SPRED2 deficiency may lead to lung ischemia-reperfusion injury via ERK1/2 signaling pathway activation

Masanori Okada[1], Masaomi Yamane[1], Sumiharu Yamamoto[1], Shinji Otani[2], Kentaroh Miyoshi[1], Seiichiro Sugimoto[1], Akihiro Matsukawa[3] Shinichi Toyooka[1], Takahiro Oto[2], and Shinichiro Miyoshi[1]

[1]General Thoracic Surgery and Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.

[2]Department of Organ Transplant Center, Okayama University Hospital, Okayama, Japan.

[3]Department of Pathology and Experimental Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.

700-8558 2-5-1 Shikata-cho, Kita-ku, Okayama-shi

Corresponding author: Masaomi Yamane, MD, PhD, Associate Professor

General Thoracic Surgery and Breast and Endocrinological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences,

2-5-1, Shikata-cho, Okayama City Kita-ku, Okayama, 700-8558, JAPAN

TEL: +81-86-235-7265

FAX: +81-86-235-7269

Email: yamane-m@cc.okayama-u.ac.jp

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ABSTRACT

Purposes: Inflammatory changes during lung ischemia-reperfusion injury (IRI) are related to the activation of the extracellular signal-regulated kinase (ERK) 1/2 signaling pathway. Sprouty-related EVH1 (enabled/vasodilator-stimulated phosphoprotein homology 1)-domain-containing proteins (SPREDs) are known inhibitors of ERK1/2 signaling. The role of SPRED2 in lung IRI was examined in a left hilar clamp mouse model.

Methods: C57BL/6 wild type (WT) and *Spred2*$^{-/-}$ mice were used in the left hilar clamp model. Experimental groups underwent 30 min of left hilar clamping followed by 1 h of reperfusion. U0126, an ERK1/2 inhibitor, was administered to *Spred2*$^{-/-}$ mice with reperfused lungs.

Results: The partial pressures of oxygen of the *Spred2*$^{-/-}$ mice **after reperfusion** were significantly worse than those of WT mice ($p < 0.01$). *Spred2*$^{-/-}$ mice displayed more severe injuries, with increased neutrophil infiltration observed by histological evaluation and flow cytometry ($p < 0.001$). This severe inflammation was inhibited by U0126. ERK1 activation was significantly higher in the lungs of *Spred2*$^{-/-}$ mice **after reperfusion**, according to western blot analysis ($p < 0.05$).

Conclusion: Activation of the ERK1/2 signaling pathway influences the severity of lung IRI, causing inflammation with neutrophil infiltration. SPRED2 could be a promising target for the suppression of lung IRI.

INTRODUCTION

Primary graft dysfunction (PGD) is a major cause of morbidity and mortality after lung transplantation. Ischemia-reperfusion injury (IRI) of the lungs is the most common cause of PGD. Therefore, elucidation of the mechanism of IRI is required for better outcomes soon after transplantation [1, 2].

Lung IRI involves histological damage due to several complex biochemical changes. Hypoxia and reoxygenation can activate inflammatory cascades that destroy lung endothelial and alveolar epithelial barrier integrity and result in neutrophil recruitment [2-9]. In this situation, signaling pathways modulate several cellular events in the response to external stimuli and ensure that cells act appropriately. In particular, the mitogen-activated protein kinases (MAPK) constitute a large kinase network that regulates a variety of fundamental cellular processes [10-12].

Among the MAPK family, three “classical MAPK” (extracellular signal-regulated kinase (ERK) 1/2, c-jun N-terminal kinase (JNK), and p38) pathways have thus far been characterized in detail. Important roles in IRI have been reported for the JNK and p38 pathways [2, 13-15]. While the ERK1/2 pathway is activated mainly in response to mitogens and growth factors, with pro-survival effects, ERK1/2 can function in a pro-apoptotic manner under some circumstances, and ERK1/2 activation is related to inflammatory changes including IRI [16-18]. In animal

models, ERK1/2 activation leads to severe IRI in the kidneys [19, 20], liver [21], and heart [22]. In clinical practice, ERK1/2 is activated during human lung transplantation [23]. However, the role and mechanism of ERK1/2 activation in lung IRI remains unclear.

In the ERK1/2 pathway, external stimuli activate rat sarcoma virus oncogene (RAS) via the activation and phosphorylation of receptor tyrosine kinases. RAS then activates v-raf-leukemia viral oncogene 1 (RAF1), which activates MAPK/ERK kinase (MEK) 1/2, starting a phosphorylation cascade that activates ERK1/2. Sprouty-related EVH1 (enabled/vasodilator-stimulated phosphoprotein homology 1)-domain-containing proteins (SPREDs) can inhibit RAF activation, resulting in ERK1/2 inactivation [24-27]. SPRED2 is ubiquitously expressed in various tissues, including the lungs, and controls the development of lipopolysaccharide-induced lung inflammation by negatively regulating the ERK1/2 pathway [28]. We hypothesized that lung IRI is induced by the ERK1/2 pathway when SPRED2 is suppressed. In this experimental study, we aimed to assess the role of SPRED2 on the ERK1/2 pathway in lung IRI utilizing a mouse left hilar ligation model.

MATERIALS AND METHODS

Animals

We used C57BL/6 wild type (WT) and *Spred2*$^{-/-}$ mice (7–10 weeks old, approximately 25–35 g).

Spred2$^{-/-}$ mice were generated as previously described [27, 28]. These mice were bled and maintained in specific pathogen-free conditions at the Department of Animal Resources, Okayama University (Okayama, Japan). The Animal Care Committee at Okayama University reviewed and approved all aspects of our experimental protocol before experimentation. All experimental mice received humane care in accordance with the "Principles of Laboratory Animal Care" of the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals," prepared by the National Academy of Science and published by the National Institutes of Health (NIH).

Experimental design

Five groups (n=5 mice each) were included in the study. WT and *Spred2*$^{-/-}$ mice underwent either the hilar clamp procedure (experimental groups) or median thoracotomy alone (control groups). The 5th group was comprised of *Spred2*$^{-/-}$ mice, which were intraperitoneally administered U0126 (30 mg/kg; Cell Signaling Technology, Danvers, MA, USA), an inhibitor of ERK1/2 phosphorylation, 2 h before hilar clamping.

Mouse hilar clamp procedure

After anesthesia by intraperitoneal administration of ketamine (Daiichi Sankyo Propharma, Tokyo, Japan) and xylazine (Intervet, Tokyo, Japan), mice were intubated with a 20-gauge angiocatheter via tracheotomy and put on a ventilator (Harvard, Holliston, MA, USA). Mice

were ventilated at a fraction of inspiratory oxygen (FiO_2) of 1.0, a tidal volume of 0.5 mL, and a respiratory rate of 120 breaths/min. The left hilum was approached via median thoracotomy in the supine position and clamped en bloc with a microclip, taking care not to injure the lung. After 30 min of ischemia, the microclip was slowly released. **Then, 60 min after reperfusion, mice were sacrificed just before the arterial blood was drawn from the left ventricle and the left lung was resected.** The harvested lungs were separated into 3 sections for subsequent analytical techniques.

Pulmonary function assessment

We measured the arterial partial pressure of oxygen (PaO_2) to assess pulmonary functional changes **after reperfusion**. PaO_2 was measured by blood gas analysis using a Rapid Lab 348 apparatus (Siemens Healthcare Diagnostics, Tokyo, Japan) immediately after drawing oxygenated blood from the left ventricle.

Histopathology

We confirmed lung changes during IRI by histopathological evaluation. Lung samples were fixed in 10% formaldehyde, sectioned, and stained with hematoxylin and eosin. The total number of neutrophils per high power field (HPF) was counted using a microscope.

Flow cytometry

Flow cytometry was performed to quantify the neutrophils in the lungs. Cells were stained with

fluorochrome-labelled anti-granulocyte-differentiation antigen-1 (Gr-1; BD Biosciences, San Jose, CA, USA) and anti-CD11c (BD Biosciences) antibodies. The amounts of $CD11c^{low}/Gr\text{-}1^{high}$ cells, indicating neutrophils, were assessed on a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo (FlowJo LLC, Ashland, OR, USA).

Western blot analysis

Western blotting was performed to examine MAPK activation. Membranes were incubated with primary antibodies, anti-phosphorylated ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), anti-phosphorylated JNK (Cell Signaling Technology), anti-phosphorylated p38 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-actin (Cell Signaling Technology)) overnight at 4°C with shaking. After washing, the membranes were incubated with secondary antibodies (Santa Cruz Biotechnology) for 1 h at 25°C. Proteins were visualized by enhanced chemiluminescence using the ECL Advanced Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ, USA). Images were analyzed in ImageJ (version 1.45; NIH, Bethesda, MD, USA). MAPK activation levels were quantified as the ratios of phosphorylated/total MAPK density.

Statistical analysis

When the results were significant according to one-way analysis of variance, differences between series were determined using Tukey's test. Values are expressed as the mean ± standard

deviation (SD). $P < 0.05$ was considered statistically significant. All analyses were performed in BellCurve for Excel (Social Survey Research Information, Tokyo, Japan).

RESULTS

Pulmonary function assessment

The PaO_2 levels of the *Spred2*$^{-/-}$ control mice were equivalent to those of the WT control mice (Fig. 1). The hilar clamp procedure caused decreased PaO_2 levels, and the PaO_2 level of the *Spred2*$^{-/-}$ mice significantly decreased ($p = 0.004$). When U0126 was administered, however, the PaO_2 level of the *Spred2*$^{-/-}$ mice was significantly improved, even **after reperfusion** ($p = 0.009$).

Histopathology

The pulmonary parenchyma showed inflammatory changes such as septal thickening, congestion, edema severity, hemorrhage, and neutrophil infiltration **after reperfusion** (Fig. 2). *Spred2*$^{-/-}$ mice subjected to hilar clamping displayed particularly severe injuries. Conversely, the lungs of *Spred2*$^{-/-}$ mice injected with U0126 showed less inflammatory changes **after reperfusion**.

The average number of neutrophils/HPF in the lung of *Spred2*$^{-/-}$ mice **after reperfusion** was significantly higher ($p < 0.001$). However, this increase was suppressed upon administration of U0126 ($p < 0.001$).

Flow cytometry

The percentage of $CD11c^{low}/Gr\text{-}1^{high}$ cells was significantly higher in the lungs of *Spred2*$^{-/-}$ mice **after reperfusion** ($p < 0.001$; Fig. 3). This increase was also inhibited by U0126 ($p < 0.001$), consistent with the histologic evaluation of neutrophil accumulation.

Western blotting analysis

ERK1 activation was significantly higher in the lungs of *Spred2*$^{-/-}$ mice **after reperfusion** (WT vs. *Spred2*$^{-/-}$, $p = 0.048$; *Spred2*$^{-/-}$ vs. *Spred2*$^{-/-}$ + U0126, $p = 0.031$; Fig. 4). Conversely, there were no significant changes in the activation levels of JNK (WT vs. *Spred2*$^{-/-}$, $p = 0.895$; *Spred2*$^{-/-}$ vs. *Spred2*$^{-/-}$ + U0126, $p = 0.989$) nor p38 (WT vs. *Spred2*$^{-/-}$, $p = 0.087$; *Spred2*$^{-/-}$ vs. *Spred2*$^{-/-}$ + U0126, $p = 1.000$).

DISCUSSION

In transplant recipients, lung IRI is a major complication leading to pulmonary dysfunction. However, the details of its molecular mechanisms remain unknown. In this study, we found that the SPRED2-RAF pathway had a strong impact on lung IRI, resulting in ERK1/2 activation and neutrophil infiltration in reperfused lungs. With inhibition of ERK1/2 activation, the severity of lung IRI was ameliorated.

Lung IRI is characterized by increased microvascular permeability, increased pulmonary

vascular resistance, pulmonary edema, impaired oxygenation, and pulmonary hypertension [1, 2]. Critical steps in the pathophysiology of these conditions are the activation of alveolar macrophages, the release of various pro-inflammatory molecules, and the accumulation of neutrophils, resulting in excessive and uncontrolled inflammation and pulmonary tissue damage [3, 7, 8]. Consistent with our research results, previously reported animal models have demonstrated that the inflammatory response in lung IRI is characterized by neutrophil infiltration in the lungs, and that inhibiting the neutrophil reaction improves lung injury [4, 8]. Moreover, the inflammation in lung IRI is more severe and complicated than that observed in other organs, because loss of ventilation during ischemia induces the formation of reactive oxygen species [5, 6].

Various signaling pathways play key roles in lung IRI, including all three classical MAPK pathways. The JNK and p38 pathways are related to apoptosis and cell death [11, 12, 18], and are therefore thought to play major roles in lung IRI. Conversely, in this study, SPRED2 control of the ERK1/2 pathway resulted in lung IRI with marked neutrophil infiltration, while p38 and JNK activation did not significantly change. This difference may be attributable to the different observation times used after ischemia or reperfusion [4, 29]. It was reported that p38 was phosphorylated mainly during ischemia, while ERK1/2 and JNK were mainly phosphorylated during and following reperfusion [29]. The conditions of our study may have

promoted the specific detection of ERK1/2 activation.

In a rat lung transplantation model, inhibition of JNK activity led to decreased expression of jun proto-oncogene, an activator of several pro-inflammatory cytokines, leading to decreased release of tumor necrosis factor (TNF)- α into the bronchoalveolar lavage fluid and decreased lung IRI [13]. The activity of p38 was suppressed by FR167653, a potent inhibitor of TNF-α and interleukin-1 production, resulting in attenuated lung IRI in a rat hilar ligation model [14]. In addition, inhibition of p38 with carbon monoxide, which modulates caspase 3 and protects against apoptosis, led to attenuated lung IRI [15]. While ERK1/2 activation generally contributes to cell proliferation and survival, ERK1/2 can also have pro-apoptotic functions under some circumstances, including in lung IRI [17, 18]. ERK1/2 is dramatically activated during human lung transplantation [23], but how this activation relates to lung IRI remains unknown. The effects of ERK1/2 activation may have as great an impact on lung IRI as those of JNK and p38. In our study, the inhibition of ERK1/2 dramatically attenuated lung IRI, with decreased lung damage and improved oxygenation. This may be due to JNK activation by MAP kinase kinase 4/7, which were in turn activated by over-activated RAS in SPRED2 knockout mice [12], but did not induce heavy lung injury. Additionally, though the anti-apoptotic effects of ERK1/2 can be regulated indirectly by p38 signaling [30], the activation level of p38 was not different between the experimental groups, suggesting that

ERK1/2 activation was not affected by p38. Therefore the increased activation of ERK1/2 in our study might not be due to crosstalk from JNK and p38.

Inhibition of the ERK1/2 signaling pathway may represent a possible therapeutic strategy for the treatment of lung IRI. ERK1/2 is activated approximately 1–2 h after reperfusion rather than during ischemia [4, 29], so in clinical practice, it may alleviate lung IRI to perform reperfusion in stages. Additionally, as shown in this study, it may be effective to control ERK1/2 activation by the administration of an ERK1/2 signaling inhibitor such as U0126. However, excessive suppression of ERK1/2 led to increased intestinal permeability, neutrophil infiltration, and lung injury in mice injected with U0126 [31], as the ERK1/2 signaling pathway, a regulator of cell proliferation, is required for survival. SPRED2 may also represent a therapeutic target, as it controlled the development of lipopolysaccharide-induced lung inflammation by negatively regulating the ERK1/2 pathway in a SPRED2 knockout mouse model [28].

This study has some limitations. First, it has not resolved how lung IRI affects chemical mediators and neutrophil extracellular traps (NETs) after activation of the ERK1/2 pathway, which interacts not only with other MAPK pathways but also the nuclear factor κB, Toll-like receptors, and phosphatidylinositol 3-kinase-Akt pathways [2]. Future studies examining the effects on these pathways will be required. Moreover, a recent study reported that

NETs, which consist of neutrophilic DNA and proteins, are formed in the injured lung **after reperfusion** in animal models, and that their disruption reduces lung injury [7]. NET formation is controlled by various intracellular signaling pathways, including the ERK1/2 pathway [32]. Therefore, activation of ERK1/2 signaling after ischemia and reperfusion may promote NET formation, resulting in lung injury. This will also require further investigation.

In addition, we showed these results without lung transplantation. These results of the left hilar ligation model in this research do not completely represent those of the transplantation model. For IRI in lung transplantation, we also have to consider other factors, including innate immune responses and lymphocyte alloreactivities. Further studies on the transplantation model will be required.

In conclusion, activation of the ERK1/2 signaling pathway leads to severe lung IRI, causing inflammation and edema with neutrophil infiltration. Inhibition of the ERK1/2 pathway may attenuate neutrophil recruitment, **partially** suppressing primary graft dysfunction during or just after lung transplantation. Meanwhile, the ERK1/2 pathway also plays an important role for life maintenance by regulating cell growth, proliferation, and survival. Further studies regarding the mechanisms of ERK1/2 signaling in lung IRI may provide helpful clues towards effective treatments to prevent PGD after lung transplantation.

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In case of no conflict of interest:

Conflict of interest statement: Masanori Okada and other co-authors have no conflict of interest.

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LEGENDS

[Fig. 1] Pulmonary function of the left lung after ischemia-reperfusion Mice were subjected to hilar clamping, and then oxygenated blood was drawn from the left ventricle. The mice were ventilated with 100% oxygen. -/- = *Spred2*$^{-/-}$ mice, C = Control mice not undergoing hilar clamping, IR = ischemia-reperfusion experimental group undergoing hilar clamping, IR+U = ischemia-reperfusion experimental group injected with U0126, WT = wild type.

[Fig. 2] Histologic evaluation of the lungs Comparison of hematoxylin and eosin-stained pulmonary parenchyma sections from each mouse **after reperfusion**. -/- = *Spred2*$^{-/-}$ mice, C = Control group not undergoing hilar clamping, IR = ischemia-reperfusion experimental group undergoing hilar clamping, IR+U = ischemia-reperfusion experimental group injected with U0126, WT = wild type.

[Fig. 3] Flow cytometry analysis of lung neutrophil infiltration Flow cytometry was performed to quantify the percentage of CD11c^{low}/Gr-1^{high} cells, indicating neutrophils. -/- = *Spred2*$^{-/-}$ mice, C = Control group not undergoing hilar clamping, IR = ischemia-reperfusion experimental group undergoing hilar clamping, IR+U = ischemia-reperfusion experimental

group injected with U0126, WT = wild type.

[Fig. 4] ERK activation levels MAPK activation levels were quantified as the ratios of phosphorylated/total MAPK density in western blots. -/- = *Spred2*$^{-/-}$ mice, C = Control group not undergoing hilar clamping, IR = ischemia-reperfusion experimental group undergoing hilar clamping, IR+U = ischemia-reperfusion experimental group injected with U0126, WT = wild type.