Effects of lysophosphatidic acid and its receptors LPA$_{1/3}$ on radiation pneumonitis

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Abstract. Radiation pneumonitis (RP) is a serious complication of radiation therapy for thoracic tumors. Lysophosphatidic acid (LPA) and its receptors LPA$_{1/3}$ were reported to participate in the processes of inflammation. We tested the hypothesis that LPA and its receptors LPA$_{1/3}$, take part in the pathogenesis of RP. In our study, irradiation increased LPA levels in the lung and expression of LPA$_{1/3}$. To further determine the role of LPA$_{1/3}$, we performed pharmacological knockout of LPA$_{1/3}$ by a specific antagonist, VPC-12249. On day 60 post-irradiation, RP was significantly alleviated in a dose-dependent manner in mice treated with VPC-12249, as shown by H&E staining, malondialdehyde (MDA, an indicator of oxidative damage) assay in lung, and concentrations of proinflammatory and profibrotic cytokines in plasma, including IL-1ß, TNF-α, and TGF-ß1. Additionally, VPC-12249 administration decreased the phosphorylation of IκB-α (the initial event that activates the NF-κB signal way), and expression of *TGF-ß1*, *CTGF*, and *α-SMA* mRNA. Our findings suggest that LPA and LPA$_{1/3}$ may play a pivotal role in RP, and LPA-LPA$_{1/3}$ may serve as novel therapeutic targets for the treatment of RP.

Introduction

Radiation therapy (RT) is a conventional treatment for thoracic cancers. However, radiation pneumonitis (RP) is a common and serious complication that sometimes may even cause death when irradiating the lung (1). As a result, RP is the main dose-limiting factor when irradiating the lung

and can affect the quality of life of survivors. Furthermore, with more concurrent chemotherapy modality being applied into clinical RT practice, an increasing incidence of RP is inevitable (2). However, the prevention and treatment of RP have long been clinical challenges in RT of thoracic cancers. New strategies for the prevention and treatment of RP are urgently needed.

RP is an acute inflammatory process that is accompanied by an increase in inflammatory cell exudation and edema, which develop 2-4 months post-RT (3). The molecular mechanisms of RP remain unclear, but it is generally believed that the process is regulated by the release and activation of various proinflammatory and profibrotic cytokines by damaged and activated cells, including transforming growth factor-ß1 (TGF-ß1), tumor necrosis factor-α (TNF-α), and interleukin-1ß (IL-1ß). In this complex process, TGF-ß1 is one of the most extensively studied cytokines and plays a particularly important role in the development of RP (4). In the clinic, an elevated plasma TGF-ß1 level during RT is a predictive factor for RP (5). Reagents that reduce these cytokines have the potential to attenuate RP.

Lysophosphatidic acid (LPA) is a small, omnipresent, bioactive phospholipid (molecular weight: 430-480 Da) with many biological functions in many cell types (6). LPA is produced from cell membrane phospholipids and is present in serum, plasma, biological fluids and tissues (7). It is well known that the effects of LPA are mediated by the binding to and activation of specific G protein-coupled receptors. There are 5 high-affinity cognate receptors that have been identified as receptors for LPA, termed LPA$_1$, LPA$_2$, LPA$_3$, LPA$_4$, and LPA$_5$. These receptors have distinct tissue distributions and effector signal pathways (8). Recently, it has been reported that LPA leads to increased inflammation in airway epithelial cells (9) and induces the expression of inflammation-related genes in human endothelial cells through its receptors, LPA$_1$ and LPA$_3$ (LPA$_{1/3}$) (10). LPA levels are significantly increased in human bronchoalveolar lavage fluid (BALF) from segmental allergen-challenged patients (11). LPA$_1$ plays an important role in bleomycin-induced lung injury (12,13). These studies led us to hypothesize that LPA may participate in the process of RP.

The objective of this study was to support the hypothesis that LPA and its receptors LPA$_{1/3}$ may participate in the

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Table I. Primers for RT-qPCR.

Gene	Forward (5'-3')	Reverse (5'-3')	Product (bp)
TGFβ1	ATGGTGGACCGCAACAAC	AGCCACTCAGGCGTATCAG	251
CTGF	CCAGACCCAACTATGATGC	ACTTAGCCCTGTATGTCTTCA	280
αSMA	CAGGGAGTAATGGTTGGAAT	TCTCAAACATAATCTGGGTCA	256
Gapdh	GGTGAAGGTCGGTGTGAACG	CTCGCTCCTGGAAGATGGTG	233

process of RP. We investigated LPA levels and the protein expression of LPA$_{1/3}$ in lung tissue after irradiation. Our results revealed that RP was accompanied by increased production of LPA and upregulation of LPA$_{1/3}$ protein levels. RP was patently attenuated in mice treated with the LPA$_{1/3}$ antagonist VPC-12249. Our findings suggest that LPA$_{1/3}$ may represent potential therapeutic targets to treat RP in the clinic.

Materials and methods

Animal model of RP. Female C57BL/6 mice, 8-10-weeks old and weighing ~20 g, were used. All animals were maintained, and experiments were performed in accordance with the guidelines approved by the Laboratory Animal Care Committee of Sichuan Province. The entire lung of each mouse was irradiated with 200 kVp X-rays at a single dose of 14 Gy. The head, abdomen, and extremities were shielded with lead strips. All mice were anesthetized before irradiation with an i.p. injection of 3.5% chloral hydrate (10 ml/kg). Mice were euthanized and bled by abdominal aorta puncture at different time points.

LPA quantification. Fresh whole lung tissue from mice were homogenized in 1 ml ice-cold saline and centrifuged at 12,000 rpm for 15 min at 4°C. LPA was extracted and quantified following the manufacturer's instructions (Chinese Twofishes Company, China).

Administration of VPC-12249. A 1 mM stock solution of VPC-12249 (Avanti Polar Lipids, Inc.) was diluted in 3% fatty acid-free bovine serum albumin/PBS solution (Sigma Aldrich). C57BL/6 mice were injected intraperitoneally with VPC-12249 solution at 0.1 and 1.0 mg/kg per dose or with the vehicle (100 µl 3% fatty acid-free bovine serum albumin/PBS solution). Injection began immediately after irradiation and was repeated 3 times per week for 2 months. The mice that were not irradiated were injected with vehicle to serve as controls. All mice were sacrificed 60 days after radiation to evaluate the potential effects on pneumonitis. The left lungs were removed and used for RNA isolation and Western blot analysis, the upper right lung was fixed in 4% paraformaldehyde for histopathology, and the remaining tissue was used for malondialdehyde (MDA) assay.

Histopathology. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and viewed on a light microscope (Oympus CX41RF).
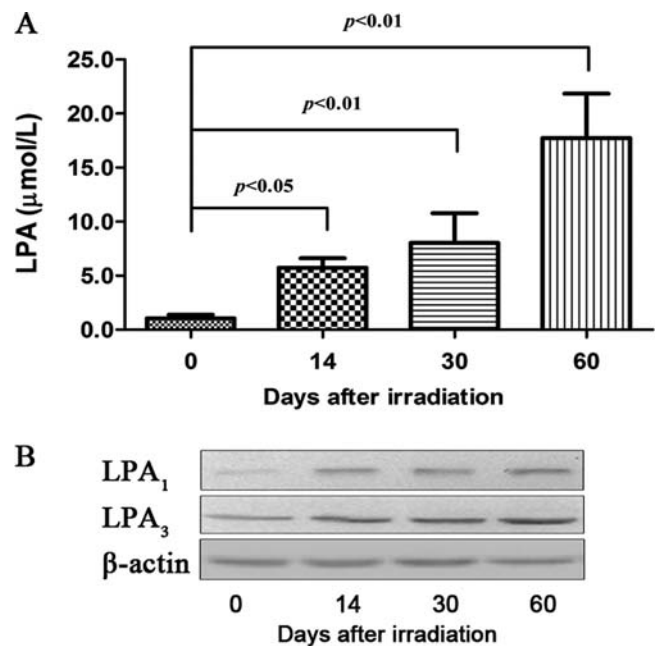

Figure 1. Radiation-induced increase of LPA levels and expression of LPA$_{1/3}$ in lung at different time points. (A) LPA levels in lung homogenate. (B) the protein expression of LPA$_{1/3}$ in lung. N=4 per time points.

Enzyme-linked immunosorbent assay (ELISA). Plasma was collected, and the concentrations of IL-1β, TNF-α, and active TGF-β1 were measured using ELISA kits (R&D Systems).

MDA activity. MDA content in lung was measured following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China).

Western blot analysis of LPA$_1$, LPA$_3$ and phospho-IκB-α (Ser32) in lung. Total protein was extracted from lung tissues as described (14), and the membrane protein was extracted by Mem-PER® Eukaryotic Membrane Protein Extration Reagent Kit (Pierce) following the manufacturer's instructions. Protein concentrations were measured using the Bradford Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions. Equivalent amounts of protein (40 µg) were separated by 15% SDS-PAGE and transferred onto a PVDF membrane (Millipore). After blocking with 5% non-fat milk in Tris-buffered saline (TBS), 0.1% Tween-20 for 1 h, the membranes were incubated with anti-LPA$_1$, anti-LPA$_3$ (Santa Cruz, 1:100), and anti-phospho-IκB-α
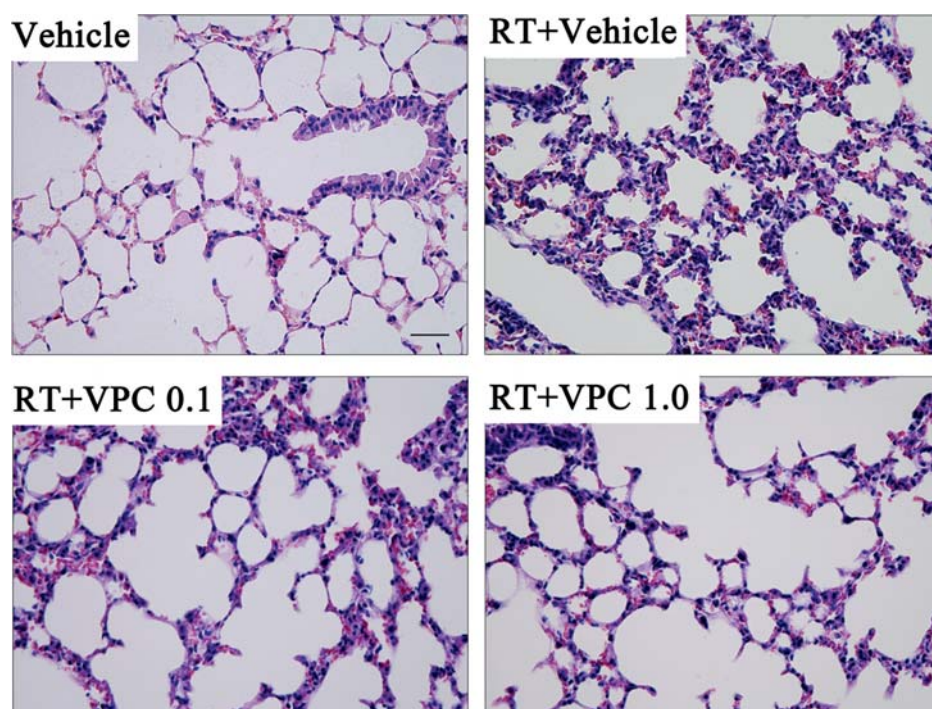
Figure 2. Histological assessment of LPA$_{1/3}$ antagonist treatment on RP at day 60, representative photomicrographs of H&E stained lung sections from experimental groups. Bar, 20 μm.

(Ser32) (Cell Signaling Technology, 1:1000) overnight. The immunoreactivites were detected using horseradish peroxidase-conjugated secondary antibody (Santa Cruz) at 1:5000 in blocking solution for 1 h at room temperature. Immunoreactivity was detected with an enhanced chemiluminescence kit (Millipore). ß-actin was used as the loading control (Santa Cruz, 1:1000).

Quantitative RT-PCR analysis. Total RNA from lung tissues was extracted, and the expression of *TGF-ß1*, connective tissue growth factor (*CTGF*), and α-smooth muscle actin (*α-SMA*) was quantified using the Chromo4 Real-time PCR System (Bio-Rad) with the SYBR RT-PCR Kit (Takara, Otsu, Japan). Relative expression levels were normalized to *Gapdh*. The primer sequences are shown in Table I.

Toxicity evaluation. To investigate the toxicity of treatment in mice, animals were observed every 3 days for weight loss, diarrhea, anorexia and skin ulcerations. The livers, spleens, kidneys and brains were stained with H&E.

Statistical analyses. Data are shown as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Dunnett's t-test, considering $P < 0.05$ significant.

Results

Radiation induces elevated LPA levels in lungs. To validate the potential role of LPA in RP, LPA was quantified in lung homogenate at different time points (Fig. 1A). LPA levels increased at all time points after irradiation (5.5-, 7.7-, and 17-fold on days 14, 30, and 60, respectively) compared with unchallenged mice (day 0). The expression levels of the
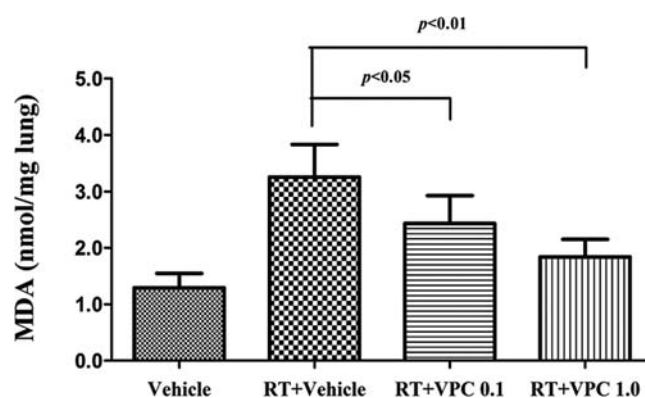

Figure 3. Measurement of MDA level in lung homogenate from experimental groups. N=5 mice per group, two repeated experiments showed similar results.

LPA$_{1/3}$ in lung after irradiation were measured by Western blot analysis, as shown in Fig. 1B; compared with unchallenged mice, the expression of LPA$_{1/3}$ increased on days 14, 30, and 60 after irradiation.

Based on these results, we hypothesized that LPA signals may participate in the process of RP through LPA$_{1/3}$. To evaluate this hypothesis, we performed pharmacological knockout of LPA$_{1/3}$ by administering a specific antagonist, VPC-12249, to radiation-exposed mice.

Alleviation of RP by LPA$_{1/3}$ antagonist treatment. On day 60 after irradiation, the lung typically exhibited pathological alveoli, with exudation of inflammatory cells in the alveolar septa (RT+Vehicle group, Fig. 2). The extent of these changes waned in mice that were treated with 0.1 mg/kg VPC-12249
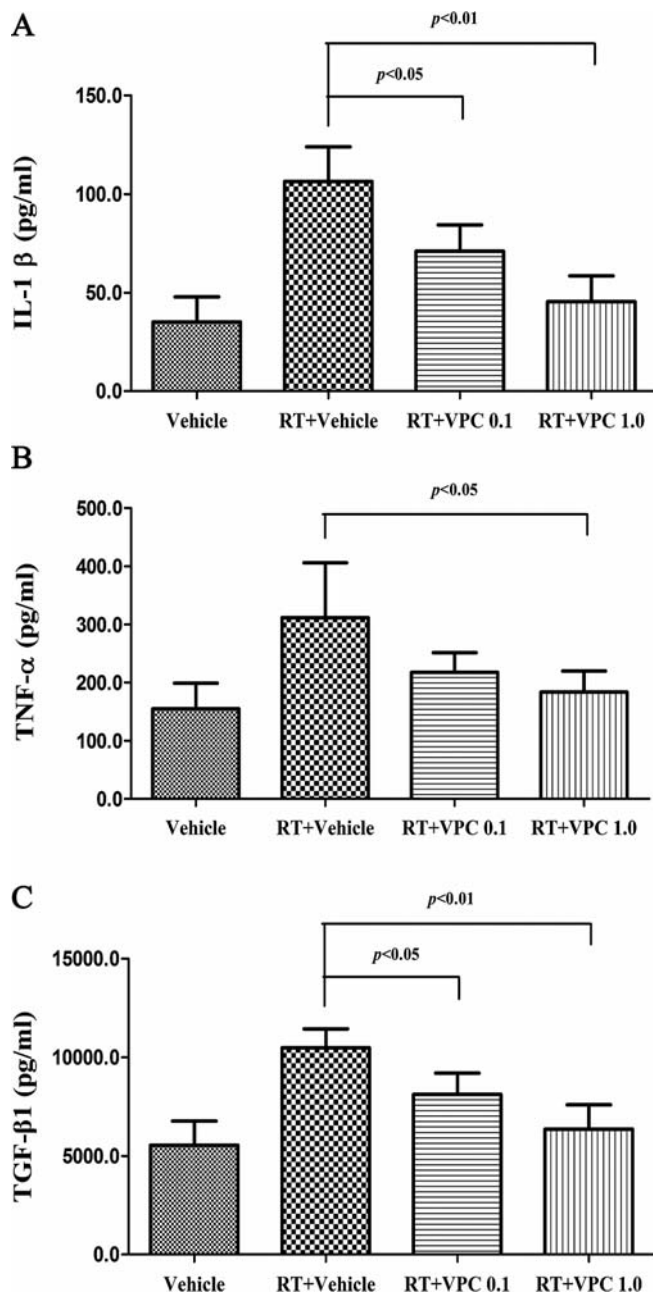
Figure 4. Measurement of cytokines in plasma from experimental groups by ELISA. (A) IL-1ß concentration. (B) TNF-α concentration. (C) TGF-ß1 concentration. N=5 mice per group, two repeated experiments showed similar results.

(RT+VPC 0.1 group), and the effect was more apparent in mice given 1.0 mg/kg VPC-12249 (RT+VPC 1.0 group).

MDA is widely used as an indicator of oxidative damage. After irradiation, there was a 2.5-fold increase in MDA levels versus non-irradiated mice. VPC 0.1 and VPC 1.0 treatment decreased MDA levels by ~25 and 43% compared with the radiation group, respectively (Fig. 3).

The concentrations of cytokines in plasma, including IL-1ß, TNF-α, and TGF-ß1, were measured by ELISA. As shown in Fig. 4, the concentrations of IL-1ß, TNF-α, and TGF-ß1 were elevated after irradiation (106, 312, and 10487 pg/ml, respectively). Administration of VPC 0.1 decreased the concentrations of these cytokines (71, 218,

and 8122 pg/ml, respectively), as did treatment with VPC 1.0 (45, 184, and 6368 pg/ml).

Attenuation of phosphorylation of IκB-α and gene expression by VPC-12249. On day 60 after irradiation, Western blot analysis was performed to detect the phosphorylation of IκB-α (the initial event that activates the NF-κB signaling pathway) in lung. As shown in Fig. 5A, treatment with VPC 0.1 attenuated the radiation-induced increases in IκB-α phosphorylation, which were more marked in mice that were treated with VPC 1.0.

mRNA expression was detected by quantitative RT-PCR analysis, including *TGF-β1*, *CTGF* (a downstream mediator of TGF-ß1), and *α-SMA* (a marker of myofibroblasts). As shown in Fig. 5B-D, treatment with VPC 0.1 and 1.0 decreased the radiation-induced mRNA expression of *TGF-β1*, *CTGF* and *α-SMA* significantly.

Toxicity of treatments. During our experiments, as compared with the RT+Vehicle group, no obvious adverse affects were observed in the treated group based on gross measures, weight loss, behavior, and feeding; furthermore, no pathological changes were observed in liver, spleen, kidney, or brain, as evidenced by H&E staining (data not shown).

Discussion

The results of the present study show that: i) irradiation induces elevated LPA levels in lung and expression of the LPA receptors LPA$_{1/3}$; and ii) RP is significantly alleviated in a dose-dependent manner in mice that are administered an LPA$_{1/3}$ antagonist, VPC-12249. These findings support the hypothesis that LPA and its receptors LPA$_{1/3}$, participate in the pathological process of RP and that inhibition of LPA$_{1/3}$ may represent a promising strategy for RP.

LPA is expressed in all mammalian cells and tissues (15), including plasma (16), BALF (11,12), and malignant effusions (17). Previous studies have shown that LPA might be involved in renal interstitial fibrosis (18) and bleomycin-induced pulmonary injury (12,13). In our study, LPA levels in lung were significantly elevated in the RP mouse model. The biological functions of LPA are mediated by activation of its receptors (19). In the present study, we found that LPA$_{1/3}$ levels increased after thoracic irradiation. These findings suggest that LPA$_{1/3}$ may play vital roles in the development of RP. This hypothesis is further supported by evidence that RP is alleviated in mice that are treated with an LPA$_{1/3}$ antagonist.

Oxidative stress, inflammatory cell infiltration, cytokine production, and related gene expression play pivotal roles in the pathogenesis of RP (20). The exact mechanisms by which RP is mitigated by LPA$_{1/3}$ antagonists are not well elucidated, but several factors may lead to this protection. First, irradiation-induced oxidative damage is the initial pathogenic event in RP, and decreased oxidative stress using extracellular superoxide dismutase can protect normal lung tissue from radiation injury (21). LPA can cause oxidative stress in nerve growth factor-differentiated PC12 cells, thus leading to apoptosis of these cells (22). In our study, irradiation-induced oxidative stress, which was assayed by
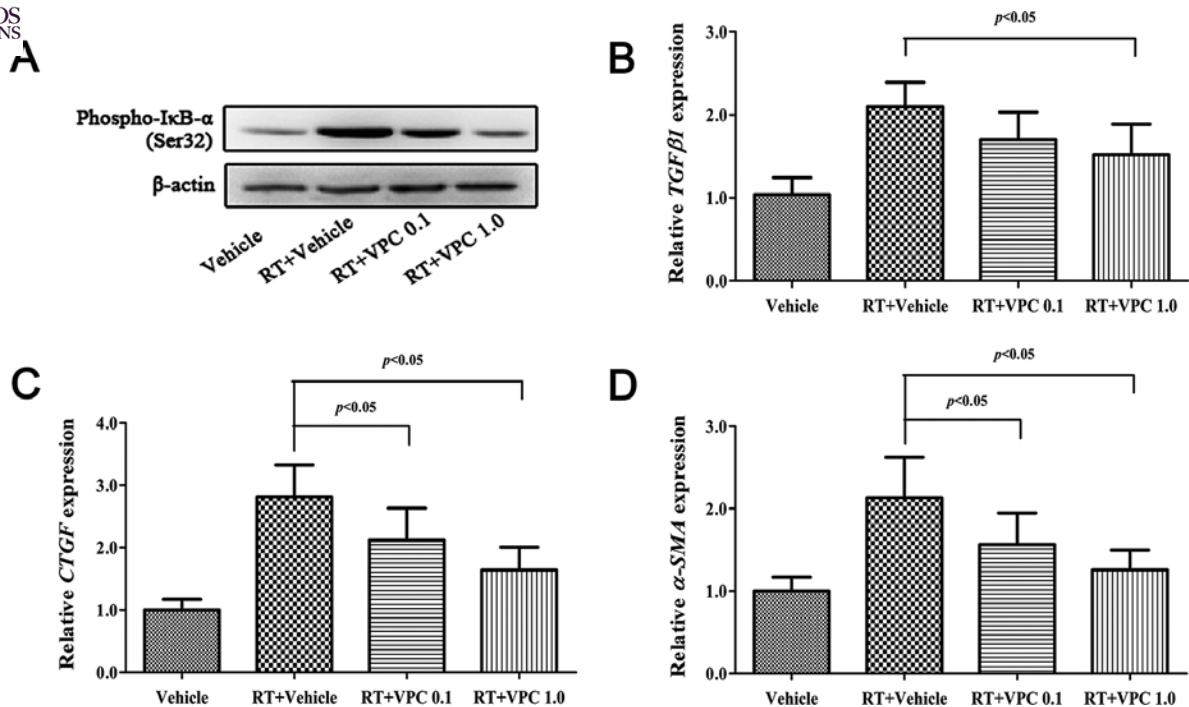
Figure 5. Detection of NF-κB activation and gene expression. (A) Western blot analysis of phosphorylation of IκB-α in lung. Quantitative RT-PCR were performed to detect the relative mRNA expression in lung, (B) *TGF-ß1* mRNA expression, (C) *CTGF* mRNA expression, and (D) *α-SMA* mRNA expression. N=5 mice per group, two repeated experiments showed similar results.

biochemical measurement of MDA levels, was reduced after VPC-12249 administration, which may result in a protective effect against RP.

Second, inflammatory cell infiltration or exudation into lung parenchyma seems to play an important role in the development of RP. Agents that decrease inflammatory cell exudation have the potential to alleviate RP. In the bleomycin challenge mouse model, vascular permeability is lowered in LPA$_1$-deficient mice, thus leading to decreased exudation (12). LPA may contribute to infiltration and activation of inflammatory cells in bronchial asthma (23). LPA also participates in inflammatory processes by enhancing chemoattraction in neutrophils and monocytes/macrophages (24). Our study demonstrated that LPA$_{1/3}$ antagonists could significantly reduce inflammatory infiltration in the alveolar septa, thus alleviating the extent of RP (as determined by H&E staining).

Third, RP is mediated by a cascade of proinflammatory and profibrotic cytokine activation (25), in which TGF-ß1 plays a pivotal role. The results have shown that these cytokines, including the proinflammatory cytokines IL-1ß and TNF-α and the profibrotic cytokine TGF-ß1 in plasma, are decreased in mice that are administered LPA$_{1/3}$ antagonists, indicating that LPA$_{1/3}$ may contribute to the inflammatory cascade of cytokines after irradiation.

Fourth, in the process of RP, the activation of transcription factors and subsequent gene expression, caused by DNA damage, participate in the inflammatory cascade response and in turn exacerbate the injury (2,26,27). LPA regulates the expression of numerous genes via activation of transcription factors, including NF-κB (15). NF-κB is present in inactive cells by binding to IκB; after stimulation, IκBα is phosphorylated and dissociates from NF-κB, leading to the translocation of NF-κB into the nucleus and the transcription of downstream genes (28). In human bronchial epithelial cells, LPA induces the phosphorylation of IκB and *IL-8* expression (29). The LPA receptors LPA$_{1/3}$ are efficient in mediating LPA-induced *IL-8* transcription (30). In MEFs, LPA induces NF-κB activation and *IL-6* expression (31). In the UUO mouse model, blocking LPA$_1$ attenuates the expression of *TGF-ß1*, *CTGF*, *α-SMA*, and *F4/80* (18). Our results show that an LPA$_{1/3}$ antagonist attenuates the irradiation-induced increases in phosphorylation of IκB-α and mRNA expression of *TGF-ß1*, *CTGF*, and *α-SMA*, suggesting that LPA$_{1/3}$ play an important role in the activation of transcription factors and gene expression after irradiation.

Our results demonstrate that irradiation upregulates LPA levels and its receptors, LPA$_{1/3}$, in lung. RP is significantly alleviated in mice that are administered VPC-12249, as shown by H&E staining; MDA assay in lung; and IL-1ß, TNF-α, and TGF-ß1 levels in plasma. Additionally, administration of VPC-12249 decreases the phosphorylation of IκB-α and mRNA levels of *TGF-ß1*, *CTGF* and *α-SMA*. The results above support the hypothesis that LPA-LPA$_{1/3}$ participate in the pathological process of RP. LPA-LPA$_{1/3}$ signal pathway may serve as potential therapeutic targets for the treatment of RP. However, before any translation of these results into the clinic, the underlying mechanisms in which LPA and LPA$_{1/3}$ are involved should be elucidated.

In summary, our results suggest for the first time, to our knowledge, that LPA and its receptors LPA$_{1/3}$, play a pivotal

role in RP. The present findings may be of importance in further exploring the potential application of LPA$_{1/3}$-targeted agents in the treatment of RP.

Acknowledgements

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