cell-line (Fig. 4D).

3. LXA_4 inhibits radiation-induced lung inflammatory responses (1)

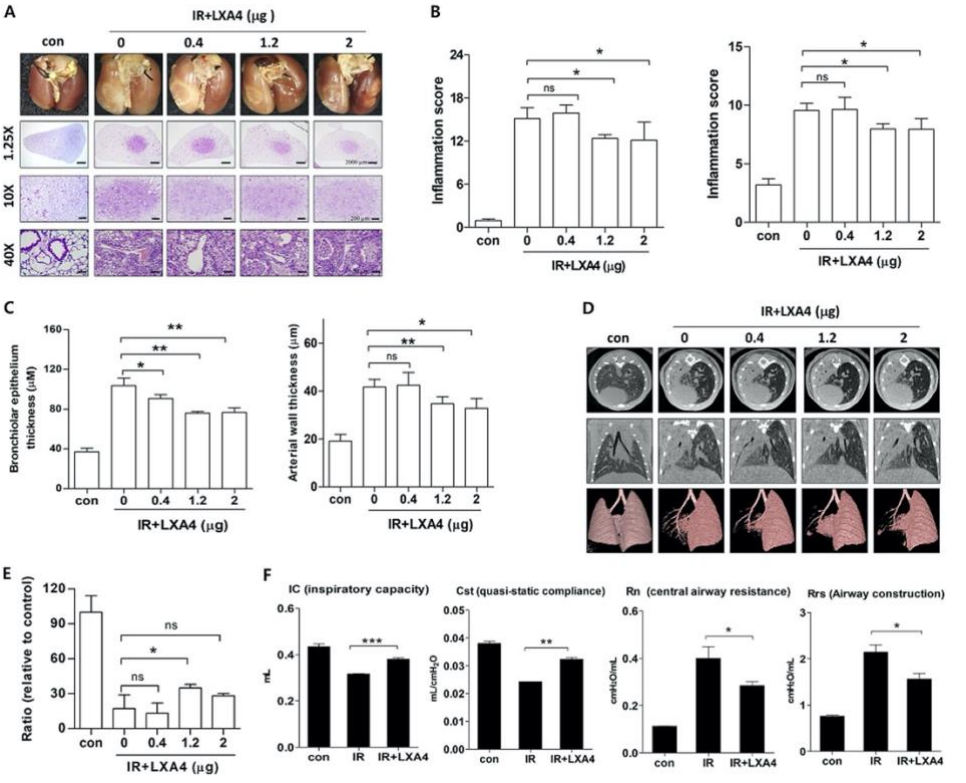

Figure 5. LXA_4 inhibits radiation-induced lung inflammatory responses (1) control, irradiation (IR) after 2wks; IR, IR with LXA_4 after 2 weeks; IR+LXA_4. **A**. Representative gross (top) and H&E (bottom) images of left lung tissues 2 weeks after 75 Gy IR with or without LXA_4 in the mouse model. Magnification, ×1.25, ×10, and ×40. **B**. Quantification of inflammatory foci at the lesion site (left) and in the surrounding lung tissue (right). **C**. Quantification of bronchiolar epithelium (left) and arterial wall (right) thickness in lung tissues. **D**. 3D micro-computed tomography (CT) of mouse lung tissue. Horizontal (top), trans-axial (middle), the automated analysis protocol is illustrated in a normal and an inflammatory lung. Areas of density between -700 and -350 HU on 3D micro-CT images are shown in pink(bottom) **E**. Normal lung volume excluding consolidation was quantified using the micro-CT images. **F**. mouse lung function

parameters were measured using the FlexiVent™ system 2 weeks after irradiation, i.e., inspiratory capacity (IC), quasi-static compliance (Cst), central airway resistance (Rn), and airway construction (Rrs). Data are expressed as the mean ± S.E.M. (n=3, *p<0.05, **p<0.01, ***p < 0.001 versus control).

Using a small animal radiation model, we evaluated the effects of LXA_4 on pneumonitis 2 weeks after 75 Gy IR by evaluating the lung morphology and performing H&E staining, imaging (micro-CT), and pulmonary function assays (FlexiVent™). In gross specimen, the normal lung was brown in color, whereas the irradiated lung after 2 weeks exhibited a ring-shaped boundary with white-colored adjacent areas (Fig. 5A). The IR group displayed significantly more inflammation at the lesion site than the control group, and inflammation was significantly blocked by LXA_4 (1.2 and 2 μg per mouse) in the IR + LXA_4 group, which displayed less damage than the IR group (Fig. 5B, Left). The Inflammatory scores changed in the tissues surrounding the irradiated lesion were also significantly reduced in the LXA_4-treated groups (1.2 and 2 μg/mouse) compared to those in the IR group (Fig. 5B, Right).

Micro-CT images taken 2 weeks after IR show that pulmonary consolidation. The consolidation occurs when the air that fills the airways in your lungs is replaced with something else. The 3-dimensional reconstructed image can measure high or low air-to-tissue contrast volume. The lung consolidation volume was decreased in the LXA_4 group (1.2 μg) compared with the control group. (Fig. 5D, 5E).

We analyzed pulmonary function—based on six parameters—using the FlexiVent™ system. Four of these parameters showed significant improvement in the IR + LXA_4 group, i.e., IC (inspiratory capacity; IR vs. IR + LXA_4, 0.316 ± 0.0017 mL vs. 0.3812 ± 0.0044 mL), Cst (quasi-static compliance; IR vs. IR + LXA_4, 0.02428 ± 0.00048 mL/ cmH2O vs. 0.03231 ± 0.0006852 mL/cmH2O),

Rn (airway construction; IR vs. IR + LXA_4, 0.4000 ± 0.04930 cmH2O/ mL vs. 0.2843 ± 0.01795 cmH2O/mL), and Rrs (central airway resistance; IR vs. IR + LXA_4, 2.145 ± 0.1612 cmH2O/mL vs. 1.564 ± 0.1237 cmH2O/mL) (Fig. 5F).

4. LXA_4 inhibits radiation-induced lung inflammatory responses (2)

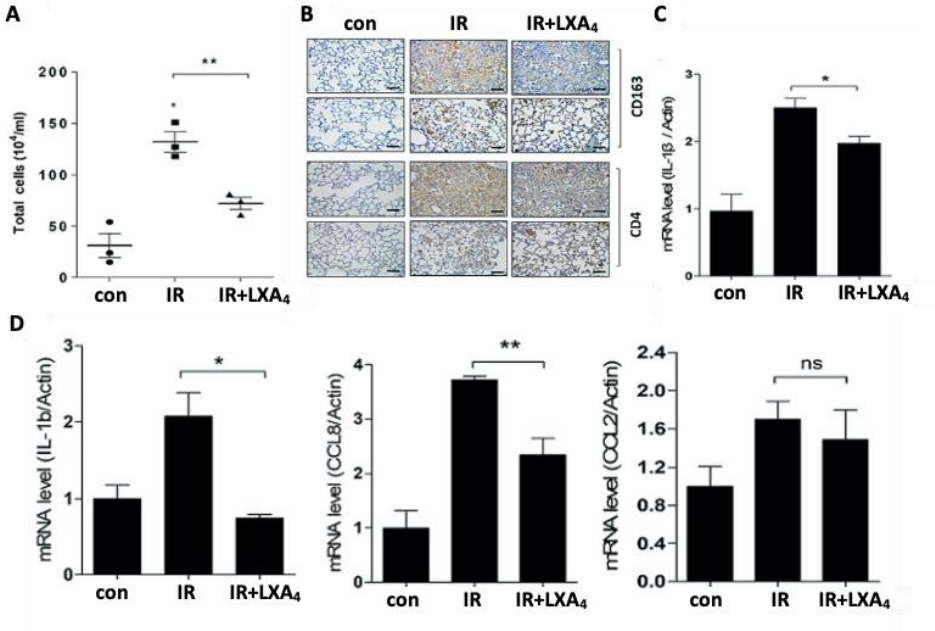

Figure 6. LXA_4 inhibits radiation-induced lung inflammatory responses (2)
control, irradiation (IR) after 2wk; IR, IR with LXA_4 after 2 weeks; IR+LXA_4. **A**. Total BAL fluid cells count in mice 2 weeks after IR. **B**. Representative immunohistochemical images of irradiated lung tissues using CD163 and CD4 antibodies. Magnification, ×40. **C**. IL-1β mRNA expression in MLE12 cells measured using quantitative real-time PCR. **D**. IL-1β, CCL8, and CCL2 mRNA expression in irradiated lung tissue measured using quantitative real-time PCR. Data are expressed as the mean ± S.E.M. (n=3, *p<0.05, **p<0.01 versus control).

IR increased the total cell counts of BAL fluid compared to those in the control group and LXA_4 group (Fig.6A). In immunohistochemistry staining with the BAL fluid, whereas LXA_4 rescued this effect and reduced the IR-induced infiltration of immune cells such as CD163+ and CD4+ cells (Fig. 6B). CD163

is a macrophage-specific protein and high CD163 expression is a characteristic of tissue responding to inflammation.[27] CD4+ cells has a roles in pathogenesis of fibrotic lung disease.[28] To confirm this anti-inflammatory effect, we evaluated inflammatory cytokine and chemokine expression by qRT-PCR. While IR significantly upregulated IL-1β, CCL2, and CCL8 expression, LXA_4 attenuated their expression in the mouse model (Fig. 6C). In vivo, LXA_4 decreased the IR-induced increase in IL- 1β expression in MLE12 cells (Fig. 6D)

5. LXA_4 inhibits radiation-induced pulmonary fibrosis

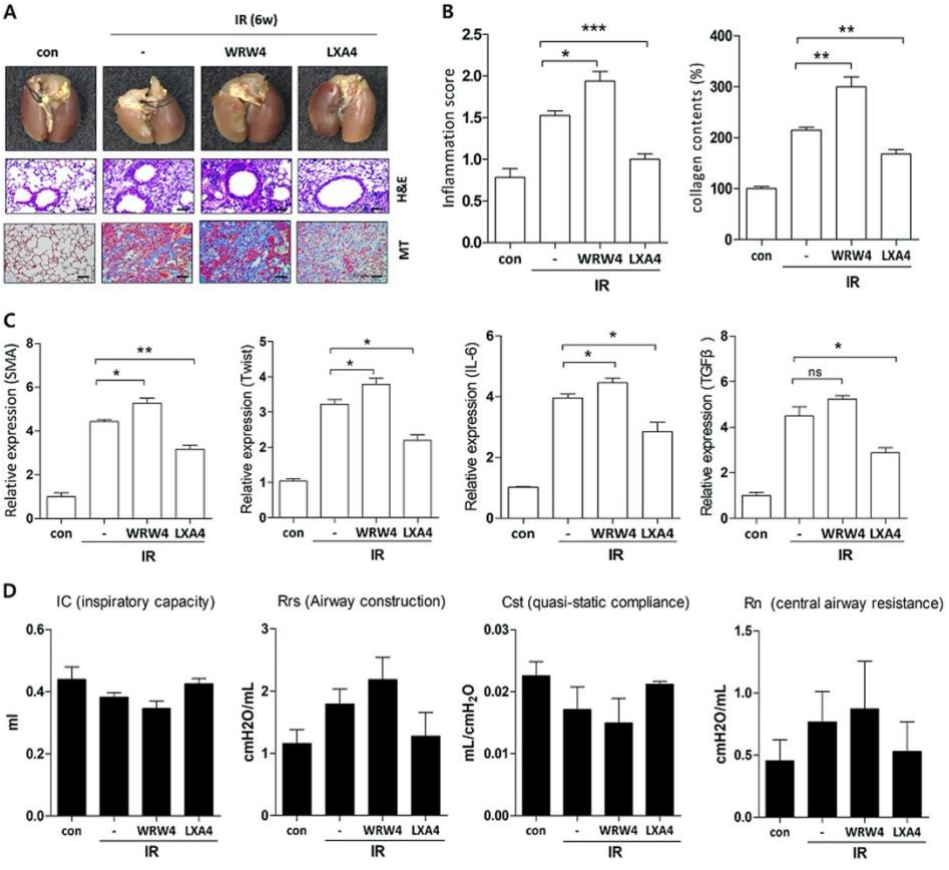

Figure 7. LXA_4 inhibits radiation-induced pulmonary fibrosis

control, irradiation (IR) after 6wks; -, IR with WRW4 after 6 weeks; WRW4, IR with LXA_4 after 6 weeks; LXA_4. **A**. Representative gross (top), H&E (middle), and MT (bottom) images of the left lung tissue 6 weeks after 75 Gy IR, IR with WRW4 which is FPR2 receptor blocker, and IR with LXA_4 in the mouse model. Magnification, ×40. **B**. Quantification of fibrotic foci (left) and collagen content (right). **C**. Quantification of relative α-SMA, Twist, IL-6, and TGF-β expression in the lung lesion site using immunohistochemistry. **D**. Functional measurement of several mouse lung parameters using the FlexiVent^{TM} system after 6 weeks IR, i.e., inspiratory capacity (IC), quasi-static compliance (Cst), central airway

resistance (Rn), and airway construction (Rrs). Data are expressed as the mean ± S.E.M. (n=3, *p<0.05, **p<0.01, ***p < 0.001 versus control).

Six weeks after IR, LXA_4 decreased the immune-cell infiltration and collagen deposition as well as bronchiolar epithelium thickness and arterial wall thickness (Fig. 7A, 7B). To minimize the effects of endogenous LXA_4, the WRW4 was used as the receptor blocker. The expression of α-SMA, Twist, IL-6, and TGF-β was inhibited by LXA_4 and increased by WRW4 (Fig. 7C).
Functionally, pulmonary functions have a tendency of rescuing by LXA_4, which reversed four of the six lung function parameters (IC, Cst, Rrs, and Rn) (Fig. 7D). These results demonstrate that LXA_4 may inhibit the development of radiation-induced fibrosis via the FPR2 receptor.

IV. DISCUSSION

Since there is no fundamental treatment for RILI, numerous studies have focused on the conservative treatments of radiation-induced lung inflammation and fibrosis. In this study, we found that lipoxygenases that involved the production of the endogenous LXA_4 were reduced after irradiation, and the total amount of endogenous LXA_4 was reduced. While the irradiation made that the expression of the FPR2 receptor was upregulated, the short of the endogenous LXA_4 proceed to the pro-inflammatory and fibrosis phase. Therefore, the irradiated mouse model which injected intravenously with the exogenous LXA_4 showed the reactions of inflammation and fibrosis were delayed and reduced cytologically, morphologically, radiologically, and functionally.

We found that the injected LXA_4 through the tail vein after irradiation that the inflammatory scores and the expression of the FPR2 receptor reached the maximum in 2 weeks after IR. We designed the LXA_4 to be injected once. In fact, when LXA_4 was administered, there was no significant difference in anti-inflammatory responses under 1.2 μg. (Fig. 4) The expression of the FPR2 receptor reached the plateau at 2 weeks after irradiation and did not increase anymore. (Fig.3) These results showed that the LXA_4 followed the dose-limiting response. The reason that receptor expression did not increase for the first 3 days in the FPR2 receptor is probably the endogenous LX was acted and degraded on the receptor.

Currently, the LX analog is in development. There are four types of analogs; the prolonged half-life and bioactivation of native LXA_4[8] which we used in this study, 3-oxa-LXA_4 analogs[29], pyridine/benzo-LXA_4 analogs[30], and imidazole related analogs.[31] There were many preclinical experiments for mice for inflammatory disease. There was a phase I study for COPD, asthma, Inflammatory bowel disease, and gingivitis (trial identifier: NCT02342691). And the phase 3 trial for uncontrolled asthma (the ASTHMIRINE trial) is ongoing (trial identifier:

NCT02906761). However, there was no study for radiation-induced side effects. Consistent with our findings, LXA_4 has emerged as an anti-inflammatory and anti-fibrotic modulator; its endogenous nature, there were no serious side effects and death in these experiments. The longer the duration of the combination with LX and the receptor, the higher the anti-inflammatory effect, so we need to expect maximized anti-inflammatory effect by controlling the number and timing of LX administration. In addition, it has been reported that lipoxin has a "reverse effect" when fibrosis has already progressed in other fibrosis models[32] such as Lipopolysaccharide or bleomycin triggered. If it is also effective in radiation-induced fibrosis, it is clinically more valuable.

A limitation of this study was that it was designed to be injected with lipoxin only once. We did not measure how long the lipoxin analog which we used in this experiment lasted. in figure 1-D, the endogenous lipoxin may be degraded after 3 days of radiation, and lipoxin seems to be continuously lacking thereafter. Therefore, it was necessary to design more sophisticated the timing of injection and the interval of continuous supply. In addition, fibrosis is known to be irreversible, but it is a pity that we did not proceed with the experiment for the revere effects. In addition, micro-CT imaging was not performed in the 6-week fibrosis model.

V. CONCLUSION

In conclusion, we demonstrated that LXA_4 exerts protective effects by inhibiting collagen production, decreasing the expression of fibrosis-related proteins and inflammatory cytokines. These results suggest that LXA_4 reduces radiation-induced lung inflammation and fibrosis and may serve as a useful therapeutic agent for inhibiting radiotherapy-induced inflammatory responses and fibrosis in normal lung tissue.

REFERENCES

1. Ghafoori P, Marks LB, Vujaskovic Z, Kelsey CR. Radiation-induced lung injury. Assessment, management, and prevention. Oncology (Williston Park) 2008;22:37-47; discussion 52-3.
2. Yazbeck VY, Villaruz L, Haley M, Socinski MA. Management of normal tissue toxicity associated with chemoradiation (primary skin, esophagus, and lung). Cancer J 2013;19:231-7.
3. Wynn RB, Mehta V. Reduction of treatment breaks and radiation-induced esophagitis and pneumonitis using amifostine in unresectable non-small cell lung cancer patients receiving definitive concurrent chemotherapy and radiation therapy: a prospective community-based clinical trial. Semin Oncol 2005;32:S99-104.
4. Garces YI, Okuno SH, Schild SE, Mandrekar SJ, Bot BM, Martens JM, et al. Phase I North Central Cancer Treatment Group Trial-N9923 of escalating doses of twice-daily thoracic radiation therapy with amifostine and with alternating chemotherapy in limited stage small-cell lung cancer. Int J Radiat Oncol Biol Phys 2007;67:995-1001.
5. Bracci S, Valeriani M, Agolli L, De Sanctis V, Maurizi Enrici R, Osti MF. Renin-Angiotensin System Inhibitors Might Help to Reduce the Development of Symptomatic Radiation Pneumonitis After Stereotactic Body Radiotherapy for Lung Cancer. Clin Lung Cancer 2016;17:189-97.
6. Wang H, Liao Z, Zhuang Y, Xu T, Nguyen QN, Levy LB, et al. Do angiotensin-converting enzyme inhibitors reduce the risk of symptomatic radiation pneumonitis in patients with non-small cell lung cancer after definitive radiation therapy? Analysis of a single-institution database. Int J Radiat Oncol Biol Phys 2013;87:1071-7.
7. Ding NH, Li JJ, Sun LQ. Molecular mechanisms and treatment of radiation-induced lung fibrosis. Curr Drug Targets 2013;14:1347-56.
8. Serhan CN, Maddox JF, Petasis NA, Akritopoulou-Zanze I, Papayianni A, Brady HR, et al. Design of lipoxin A4 stable analogs that block transmigration and adhesion of human neutrophils. Biochemistry 1995;34:14609-15.
9. Capra V, Back M, Barbieri SS, Camera M, Tremoli E, Rovati GE. Eicosanoids and their drugs in cardiovascular diseases: focus on atherosclerosis and stroke. Med Res Rev 2013;33:364-438.
10. Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M,

et al. International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. Pharmacol Rev 2009;61:119-61.

11. Zhang B, Jia H, Liu J, Yang Z, Jiang T, Tang K, et al. Depletion of regulatory T cells facilitates growth of established tumors: a mechanism involving the regulation of myeloid-derived suppressor cells by lipoxin A4. J Immunol 2010;185:7199-206.
12. Ramon S, Bancos S, Serhan CN, Phipps RP. Lipoxin A(4) modulates adaptive immunity by decreasing memory B-cell responses via an ALX/FPR2-dependent mechanism. Eur J Immunol 2014;44:357-69.
13. Martins V, Valenca SS, Farias-Filho FA, Molinaro R, Simoes RL, Ferreira TP, et al. ATLa, an aspirin-triggered lipoxin A4 synthetic analog, prevents the inflammatory and fibrotic effects of bleomycin-induced pulmonary fibrosis. J Immunol 2009;182:5374-81.
14. Bandeira-Melo C, Bozza PT, Diaz BL, Cordeiro RS, Jose PJ, Martins MA, et al. Cutting edge: lipoxin (LX) A4 and aspirin-triggered 15-epi-LXA4 block allergen-induced eosinophil trafficking. J Immunol 2000;164:2267-71.
15. Guilherme RF, Xisto DG, Kunkel SL, Freire-de-Lima CG, Rocco PR, Neves JS, et al. Pulmonary antifibrotic mechanisms aspirin-triggered lipoxin A(4) synthetic analog. Am J Respir Cell Mol Biol 2013;49:1029-37.
16. Hao H, Liu M, Wu P, Cai L, Tang K, Yi P, et al. Lipoxin A4 and its analog suppress hepatocellular carcinoma via remodeling tumor microenvironment. Cancer Lett 2011;309:85-94.
17. Janakiram NB, Mohammed A, Rao CV. Role of lipoxins, resolvins, and other bioactive lipids in colon and pancreatic cancer. Cancer Metastasis Rev 2011;30:507-23.
18. Lee TH, Crea AE, Gant V, Spur BW, Marron BE, Nicolaou KC, et al. Identification of lipoxin A4 and its relationship to the sulfidopeptide leukotrienes C4, D4, and E4 in the bronchoalveolar lavage fluids obtained from patients with selected pulmonary diseases. Am Rev Respir Dis 1990;141:1453-8.
19. Levy BD, Romano M, Chapman HA, Reilly JJ, Drazen J, Serhan CN. Human alveolar macrophages have 15-lipoxygenase and generate 15(S)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid and lipoxins. J Clin Invest 1993;92:1572-9.
20. Parkinson JF. Lipoxin and synthetic lipoxin analogs: an overview of anti-inflammatory functions and new concepts in

immunomodulation. Inflamm Allergy Drug Targets 2006;5:91-106.
21. McMahon B, Godson C. Lipoxins: endogenous regulators of inflammation. Am J Physiol Renal Physiol 2004;286:F189-201.
22. Levy BD, Serhan CN. Exploring new approaches to the treatment of asthma: potential roles for lipoxins and aspirin-triggered lipid mediators. Drugs Today (Barc) 2003;39:373-84.
23. Romano M. Lipid mediators: lipoxin and aspirin-triggered 15-epi-lipoxins. Inflamm Allergy Drug Targets 2006;5:81-90.
24. Duffy CD, Maderna P, McCarthy C, Loscher CE, Godson C, Guiry PJ. Synthesis and biological evaluation of pyridine-containing lipoxin A4 analogues. ChemMedChem 2010;5:517-22.
25. O'Sullivan TP, Vallin KS, Shah ST, Fakhry J, Maderna P, Scannell M, et al. Aromatic lipoxin A4 and lipoxin B4 analogues display potent biological activities. J Med Chem 2007;50:5894-902.
26. Dhillon T, Mauri FA, Bellezza G, Cagini L, Barbareschi M, North BV, et al. Overexpression of the mammalian target of rapamycin: a novel biomarker for poor survival in resected early stage non-small cell lung cancer. J Thorac Oncol 2010;5:314-9.
27. Etzerodt A, Moestrup SK. CD163 and inflammation: biological, diagnostic, and therapeutic aspects. Antioxid Redox Signal 2013;18:2352-63.
28. Lo Re S, Lison D, Huaux F. CD4+ T lymphocytes in lung fibrosis: diverse subsets, diverse functions. J Leukoc Biol 2013;93:499-510.
29. Guilford WJ, Bauman JG, Skuballa W, Bauer S, Wei GP, Davey D, et al. Novel 3-oxa lipoxin A4 analogues with enhanced chemical and metabolic stability have anti-inflammatory activity in vivo. J Med Chem 2004;47:2157-65.
30. Sun YP, Tjonahen E, Keledjian R, Zhu M, Yang R, Recchiuti A, et al. Anti-inflammatory and pro-resolving properties of benzo-lipoxin A(4) analogs. Prostaglandins Leukot Essent Fatty Acids 2009;81:357-66.
31. de Gaetano M, Butler E, Gahan K, Zanetti A, Marai M, Chen J, et al. Asymmetric synthesis and biological evaluation of imidazole- and oxazole-containing synthetic lipoxin A4 mimetics (sLXms). Eur J Med Chem 2019;162:80-108.
32. Roach KM, Feghali-Bostwick CA, Amrani Y, Bradding P. Lipoxin A4 Attenuates Constitutive and TGF-beta1-Dependent Profibrotic Activity in Human Lung Myofibroblasts. J Immunol 2015;195:2852-60.

<ABSTRACT (IN KOREAN)>

임상 유사 방사선 소동물 모델을 통한 방사선 유도 폐 부작용의 Lipoxin A_4에 의한 폐 섬유화 조절 연구

<지도교수 조재호>

연세대학교 대학원 의학과

김현정

방사선 유도 폐부작용은 흉강의 방사선치료에 있어서 피할 수 없는 부작용이다. 이는 치료 기간 이후에도 지속되어 비가역적인 폐섬유화로 진행되기 때문에 환자의 호흡능을 저해하여 삶의 질에도 영향을 준다. 이런 심각성에도 증상치료 이외에는 근본적 치료는 없는 상태이다. 라이폭신은 염증반응에 생성되어 항염작용하는 물질로 체내의 세포의 막에서 만들어지는 지용성 물질이다. 우리는 소동물의 방사선조사 모델을 사용하여 라이폭신을 외부에서 주입하여 방사선 유도 부작용을 줄이는 효과를 찾고자 하였다. 라이폭신은 면역세포의 동원을 억제하고 방사선으로 유도되는 염증 사이토카인과 섬유화 단백질의 형성을 폐에서 억제하였다. 또한 마이크로 시티를 이용하여 폐의 경화반응이 줄어드는 것을 확인할 수 있었으며, 플랙시벤트를 통해 폐의 기능 감소에도 적게 영향을 주었다. 결론적으로 라이폭신은 소동물폐조사 모델을 통하여 폐유도 방사선 부작용에 치료제로서 가능성을 보여주었다.

핵심되는 말 : 방사선유도 부작용; 폐; 라이폭신; LXA_4

PUBLICATION LIST

Kim H, Park SH, Han SY, Lee YS, Cho J, Kim JM. LXA4-FPR2 signaling regulates radiation-induced pulmonary fibrosis via crosstalk with TGF-beta/Smad signaling. Cell Death Dis 2020;11:653.