Attenuation of lipopolysaccharide-induced acute lung injury after (pro)renin receptor blockade

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ABSTRACT

Purpose/Aim: We performed a randomized, prospective animal study to investigate whether inhibiting the renin-angiotensin system with a (pro)renin receptor blocker (PRRB) prevents acute lung injury (ALI) in a rodent model.

Materials: We used Thirty-six male Sprague-Dawley rats. We administered lipopolysaccharide (LPS; 2 mg/kg) intratracheally with or without PRRB pretreatment (1 mg/kg/d).

Methods: We performed bronchoalveolar lavage (BAL) and lung removal at 4 h after LPS administration and measured levels of inflammatory cytokines, high mobility group box 1 (HMGB-1) protein, and total protein in bronchoalveolar lavage fluid (BALF). Myeloperoxidase (MPO) activity was detected in lung tissue homogenates using a sensitive ELISA. We performed hematoxylin and eosin staining and immunohistochemical staining for nonproteolytically activated prorenin in the left lung.

Results: The PRRB decreased leukocyte counts and total protein, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-6, and IL-10 levels in the BALF and MPO activity in lung tissue. The PRRB reduced interstitial edema, hemorrhage, and the neutrophil count in the lung tissues. Consistent with the reduction in lung tissue damage, immunohistochemical staining showed that the PRRB decreased the amount of nonproteolytically activated prorenin.

Conclusions: The PRRB blocked LPS-induced inflammatory response in the lung and protected against ALI. Therefore, it is a potential therapeutic agent for preventing ALI.

KEYWORDS acute lung injury, (pro)renin receptor blocker, prorenin, renin–angiotensin system

INTRODUCTION

Acute lung injury (ALI) is a major complication that follows invasive operations. Although many therapeutic strategies have been applied to ALI treatment, none have proven distinctly successful [1–3].

The renin-angiotensin system (RAS) plays a well-defined and essential role in maintaining blood pressure homeostasis. Underscoring this, pharmacologic blockade of the RAS has been found to have a protective effect on several organs, especially the heart and kidneys [4–6]. Increasingly, however, the role of the RAS in the pathogenesis and evolution of inflammatory responses has received attention; this is due in part to studies that have shown that the RAS is a key mediator of inflammation [7]. Furthermore, the RAS can contribute to pulmonary defects, and the activity of angiotensin converting enzyme (ACE), a key RAS component, may regulate the acute inflammatory response in the lung [8]. Angiotensin-II (ANG II) produces an inflammatory response by binding to the type-1 ANG II receptor and activating an intracellular signaling cascade that upregulates several
pro-inflammatory genes. This leads to the hypothesis that ACE inhibitors could prevent inflammation-associated ALI through blockade of ANG II production [9].

The precursor of renin, a principal component of the RAS, is prorenin. Prorenin is a pro-peptide that consists of 43 amino acid residues that are attached to the N terminus of active renin; this prosegment folds into the active site cleft of active renin to prevent productive interaction with angiotensinogen [10]. Our previous studies have shown that an N-terminal prosegment of human prorenin has a segment termed the “handle region” (HR) and a segment called the “gate region” (GR), which is not accessible by its specific antibodies until it is loosened from the active site cleft [11]. Rat prorenin contains analogous HR and GR in positions that are identical to those of human prorenin. When prorenin binds to the (pro)renin receptor (PRR), it is nonproteolytically converted into an enzymatically active state [12, 13]. This is important in the activation of the renal RAS and the development of nephropathy in diabetic rats. However, the physiological functions of the PRR remain unknown. Previous studies have confirmed that mRNA of (pro)renin receptor (PRR) developed in the entire body of the mouse, brain, testis, kidney, adrenal grand, lung, heart, skeletal muscles, liver, and fatty tissues [14]. In addition, PRR is necessary for the biosynthesis of V-ATPase; therefore, a disorder affecting the function of PRR can cause cell death. These findings suggest that PRR is a molecule with a ubiquitously essential function in cells [15].

Recent studies have shown that synthetic peptides that correspond to the handle region of prorenin bind to the PRR and thereby inhibit prorenin binding [16, 17]. Therefore, the handle region peptide (HRP) is thought to be an important PRR blocker (PRRB). PRRB is a decoy peptide that possesses the structure of the HR (HRP) that competitively binds to a prorenin-binding protein and inhibits the nonproteolytic activation of prorenin, prevents the development of nephropathy, and suppresses the increase in renal angiotensin. PRRB is more potent in these activities than ACE inhibitors [13]. In addition, the prorenin/(pro)renin receptor interaction induces extracellular signal-regulated kinase (ERK) activation related to inflammation; this is independent of the effects on ANG II [18]. This suggests that PRRB may also inhibit ERK-related intracellular signaling pathways [19].

Although PRRB was originally found to reduce chronic organ damage associated with hypertension or diabetes, PRRB might also be effective in acute systemic inflammatory diseases such as sepsis or systemic inflammatory response syndrome. Our research group previously investigated the effect of PRRB pretreatment on sepsis in a rat cecal ligation and puncture (CLP) model [20]. Treatment with PRRB improved the survival rate of the post-CLP septic rats, and PRRB also significantly reduced cytokine and high mobility group box-1 (HMGB-1) levels in the serum after CLP. On the basis of the result of our previous study, we hypothesized that it might also ameliorate acute inflammatory organ damage, such as that in ALI. Therefore, we tested the effects of PRRB in a lipopolysaccharide (LPS)-induced ALI model in rats.

**MATERIALS AND METHODS**

**Animals**

All protocols conformed to the National Institutes of Health guidelines, and the animal care was in compliance with the principles of Laboratory Animal Care. This study was approved by the Keio University Animal Care and Use Committee, Tokyo, Japan. We used male, 8-week-old, specific pathogen-free Sprague-Dawley rats weighing 250–300 g (CLEA Japan, Inc, Tokyo, Japan). The animals were allowed to acclimatize for 7 d before use. They had ad libitum access to food and water throughout the study and were housed at 23°C under a 12:12-h light-dark cycle.

**Drugs**

We used LPS (O127:B8; Sigma, St Louis, MO, USA) that was derived from *Escherichia coli* (O127) endotoxin and dissolved in sterile saline. PRRB (Rat PRRB; sequence: NH2-R10RILLKKMPSV19-COOH, purity: >90%, Gene Design Inc., Osaka, Japan) and scramble peptide (Rat scramble; sequence: NH2-M30TRISAE36-COOH, purity: >90%, Gene Design Inc) were dissolved in 5% acetic acid.

**Animal Care and Treatment**

We used a microsprayer (Penn-Century, Philadelphia, PA, USA) for bilateral administration of LPS (2 mg/kg) or PBS to the lungs via the trachea. Prior to administration, we anesthetized the animals with ketamine (30 mg/kg) and xylazine (3 mg/kg) combined with inhalation of 4.0% isoflurane; subcutaneous injection of sterile saline (1 mL) was used to avoid dehydration during anesthesia. Scramble and PRRB peptides (1 mg/kg/d) were administered intraperitoneally using an osmotic pump (Model 2004 for 28-d use; ALZET Pump, DURECT Corp, USA).
We randomly divided the rats into 3 experimental groups (n = 12 for each group): control group, rats were pretreated for 1 week with the scramble peptide before PBS was sprayed into the trachea by using a microsprayer; LPS plus PRRB group, rats were pretreated for 1 week with PRRB before LPS was sprayed into the trachea by using a microsprayer; LPS plus scramble peptide group, the rats were pretreated for 1 week with the scramble peptide before LPS was sprayed into the trachea by using a microsprayer.

We sacrificed rats from all groups under sodium pentobarbital anesthesia at 4 h after LPS or PBS administration by cutting their aorta, and bronchoalveolar lavage (BAL) was performed immediately after sacrifice to evaluate pulmonary inflammation induced by LPS. Lungs were then removed; the right lungs were sectioned and frozen with liquid nitrogen, while the left lungs were fixed by inflation with 4% paraformaldehyde at a transpulmonary pressure of 25 cm H₂O and embedded in paraffin.

We determined IFN-γ, IL-1β, IL-2, IL-6, IL-10, and TNF-α levels by using a multiplex cytokine bead array system (Bio-Plex; Bio-Rad, Hercules, CA, USA). HMGB-1 was measured using a monoclonal antibody to HMGB-1 (Shino-Test, Tokyo, Japan). The lower detection limit of this kit was 0.2 ng/mL. Total protein was measured using a dye-binding assay, with bovine serum albumin as the standard (Bio-Rad protein assay).

Paraffin sections were generated from the fixed left lungs and stained with hematoxylin and eosin (H&E) for light microscopy. We evaluated the expression of nonproteolytically activated prorenin in the lungs using immunohistochemical staining. First, a rabbit anti-rat GR antibody was applied to the tissue sections as the primary antibody. Rabbit anti-rat GR antibody was raised against the rat “gate region” (GR), which is usually buried in the main body of prorenin during its inactive state and exposed when in the nonproteolytically active state [11, 13]. This was followed by incubation with a biotin-conjugated anti-rabbit IgG secondary antibody. If the nonproteolytic activation mechanism is functional, then the prorenin will gain renin activity by binding of the prorenin receptor to its HR, and tissues that are bound by the nonproteolytically activated prorenin can be visualized by immunohistochemical staining of GR using its specific antibody [11]. The nonproteolytically activated prorenin was then visualized with a Vectastain ABC standard kit (Vector Laboratories, Burlingame, CA, USA) and an AEC Standard Kit (Dako, Carpenteria, CA, USA) according to the manufacturers’ instructions.

For analyses of immunoreactive, nonproteolytically activated prorenin, five random fields on each slide were captured with a microscope at ×400 magnification (Optiphot, Nikon Inc, Tokyo, Japan). We evaluated nonproteolytically activated prorenin expression as the average of the percentage of positive lung tissue (none = 0; 10%–25% = 1; 25%–50% = 2; and ≥50% = 3).

All results have been expressed as mean ± SE values. Comparisons between groups were examined using the Mann–Whitney U test. Statistical analysis was performed using SPSS version 15.0 (IBM Corporation, Chicago, United States). A P values <0.05 was considered statistically significant.
FIGURE 1. The PRRB decreased leukocyte recruitment to the lung in LPS-induced inflammation. (A) Total leukocyte count. Rats were pretreated for 1 week with intraperitoneal administration of the scramble peptide or PRRB, and bronchoalveolar lavage (BAL) was performed at 4 h after LPS or PBS administration. The number of leukocytes in BALF was remarkably lower in the LPS+PRRB group than in the LPS+scramble group. (B) MPO activity. Very high MPO activity was induced after LPS injection, and MPO activity was significantly lower in animals in the LPS+PRRB group than in the LPS+scramble peptide group. The values presented are the mean ± SE (n = 12 in each group).

RESULTS

The PRRB Decreased Leukocyte Recruitment to the Lung During LPS-Induced Lung Injury

We reasoned that if PRRB could attenuate inflammation-induced ALI, then inflammatory marker levels would be reduced in the BALF from PRRB-treated animals. In the LPS+scramble peptide group, the leukocyte count in the BALF was highly elevated to 150–300 × 10⁴ leucocytes, but decreased significantly in the LPS+PRRB group (P < 0.05, Figure 1A).

MPO activity was determined to assess the neutrophil accumulation within pulmonary tissues. Very high MPO activity was induced after LPS administration; however, treatment with PRRB significantly suppressed this activity (P < 0.01, Figure 1B).

Effects of the PRRB on the Concentration of Inflammatory Mediators in BALF

BALF was obtained at 4 h after LPS administration, with or without pretreatment of PRRB. IFN-γ, IL-1β, IL-2, IL-6, IL-10, TNF-α, and HMGB-1 levels were elevated in the LPS+scramble peptide group, whereas treatment with PRRB significantly reduced the concentrations of IL-1β (P < 0.01; Figure 2A), IL-2 (P < 0.01; Figure 2B), IL-6 (P = 0.031; LPS+scramble peptide group; Figure 2C), IL-10 (P < 0.01; Figure 2D), TNF-α (P < 0.01; Figure 2E), and HMGB-1 (P < 0.05; Figure 2F). In contrast, no significant differences in the levels of IFN-γ were observed among the groups (Figure 2G).

The PRRB Prevented Lung Tissue Damage After LPS-Induced Inflammation

Upon histologic examination of lung tissue, we found that the neutrophil counts in large and small airways markedly decreased following PRRB treatment. We also observed that interstitial edema formation and hemorrhage were attenuated in the presence of PRRB (Figure 3). The amount of total protein in BALF was also lower in the LPS+PRRB group than in the LPS+scramble peptide group (P < 0.05; Figure 4). There was no significant difference between the LPS+PRRB group and control group in this regard (Figure 4).

Effect of the PRRB on the Immunohistochemical Findings for Lung Tissue

Immunohistochemical analysis showed that the number of cells expressing nonproteolytically activated prorenin increased after LPS administration. A vast area of the lung was immunostained for nonproteolytically activated prorenin, mainly in alveolar epithelial cells. In stark contrast, PRRB treatment reduced the number of cells expressing nonproteolytically activated prorenin (Figure 5A). The percentage score was also significantly lower in the PRRB+scramble
FIGURE 2. Inflammatory mediator (IL-1β, IL-2, IL-6, IL-10, TNF-α, and HMGB-1) levels were elevated in the LPS + scramble peptide group; however, treatment with the PRRB significantly reversed this effect. A, IL-1β; B, IL-2; C, IL-6; D, IL-10; E, TNF-α; F, HMGB-1. The values presented are the mean ± SE (n = 12 in each group).
Histologic Analysis

FIGURE 3. Effects of the PRRB on lung injury in LPS-treated rats. The figure shows representative lung specimens obtained from the control group (A: ×100 magnification), LPS+PRRB group (B: ×100 magnification), and LPS+scramble peptide group (C: ×100 magnification) (hematoxylin and eosin staining). The neutrophil counts in large and small airways, edema formation, and hemorrhage markedly decreased on PRRB treatment.

FIGURE 4. The amount of total protein in BALF was significantly lower in the LPS+PRRB group than in the LPS+scramble group (P < 0.01; Figure 5B).

DISCUSSION

In the present study, we have demonstrated that pretreatment with PRRB significantly reduced LPS-induced ALI and was associated with reduced neutrophil counts, total protein leakage, MPO activity, and inflammatory cytokine and HMGB-1 levels. Furthermore, PRBB reduced the number of cells expressing nonproteolytically activated prorenin in lung tissues.

The PRR was initially implicated in the pathogenesis of cardiovascular diseases since it facilitated activation of the local RAS [12]. Renin activates the RAS cascade by transforming angiotensinogen to Ang I, which is subsequently converted by the ACE to produce Ang II. Upon prorenin binding to the PRR, the catalytic activity of prorenin and renin increases, resulting in increased RAS activation [12].

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FIGURE 5. Changes in nonproteolytically activated prorenin expression in lung tissue specimens after LPS administration in rats. 5A: The figure shows representative lung specimens obtained from the control group (a: \( \times 400 \) magnification), LPS+PRRB group (b: \( \times 400 \) magnification), and LPS+scramble peptide group (c: \( \times 400 \) magnification). The number of cells expressing nonproteolytically activated prorenin in the PRRB-treated group significantly reduced and was comparable to that for the control group. Arrows show positive staining for nonproteolytically activated prorenin. 5B: Quantitative analysis of nonproteolytically activated prorenin-positive areas. The percentage score was also lower in the PRRB+scramble group than in the LPS+scramble group. The values presented are the mean ± SE (\( n = 12 \) in each group).
The involvement of the RAS in the pathogenesis of ALI, sepsis, and the systemic inflammatory response syndrome has recently been reported [7, 21]. These studies demonstrated that ACEs, Ang II, and Ang II Type 1 receptor promote severe ALI [7, 21]. Moreover, ACE inhibitors protect against the ALI associated with LPS-induced sepsis in rats and can prevent LPS-induced increases in HMGB-1 levels [22, 23]. However, to the best of our knowledge, the present study is the first to report an association between PRR, nonproteolytically activated prorenin and ALI.

Our results suggest that the PRRB attenuates neutrophil recruitment to the lung after exposure to LPS. By blocking this early feature of ALI pathogenesis, PRRB significantly reduces the lung tissue damage.

Inflammatory cytokines such as TNF-α, IL-1β, IL-2, IL-6, and IFN-γ are secreted in the early phase of the inflammatory response and play an important role in the development of ALI [24]. In this study, we found that PRRB pretreatment significantly suppressed the induction of most of these cytokines, which was associated with a marked reduction in inflammation-associated lung pathology. The levels of other inflammatory cytokines substantially increased with LPS administration; however, the level of IFN-γ did not increase in the present study. Although it remains unclear why IFN-γ did not increase, whether IFN-γ in the lung increases in rats with LPS-induced lung injury remains controversial [25].

HMGB-1 causes acute lung inflammation when administered intratracheally [26] and plays an important role in the development of LPS-induced lung injury [27, 28]. We demonstrated that pretreatment with PRRB significantly reduces the level of HMGB-1 in BALF. This clearly correlates with the PRRB-dependent reduction in lung pathology, although whether PRRB exerts all its beneficial effects due to the suppression of HMGB-1 remains unclear.

We also found a high accumulation of activated prorenin in the cells of the bronchiolar wall and alveoli (mainly in the alveolar epithelial cells) of LPS-induced damaged lung tissue. Pretreatment with PRRB significantly reduces this effect, which correlated well with the reduction in interstitial edema, thickness of the bronchiolar walls, and hemorrhage. Together, these results suggest that nonproteolytically activated prorenin stimulates lung damage.

Our results indicate that nonproteolytical activation of prorenin may lead to a surge in the activity of the RAS in the lung during ALI; this likely also triggers a cytokine response in the lung tissue. The RAS is implicated in the mediation of the cytokine storm [29]. Thus, the pro(rein)-RAS cascade may play a major role in ALI pathology. However, prorenin binding to the PRR can activate MAPK and ERK signaling cascades, leading to increased expression of inflammatory and fibrotic molecules [30, 31]. These signaling events are independent of the generation of Ang II, leading to the hypothesis that the PRR may directly promote tissue damage [32]. Therefore, we suggest that PRRB can attenuate inflammation-associated lung damage by inhibiting both RAS-dependent and RAS-independent intracellular signaling pathways.

It is essential that the mechanism of intracellular signaling pathways caused by prorenin/prorenin receptor interaction be elucidated. We plan to conduct a research study to determine this particular mechanism.

**CONCLUSION**

Here, we have shown that PRRB treatment reduces nonproteolytic activated prorenin in the lung tissue and protects against ALI induced by LPS. These findings suggest that activated prorenin plays an important role in acute lung damage and that it may be an important new therapeutic target for the treatment of ALI. In terms of clinical utility, it will be important to determine whether PRRB treatment is also effective when administered after the onset of ALI; this is the subject of ongoing research within our group.

**Declaration of interest**: Kenjiro Ishii and other co-authors have no conflict of interest to declare. The authors alone are responsible for the content and writing of the paper.

**REFERENCES**


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