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Fasudil inhibits neutrophil-endothelial cell interactions by regulating the expressions of GRP78 and BMPR2

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ABSTRACT

Regulation of leukocyte-endothelial cell interactions and of vascular permeability plays a critical role in the maintenance of functional pulmonary microvascular barriers. Little is yet known about the effect of the Rho-associated protein kinase (ROCK) inhibitor fasudil on leukocyte-endothelial cell interactions or the underlying mechanism. In the present study, as evaluated using co-culture systems of neutrophils and human pulmonary microvascular endothelial cells (HPMECs), fasudil dose-dependently suppressed neutrophil chemotaxis by decreasing the production of chemotactic factors in lipopolysaccharide (LPS)-treated HPMECs. The inhibitory role of fasudil in neutrophil chemotaxis was mediated by down-regulation of the chaperone glucose-regulated protein 78 (GRP78), since the inhibition was abolished by 4-phenyl butyric acid (a chemical chaperone mimicking GRP78). In addition, fasudil inhibited LPS-induced neutrophil-endothelial adhesion by reducing the expression of intercellular adhesion molecule (ICAM)-1. By use of lentiviral transfection in HPMECs, bone morphogenic protein receptor 2 (BMPR2) overexpression suppressed the LPS-induced increase of both ICAM-1 expression and neutrophil-endothelial adhesion, whereas knocking down BMPR2 abolished the inhibitory role of fasudil in both ICAM-1 expression and neutrophil-endothelial adhesion. Moreover, fasudil alleviated LPS-induced hyperpermeability of HPMEC monolayers by leading to the recovery of intercellular junctions, thereafter reduced neutrophil transendothelial cell migration. Therefore, fasudil inhibited leukocyte-endothelial cell interactions and vascular hyperpermeability through modulation of GRP78 and BMPR2 expression, suggesting a potential role for ROCK as a switch for inhibiting leukocyte-endothelial cell interactions.

1. Introduction

In pulmonary microvasculature, extravasation of leukocytes across the pulmonary alveolar-capillary barrier leads to pulmonary edema, deficient gas exchange, arterial hypoxemia, and eventually, respiratory failure [1–3]. Therefore, the interplay between leukocytes and microvascular cells under pathological conditions, together with the emerging roles of leukocytes in the modulation of the inflammatory response, makes leukocyte–endothelial cell interactions prime targets for therapeutics to potentially treat a wide range of pulmonary diseases [4], including acute lung injury (ALI), chronic alveolar inflammation, pulmonary hypertension, and interstitial fibrosis.

Leukocyte extravasation under pathological conditions is a dynamic process consisting of leukocyte-endothelial chemotaxis, adhesion and sequential transendothelial migration [5]. The activated endothelial cells, which result from mechanical, chemical, or biological stimuli [6], produce substantial chemotactic factors (*e.g.*, chemokine [C-X-C motif] ligand [CXCL] 1, CXCL2 and CXCL8) and adhesion molecules (*e.g.*, intercellular adhesion molecule [ICAM]-1 and vascular cell adhesion molecule [VCAM]-1), which in turn result in leukocyte-endothelial chemotaxis and adhesion [6]. The dysfunction of the alveolar-endothelial barrier results from the loss of inter-endothelial junctions, including tight junctions (TJs) and adherens junctions (AJs), leads to leukocyte transendothelial migration. Therefore, inhibition of leukocyte-endothelial cell interactions *via* modulation of endothelial function might be a prime target for therapeutics.

Recent investigations suggested that increased levels of glucose-regulated protein 78 (GRP78) during the early phase of endoplasmic reticulum stress enhanced the inflammatory response [6]. Knockdown of GRP78 successfully attenuated LPS-induced pancreatic inflammation [7]. An autoantibody promoted tumor necrosis factor α production in monocyte/macrophages by binding to GRP78 [8]. Thus, GRP78 could promote the activation of inflammatory signaling and might be involved in the regulation of leukocyte-endothelial cell interactions. In

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addition, studies have documented that dysfunctional bone morphogenetic protein receptor type 2 (BMPR2) is involved in inflammatory responses. For example, BMPR2 reduction prompted the secretion of cytokines such as interleukin (IL)-6 and IL-8 *in vivo* and *in vitro* [9]. BMPR2 deficiency in pulmonary artery endothelial cells also resulted in a high level of IL-8 in response to transforming growth factor-β1 (TGF-β1) and promoted the transmigration of mononuclear cells and neutrophils [10]. Similarly, pulmonary microvascular endothelial cells from patients with a BMPR2 mutation showed enhanced monocyte recruitment ability [11]. Whether BMPR2 is involved in leukocyte-endothelial cell interactions needs further exploration.

Rho-associated protein kinase (ROCK), a serine/threonine kinase, is a downstream effector of the Rho family of small GTPases. Mounting evidence has shown that ROCKs act as determinant molecular switches controlling several critical cellular functions, such as cytoskeletal rearrangement, cell adhesion and migration, inflammation, and reactive oxygen species formation [12–14]. We previously demonstrated that fasudil, a selective ROCK inhibitor, ameliorates pulmonary neutrophil infiltration during acute lung injury *in vivo* [15]. However, the underlying mechanism remains unclear. In the present study, we established a co-culture system using neutrophils and human pulmonary microvascular endothelial cells (HPMECs) and modulated BMPR2 expression in HPMECs with lentiviral transfection to investigate whether fasudil could alleviate lipopolysaccharide (LPS)-induced leukocyte-endothelial cell interactions by regulating the expression of GRP78 and BMPR2.

2. Materials and methods

2.1. Cell culture

HPMECs were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Cells were cultured at 37 °C with 5% CO_2 in endothelial cell growth medium (EGM, ScienCell, USA) containing 1% penicillin, streptomycin and endothelial cell growth factors and 5% fetal bovine serum (ScienCell, USA). Cells between passage 4 and 6 were used for experiments. After starvation for 6 h, cells were exposed to LPS (1 μg/ml, Sigma-Aldrich, MO, USA) with or without fasudil (Sigma-Aldrich, USA) at different concentrations (0.04 μg/ml, 0.2 μg/ml, 1 μg/ml) for specific time intervals.

2.2. Transfection of HPMECs with lentiviral (LV)-BMPR2-shRNA and LV-BMPR2

For BMPR2 knockdown and overexpression, HPMECs were transfected with either LV-shRNA-BMPR2 (GeneChem Co., Shanghai, China) or LV-BMPR2, while transfection with a lentiviral vector was used as the control (LV-NC). When HPMECs reached approximately 30% confluency, lentivirus dissolved in medium containing enhanced infection solution and polybrene were added to each well. After 8 h, the transfection medium was replaced with normal medium, and the cells were cultured for another 72 h. Down-regulation and overexpression of BMPR2 were confirmed by western blotting.

2.3. Neutrophil chemotaxis assay

Human neutrophils were isolated from the peripheral blood of healthy volunteers with Ficoll-Paque Plus (GE Healthcare, Pittsburgh, USA) as described previously [16]. The volunteers were recruited from the First Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from all participants, and their privacy rights were observed. This experiment was approved by the Medical Ethical Review Committees at the First Affiliated Hospital of Nanjing Medical University (2017-SR-198). Neutrophil chemotaxis was performed in a 24-well Corning transwell system (3 μm pore size, 6.5 mm diameter). When HPMECs in the lower chamber reached 70–80% confluency, cells were pretreated with fasudil for 30 min with/without 4-phenyl butyric acid (PBA, at 0.2 mM, 1 mM or 5 mM) (Sigma-Aldrich) and then exposed to LPS (1 μg/ml) for 24 h. After that, neutrophils (10^5 cells in 100 μl medium) were added into the upper chambers. The transwell chamber system was incubated at 37 °C for 45 min. Then, the top side of the upper chamber was wiped carefully. The cells that migrated through the pores onto the lower side of the membrane were fixed and stained with Diff-Quick. After that, they were counted under a phase contrast microscope (Nikon, Tokyo, Japan) and recorded in more than ten random fields in each group.

2.4. Neutrophil-endothelial adhesion assay

Neutrophil-endothelial adhesion was evaluated using an established co-culture system [17]. After pretreatment with fasudil at different concentrations (0.04 μg/ml, 0.2 μg/ml, 1 μg/ml) for 30 min, HPMECs were exposed to LPS (1 μg/ml) for 12 h. The isolated neutrophils were resuspended (10^6 cells/ml) and stained with 4 ng/ml calcein-AM (BD Biosciences) for 30 min. Then, the labeled neutrophils (2×10^5 cells/ml, 500 μl per well) were co-incubated with HPMECs for 1 h at 37 °C. Non-adherent neutrophils were washed out, and the number of adherent neutrophils in ten random areas of each well was counted under a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.5. Neutrophil transendothelial cell migration assay

Neutrophil transendothelial cell migration assay were performed according to the protocol described previously [18,19]. Briefly, HPMECs (2×10^4 cells in 200 μl EGM) were cultured on bobine plasma fibronectin (ScienCell Research Laboratories)-coated Corning transwell inserts (6.5 mm diameter, 3 μm pore size) and grown for 3 days until confluent. On day 4, endothelial cells on the inserts were treated with LPS (1 μg/ml) for 24 h with/without fasudil pretreatment (1 μg/ml). Meanwhile, another endothelial cells cultured in 25 cm^2 culture-flask were treated with LPS, the cellular supernatant was collected as conditioned medium (CM) 24 h later. Thereafter, inserts were washed with PBS and transferred into fresh 24-wells plate. Then, 100 μl of the neutrophil suspension (10^6 cells/ml) was added in the upper chamber when the lower chamber was filled with 600 μl CM or basic culture medium (BCM). The transendothelial cell migration assay system was incubated at 37 °C for 3 h. Thereafter, migrated neutrophils were collected from the bottom of the well, and counted microscopically.

2.6. Assessment of HPMEC monolayer permeability

HPMECs seeded onto 12-well inserts (0.4 μm pore size) were treated with/without fasudil (1 μg/ml) for 30 min once they were completely confluent. After that, cells were incubated with LPS for 24 h. Then, 250 μg FITC-dextran (molecule weight, 4000 kDa; Sigma-Aldrich) was added into the upper chamber. Two hours later, leakage of FITC-labeled dextran was quantified by measuring the fluorescence intensity of 200 μl of each sample from the lower chamber with a fluorescence microplate reader (Biotek, USA).

2.7. RhoGTPase activation assay

The RhoGTPase activation was detected using Rho Activation Assay Kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Briefly, cells were lysed using Mg^{2+} lysate containing 1 mM phenylmethylsulfonyl fluoride and a cocktail of protease inhibitors. Then, cleared lysates were incubated with 25 μg GST-fusion protein of the Rhotekin-binding domain (RBD) along with glutathione agarose resin to specifically pull down active Rho (GTP-bound). Active Rho (Rho-GTP) was detected by Western blot analysis using antibody specific for Rho.