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ABSTRACT
Background and objective: As a member of the epidermal growth factor family, amphiregulin contributes to the regulation of cell proliferation. Amphiregulin was reported to be upregulated in damaged lung tissues in patients with chronic obstructive pulmonary disease and asthma and in lung epithelial cells in a ventilator-associated lung injury model. In this study, we investigated the effect of amphiregulin on lipopolysaccharide (LPS)-induced acute lung injury in mice.

Methods: Acute lung injury was induced by intranasal instillation of LPS in female C57BL/6 mice, and the mice were given intraperitoneal injections of recombinant amphiregulin or phosphate-buffered saline 6 and 0.5 h before and 3 h after LPS instillation. The effect of amphiregulin on apoptosis and apoptotic pathways in a murine lung alveolar type II epithelial cell line (LA-4 cells) were examined using flow cytometry and western blotting, respectively.

Results: Recombinant amphiregulin suppressed epithelial cell apoptosis in LPS-induced lung injury in mice. Western blotting revealed that amphiregulin suppressed epithelial cell apoptosis by inhibiting caspase-8 activity.

Conclusion: Amphiregulin signaling may be a therapeutic target for LPS-induced lung injury treatment through its prevention of epithelial cell apoptosis.

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1. Introduction

Acute respiratory distress syndrome (ARDS) was first proposed by Ashbaugh et al., in 1967 and was defined by the American-European Consensus Conference in 1994 [1]. Acute lung injury is characterized by neutrophil inflammation and epithelial apoptosis [2]. Common causes of clinical disorders associated with the development of ARDS are pneumonia, gastric content aspiration, sepsis, and severe trauma [2]. Although many studies have investigated the mechanisms and risks of ARDS, the mortality rate remains high [3,4].

Lipopolysaccharide (LPS) is strongly associated with lung injury and ARDS. In type II alveolar epithelial cells, LPS induced apoptosis and cytokine production [5]. Intratracheal LPS instillation induced acute lung injury with neutrophil emigration in mice [6,7]. Neutrophil accumulation and cytokine production were reported to be caused by intranasal and aerosol LPS administration [8]. Intravenous LPS induced disseminated endothelial apoptosis [9] as well as non-endothelial tissue damage in the lung, and epithelial cell apoptosis was detected in diffuse alveolar damage [10]. We previously reported that intravenous LPS induced apoptosis of endothelial and alveolar epithelial cells, and that a broad-spectrum caspase inhibitor prevented apoptosis and lung injury in mice [11].

Amphiregulin was first described as a member of the epidermal growth factor family in 1989 [12]. It was reported to be upregulated in a ventilator-associated acute lung injury model [13]. Amphiregulin expression was also increased in damaged lung tissues in patients with chronic obstructive pulmonary disease and asthma [14–16] recently, amphiregulin has been reported to protect against lipopolysaccharide (LPS)–induced acute lung injury in mice [17]. However, the molecular mechanisms have not been well documented. Given that lung epithelial cell damage plays an important role in the pathogenesis of acute lung injury, this study was to determine the effect of amphiregulin on the apoptosis in the...
**2. Materials and methods**

**2.1. Animal treatment**

This study was conducted in accordance with the Animal Care and Use Committee of Kyushu University guidelines, approved by the Ethics Committee of Kyushu University Faculty of Medicine (No. A26-130-0), and performed according to the guidelines of the American Physiological Society. Seven-week-old female C57BL/6 mice were purchased from SLC, Inc., Shizuoka, Japan and used in all experiments. The mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (Schering-Plough, Kenilworth, NJ, USA) and intranasally administered 0.5 mg/kg LPS from *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO, USA) and intraperitoneally injected 7.5 mg/kg Escherichia coli 0111:B4 (Sigma, St. Louis, MO, USA) and intranasally administered 0.5 mg/kg LPS from *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO, USA). Control mice were administered sterile saline instead of the LPS solution. The recovered fluids were filtered through a single layer of gauze to remove the mucus. The cells present in the lavage fluid were counted using a hemocytometer. Differential counts of BAL cells were performed on 200 cells stained with Diff-Quick (Baxter Diagnostics, Dearfield, IL, USA). Total protein concentrations in BAL fluid (BALF) were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

**2.2. Histopathology of lung tissue**

Preparation of lung tissue was performed as previously described [20]. Briefly, 10% formalin was infused into the airways at a pressure of 20 cm H2O, as recommended for lung histology assessment in animal experiments, and fixed lung samples were embedded in paraffin. The lung sections were stained with hematoxylin and eosin. The pathological grades of lung injury in the whole area of the mid-sagittal sections were evaluated under 40× magnification by two observers in a blinded manner. The pathological grade was scored on a scale of 0–3 as previously described [20,21]. Briefly, the grade criteria were as follows: 0, no lung abnormality; 1, presence of lung injury involving <25% of the lung; 2, lesions involving 25–50% of the lung; or 3, lesions involving >50% of the lung.

**2.3. Immunohistochemistry of lung tissue**

Paraffin sections (3 μm thick) were adhered to slides pretreated with poly-l-lysine. Following deparaffinization, the sections were treated with 0.3% methanol for 30 min to inhibit any endogenous peroxidase activity. Immunohistochemistry was performed using a Histofine SAB-PO kit (Nichirei Corporation, Tokyo, Japan). Nonspecific protein staining was blocked with rabbit or goat serum for 30 min at room temperature. Sections were immunostained with rabbit polyclonal anti-amphiregulin (Sigma) or phosphate buffered saline (PBS) 6 and 0.5 h before and 3 h after LPS instillation, as previously reported [18]. The treatment schedule and doses of amphiregulin were determined according to previous studies [18,19]. The mice were sacrificed with an overdose of anesthesia 6 or 24 h after LPS instillation.

**2.4. Bronchoalveolar lavage fluid analysis**

Bronchoalveolar lavage (BAL) and analysis were performed as previously described [20]. Briefly, after insertion of a tracheal tube, the trachea was lavaged twice with 1 mL sterile saline at room temperature. The recovered fluids were filtered through a single layer of gauze to remove the mucus. The cells present in the lavage fluid were counted using a hemocytometer. Differential counts of BAL cells were performed on 200 cells stained with Diff-Quick (Baxter Diagnostics, Dearfield, IL, USA). Total protein concentrations in BAL fluid (BALF) were measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

**2.5. DNA damage and apoptosis in lung tissue**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end label (TUNEL) staining was performed using a DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI, USA) as previously described [18]. The number of TUNEL-positive cells was counted (the whole field of 10 randomly selected fields) in each section under a microscope using 200× magnification.

**2.6. Effects of LPS on a mouse lung epithelial cell line**

The mouse lung epithelial cell line, LA-4, derived from lung adenoma, was purchased from ATCC (Manassas, VA, USA). The cells were cultured in F12K medium with 10% fetal bovine serum and 1% penicillin–streptomycin. The cultures were incubated at 37 °C in a humidified, 95% air/5% CO2 atmosphere. The cells were treated with 0, 10, or 100 nM amphiregulin for 3 h and then washed with PBS. The cells were then incubated with 0 or 50 μg/mL LPS for 24 h to induce apoptosis and then harvested to prepare for flow cytometry or western blot analysis. The supernatants were used for cytokine/chemokine measurements.

**2.7. Apoptotic analysis by flow cytometry**

LA-4 cell apoptosis was analyzed using an Annexin V-FLUOS staining kit (Roche Diagnostics, Penzberg, Germany). Cells were washed in PBS and re-suspended in an incubation buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 5 mM CaCl2] with Annexin V–fluorescein isothiocyanate (FITC) and propidium iodide. After incubating for 20 min on ice, fluorescence was measured using a Coulter EPICS XL flow cytometer (Coulter, Miami, FL). Three samples from each group were analyzed.

**2.8. Western blot analysis**

Cells were lysed in a sample buffer [50 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 0.6% mercaptoethanol], and boiled for 2 min. The samples were subjected to western blot analysis as previously described [13]. The following primary antibodies were used: anti-caspase 11 (Santa Cruz Biotechnology), anti-cleaved caspase-8 (Cell Signaling), anti-cleaved caspase-3 (Cell Signaling), and anti-β-tubulin (Millipore). Signals were measured using ImageJ public-domain software (imagej.nih.gov/ij/) and standardized to β-tubulin.

**2.9. Enzyme-linked immunosorbent assay**

Interleukin (IL)-1β, IL-6, chemokine (C-C motif) ligand 2 (CCL2), tumor necrosis factor (TNF)-α, and intercellular adhesion molecule (ICAM)-1 were measured using enzyme-linked immunosorbent assay (ELISA) kits obtained from Thermo Scientific (USA). Chemokine (C-X-C motif) ligand 1 (CXCL1) levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

IL-1β, IL-6, CXCL1, and CCL2 in the supernatant of LPS-stimulated LA-4 cells were measured using the Bio-Plex Pro assay kit on the basis of xMAP suspension array technology (Bio-Rad Laboratories Inc., Hercules, CA, USA).
Fig. 1. Amphiregulin ameliorated LPS-induced acute lung injury in mice. (A) Hematoxylin and eosin-stained lung tissues from mice treated with saline (control), LPS + PBS, or LPS + AREG. Scale bars: 50 μm. (B) Effect of amphiregulin on the pathological grade of LPS-induced pneumopathy in mice. Each circle, triangle, and diamond corresponds to the data for one mouse. (C) Total cell count and number of neutrophils in BAL fluid. (D) Total protein concentration in BAL fluid. Data are shown as the mean ± SEM from four to six mice per group. *P < 0.05, **P < 0.01. AREG, amphiregulin; BAL, bronchoalveolar lavage; LPS, lipopolysaccharide; PBS, phosphate buffered saline; SEM, standard error of the mean.
2.10. Statistical analysis

Pathological grades were compared using the Mann–Whitney U test. Student’s t-test was used to compare the number of BALF cells, BALF protein concentrations, BALF cytokine levels, number of TUNEL-positive cells, apoptosis rate of LA-4 cells, and relative intensity of western blot products. P values of <0.05 were considered significant. Statistical analysis was performed with JMP version 8 (SAS Institute, Cary, NC, USA). Error bars denote ± standard error of the mean.

3. Results

3.1. Extrinsic amphiregulin partially ameliorates LPS-induced acute lung injury

Lung tissue from mice that were administered saline as a control demonstrated normal lung parenchyma. In contrast, mice administered LPS displayed inflammatory cell infiltration, parenchymal edema, and slight alveolar hemorrhage in lung tissues. These changes were decreased by amphiregulin treatment (Fig. 1A and B).

The numbers of total cells and neutrophils in the BALF of the LPS-injected mice were significantly higher than those in the BALF of the control mice. Amphiregulin treatment significantly decreased the numbers of total cells and neutrophils at 24 h (Fig. 1C). At 24 h after injection, the total protein concentration in the BALF (which reflects alveolar permeability) was significantly higher in the LPS-administered mice than in the control mice. This elevation in protein concentration was suppressed by amphiregulin treatment (Fig. 1D). In immunohistochemical analysis, phosphorylated EGFR was not detected in the lung epithelial cells of the LPS-administered mice, and this was significantly upregulated by amphiregulin treatment (Supplemental Fig. 1).

3.2. Extrinsic amphiregulin suppresses epithelial cell apoptosis in LPS-induced lung injury

TUNEL staining was performed to detect apoptotic cells, as their presence reflects the degree of lung injury. LPS administration caused epithelial cell apoptosis (Fig. 3A). However, extrinsic amphiregulin treatment markedly decreased the number of apoptotic cells (Fig. 3A and B). These results indicate that amphiregulin suppressed LPS-induced epithelial cell apoptosis.

3.3. Amphiregulin suppresses epithelial cell apoptosis via the caspase-8 pathway

To explore the underlying mechanisms, we used mouse epithelial cell line LA-4. Treatment with LPS for 24 h induced apoptosis in 12.39 ± 0.58% of LA-4 cells. Pre-treatment with 100 nM amphiregulin significantly suppressed this effect (8.56 ± 0.81%) (Fig. 4A).

Next, the apoptotic pathways in the LA-4 cells were analyzed. At
24 h after LPS treatment, caspase-11 and activation fragments of caspase-8 and caspase-3 were upregulated. Although amphiregulin pre-treatment did not change the caspase-11 level, it significantly suppressed cleaved caspase-8 and cleaved caspase-3 levels (Fig. 4B and C).

These results indicate that amphiregulin suppressed epithelial cell apoptosis via the caspase-8 pathway (Fig. 4D).

4. Discussion

Amphiregulin is a member of the EGF family and contributes to the regulation of cell proliferation. It is reported to have a less potent growth stimulatory effect through EGFRs, except in murine keratinocytes [12]. Moreover, amphiregulin plays an important role in protection against liver injury and bleomycin-induced lung injury. EGF is also reported to protect against sensitization to bacterial LPS [22]. The cellular response to LPS is mediated by Toll-like receptor 4 (TLR4) activation [23]. Many studies have demonstrated an association between TLR and EGFR; for example, TLRs induced IL-8 and VEGF via the EGFR phosphorylation pathway [24]. A pro-apoptotic response via EGFR has also been reported [25].

We demonstrated that amphiregulin had a protective effect on LPS-induced lung injury in mice. Amphiregulin decreased apoptosis in lung epithelial cells. In vitro results showed that amphiregulin suppressed LPS-induced epithelial cell apoptosis through inhibiting caspase-8 activity. However, caspase-11, which regulates both inflammatory response and apoptosis, was not inhibited by amphiregulin. This suggests that amphiregulin has a central but not absolute role in inhibiting LPS-induced epithelial cell apoptosis. In fact, Zhou et al. showed that amphiregulin contributed to pulmonary fibrosis in transforming growth factor-β-induced pulmonary fibrosis [26]. This contradictory report may reflect the differing nature of injury between the different experimental settings, and the time course. Amphiregulin orchestrates tissue repair and homeostasis, but a prolonged, excessive amphiregulin effect may contribute to lung fibrosis.

ARDS is characterized by neutrophil inflammation and epithelial cell apoptosis. According to a previous report, extrinsic amphiregulin suppressed inflammatory cytokines in BALF in LPS-induced lung injury [17]. However, our results showed that extrinsic amphiregulin did not suppress inflammatory cytokines (IL-1β, IL-6, and TNFα) or chemokines (CXCL1 and CCL2) in vivo. This may be due to the better efficiency of LPS intranasal administration compared with intratracheal administration.

Martins et al. reported that lung ICAM-1 mRNA levels were elevated via the nuclear factor kappa B (NF-κB) signaling pathway in LPS-induced lung injury, resulting in neutrophil migration [27]. We showed that amphiregulin suppressed ICAM-1 expression and neutrophil accumulation in vivo; however, the mechanism for this is unknown. Further work is needed to explore this effect.

In conclusion, amphiregulin treatment ameliorates LPS-induced lung injury in the acute phase by controlling apoptosis. Though
amphiregulin is a double-edged sword in terms of lung injury and fibrosis, the amphiregulin pathway may be a therapeutic target for ARDS treatment.

Disclosure statement
The authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.01.142.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.01.142.

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