Pulmonary Pharmacology & Therapeutics 38 (2016) 27–35

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

# Mitigation of tight junction protein dysfunction in lung microvascular endothelial cells with pitavastatin

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ARTICLE INFO

*Article history:*
Received 23 January 2016
Received in revised form
17 April 2016
Accepted 28 April 2016
Available online 11 May 2016

*Keywords:*
COPD
Statin
Exacerbation
Tight junction
AmotL1

ABSTRACT

*Background:* Statin use in individuals with chronic obstructive pulmonary disease (COPD) with coexisting cardiovascular disease is associated with a reduced risk of exacerbations. The mechanisms by which statin plays a role in the pathophysiology of COPD have not been defined. To explore the mechanisms involved, we investigated the effect of statin on endothelial cell function, especially endothelial cell tight junctions.

*Method:* We primarily assessed whether pitavastatin could help mitigate the development of emphysema induced by continuous cigarette smoking (CS) exposure. We also investigated the activation of liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) signaling, which plays a role in maintaining endothelial functions, important tight junction proteins, zonula occludens (ZO)-1 and claudin-5 expression, and lung microvascular endothelial cell permeability.

*Results:* We found that pitavastatin prevented the CS-induced decrease in angiomotin-like protein 1 (AmotL1)-positive vessels *via* the activation of LKB1/AMPK signaling and IFN-γ-induced hyperpermeability of cultured human lung microvascular endothelial cells by maintaining the levels of AmotL1, ZO-1, and claudin-5 expression at the tight junctions.

*Conclusion:* Our results indicate that the maintenance of lung microvascular endothelial cells by pitavastatin prevents tight junction protein dysfunctions induced by CS. These findings may ultimately lead to new and novel therapeutic targets for patients with COPD.

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## 1. Background

Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that belong to the class of lipid-lowering medications. Numerous studies have suggested that statins reduce the risk of acute cardiac events and death [1]. In addition, the antithrombotic and anti-inflammatory effects of statins have been shown to contribute to the overall beneficial activity of these drugs [2]. Indeed, statin therapy improves endothelial function by virtue of its antioxidant and anti-inflammatory effects [3,4], as well as by its ability to regulate endothelial nitric oxide synthase (eNOS) [5] and AMP-activated protein kinase (AMPK) [6] pathways to increase NO bioavailability, which in turn, suppresses the production of endothelial reactive oxygen species (ROS) [7]. According to pharmaceutical database studies, statin may wield some beneficial effect on exacerbations in chronic obstructive pulmonary disease (COPD) [8,9]. However, according to a recent large, randomized trial, such treatment showed no effect on exacerbations in patients with moderate-to-severe COPD who were at high risk for exacerbation [10]. More recently, statin use was associated with reduced odds of exacerbations in patients with COPD from the general population. However, this was not apparent in those with the most severe forms of COPD and those without cardiovascular comorbidity [11]. Though the factors responsible for these results have not been elucidated, it seems that there might be a common pathophysiology between cardiovascular disease and exacerbation in COPD patients. The probable association between these two events

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http://dx.doi.org/10.1016/j.pupt.2016.04.003
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suggests that the basic mechanisms involved in COPD exacerbation should be better clarified to help identify novel therapeutic targets. We previously demonstrated that the number of angiomotin-like protein 1 (AmotL1)-positive blood vessels, which are involved in angiogenesis *via* regulation of endothelial cell function, is decreased in emphysematous lungs compared with normal and bronchial asthmatic lungs [12]. IFN-γ may exert anti-angiogenic effects by regulating the expression of TNF-α-induced AmotL1 *via* NFκB in emphysematous lungs. Moreover, AmotL1 controls the tight junctions (TJs) of endothelial cells and regulates sprouting angiogenesis by affecting tip cell migration and controlling the cell–cell junctions of stalk cells [13].

We tested the hypothesis that statins play an important role in maintaining the expression and function of lung microvascular endothelial cells, particularly TJ proteins, resulting in the prevention of the progression of emphysema. In particular, we evaluated the impact of pitavastatin on AmotL1, zonula occludens (ZO)-1, and claudin-5 expression.

## 2. Methods

### 2.1. Animal care and cigarette smoke exposure

All of the animal protocols used in this study were approved by the National Institute of Environment Health Sciences' Animal Care and Use Committee and followed the Helsinki Convention standards for the use and care of animals. The experimental procedures were approved by the institutional animal care and use committees at Iwate Medical University (approval number 24-028) in accordance with the university's animal experiments regulations. The mice used would need to have been exposed for more than six months to cigarette smoke (CS) in order for them to develop emphysema. However, in our preliminary studies, we could not exclude the possibility of complicating age-associated vessel structure phenotypes that typically occur after more than six months of CS exposure. Therefore, we adopted the emphysema model using *Nrf2*$^{-/-}$ mice, which downregulates antioxidant defenses and increases lung inflammation, thereby leading to the development of emphysema in four weeks instead of the usual six months [12]. In this way, we minimized the aforementioned concern.

The *Nrf2*$^{-/-}$ mice, which were backcrossed >20 times onto a C57BL/6J background, were purchased from the RIKEN BioResource Center (Ibaraki, Japan). The mice were genotyped for *Nrf2* status using a polymerase chain reaction (PCR) amplification of genomic DNA, which had been extracted from the blood as previously described [14]. All the mice used in this study were aged 9–10 weeks, had a body weight of 20 g, and were maintained under specific pathogen-free conditions. The mice were divided into four groups ($n = 11$ per group): control *Nrf2*$^{-/-}$ mice treated with or without pitavastatin and CS-exposed *Nrf2*$^{-/-}$ mice treated with or without pitavastatin. The control groups were maintained in a filtered air environment, and the CS-exposed groups were subjected to CS exposure, which was performed 5 h/day, five days/week for four weeks by burning 3R4F cigarettes (purchased from the Tobacco Research Institute, University of Kentucky) using a smoking apparatus (SIC-CS type, SG-200, Shibata Kagaku, Saitama, Japan). Each smoldering cigarette was puffed for 2 s at five puffs (175 ml) per min, with a flow rate of 5375.5 mL/min diluted with compressed air (3% of final concentration). The air (1700 mL/min) then forced the cigarette smoke into the sealed mouse container (3600 $cm^3$) [12]. Pitavastatin was dissolved in DMSO and then diluted in PBS. For the pitavastatin treatment groups, the mice were administered *via* 20 ng/100 μL (PBS containing 1% DMSO) of pitavastatin, or vehicle control administered intranasally 30 min before air or CS exposure. When administered 20 ng/100 μL of pitavastatin intranasally, the concentration in the lung tissue of the mice was equivalent to that measured in human pitavastatin users [15,16].

### 2.2. Lung histology and immunohistochemistry in an experimental mouse model of emphysema

The tracheas and lungs of anesthetized mice were terminally removed and inflated with 4% paraformaldehyde in PBS to a pressure of 12 cm $H_2O$. The tissues were then embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin and eosin (HE). Immunolocalization of AmotL1, ZO-1, and claudin-5 in the lung tissues was evaluated by using antibodies against AmotL1 (Assay Biotechnology, CA, USA), ZO-1 (Proteintech, IL, USA), and claudin-5 (Santa Cruz Biotechnology, TX, USA), respectively. All of the samples were then incubated with an Alexa Fluor 488-conjugated secondary antibody (Life Technologies) and imaged using a confocal laser-scanning microscope (C1si; Nikon, Tokyo, Japan).

### 2.3. Immunoblotting analysis and immunocytochemistry of AmotL1, ZO-1, and claudin-5 in human lung microvascular endothelial cells

Normal human lung microvascular endothelial cells (HLMVECs) were purchased from Takara (Tokyo, Japan) and maintained in an EGM-2-MV BulletKit (Takara) according to the manufacturer's instructions. Cover slips were placed on the bottom of 10-cm dishes, and the HLMVECs ($1 \times 10^5$/well) were then seeded onto the dishes. The HLMVECs were transfected with or without a short hairpin RNA (shRNA) plasmid [12] for LKB1 (Santa Cruz Biotechnology) or AMPKα1 (Santa Cruz Biotechnology), followed by treatment with or without pitavastatin and stimulated with 100 ng/mL IFN-γ (R&D Systems). The cover-slips, which contained HLMVECs on their surface, were subjected to immunocytochemistry and imaged using a confocal laser-scanning microscope (C1si; Nikon). The cultured HLMVECs on the bottoms of the 10-cm dishes were collected and lysed in TNE buffer, as previously described [17]. Twenty micrograms of protein were resolved using 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, CA, USA). Each membrane was then incubated with anti-human pLKB1 ($Ser^{431}$; Santa Cruz Biotechnology), LKB1 (Santa Cruz Biotechnology), GAPDH (Proteintech), pAMPK ($Thr^{172}$; Cell Signaling Technology, Tokyo, Japan), AMPK (Cell Signaling Technology), AmotL1 (Proteintech), ZO-1 (Proteintech), or claudin-5 (Santa Cruz Biotechnology) antibody. The membranes were then analyzed using the Odyssey Infrared Imaging System (LI-COR, NE, USA) according to the manufacturer's instructions. Where relevant, the signal intensity was determined using LI-COR imaging software.

### 2.4. Tight junction functional assays

HLMVECs were seeded onto the cell culture inserts in 24-well multiwell plates with 0.4 μm pore sizes (Greiner Bio-One Co. Ltd., Tokyo, Japan) and then allowed to grow to confluence. Seventy-two hours after visual confluence was obtained, the culture medium was removed and replaced with the experimental medium containing 200 μL phenol-free EBM-2 with or without 100 nM of pitavastatin added to the upper chamber, and 500 μL of medium without pitavastatin were added to the lower chamber. After a 24-h stabilization period, the medium in the upper chamber was replaced with 200 μL of medium with or without 100 ng/mL of IFN-γ for 1 h prior to the addition of 10 μL of 10 mg/mL Alexa Fluor® 488 FITC-labeled dextran (10 molecular mass/kDa; Thermo Fisher

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