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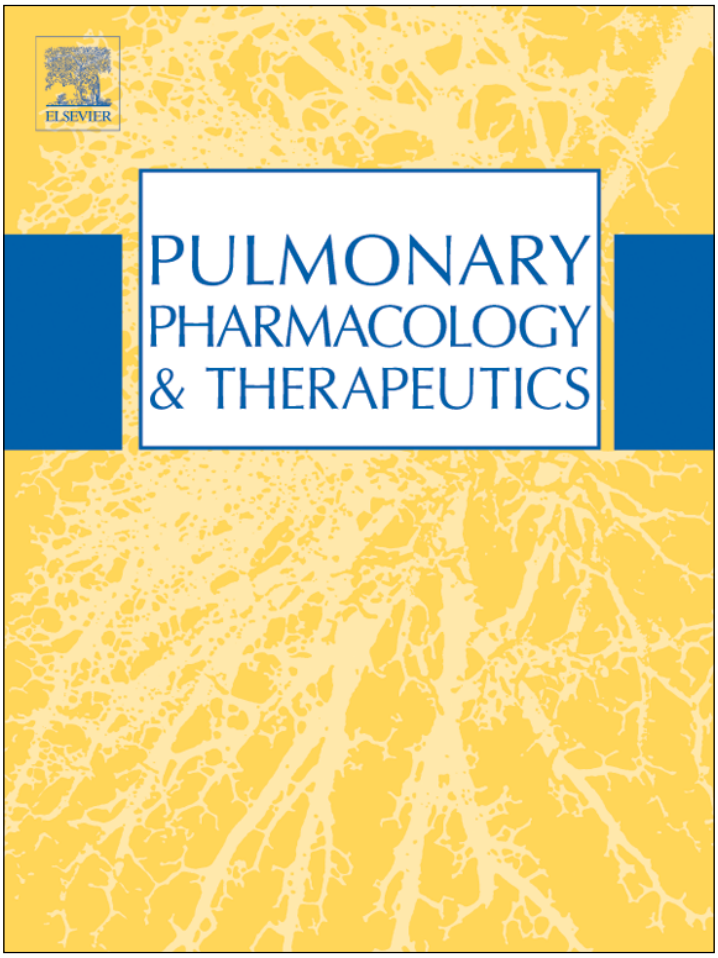

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Mitigation of tight junction protein dysfunction in lung microvascular endothelial cells with pitavastatin

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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.

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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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 (1×10^5/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

Scientific, MA, USA) or 1 μL of 100 mg/mL IRDye 680 anti-Rabbit IgG F(ab')$_2$ (140 molecular mass/kDa; LI-COR) to the upper chamber. After a 60-min incubation at 37 °C, 10-μL aliquots of medium were removed from the lower chamber, mixed with 90 μL of double-distilled water, and placed in a 48-well plate. The plates were read using an infrared imaging system (LI-COR) according to the manufacturer's instructions. All experiments were repeated five times.

2.5. Statistical analysis

Statistical analyses were performed using JMP version 11 (SAS Institute Inc., Tokyo, Japan). All data were expressed as the mean ± standard error of the mean. Comparisons of the levels of immunoblotting intensity between the four or five groups were performed using a two-tailed one-way analysis of variance (ANOVA). P values < 0.05 were considered significant. *Post hoc* multiple comparisons were performed using the Tukey-Kramer test for differences among all groups.

3. Results

3.1. Effects of pitavastatin on emphysema in Nrf2 $^{-/-}$ mouse lung histological studies

All 34 mice in the four groups survived for the full four weeks of

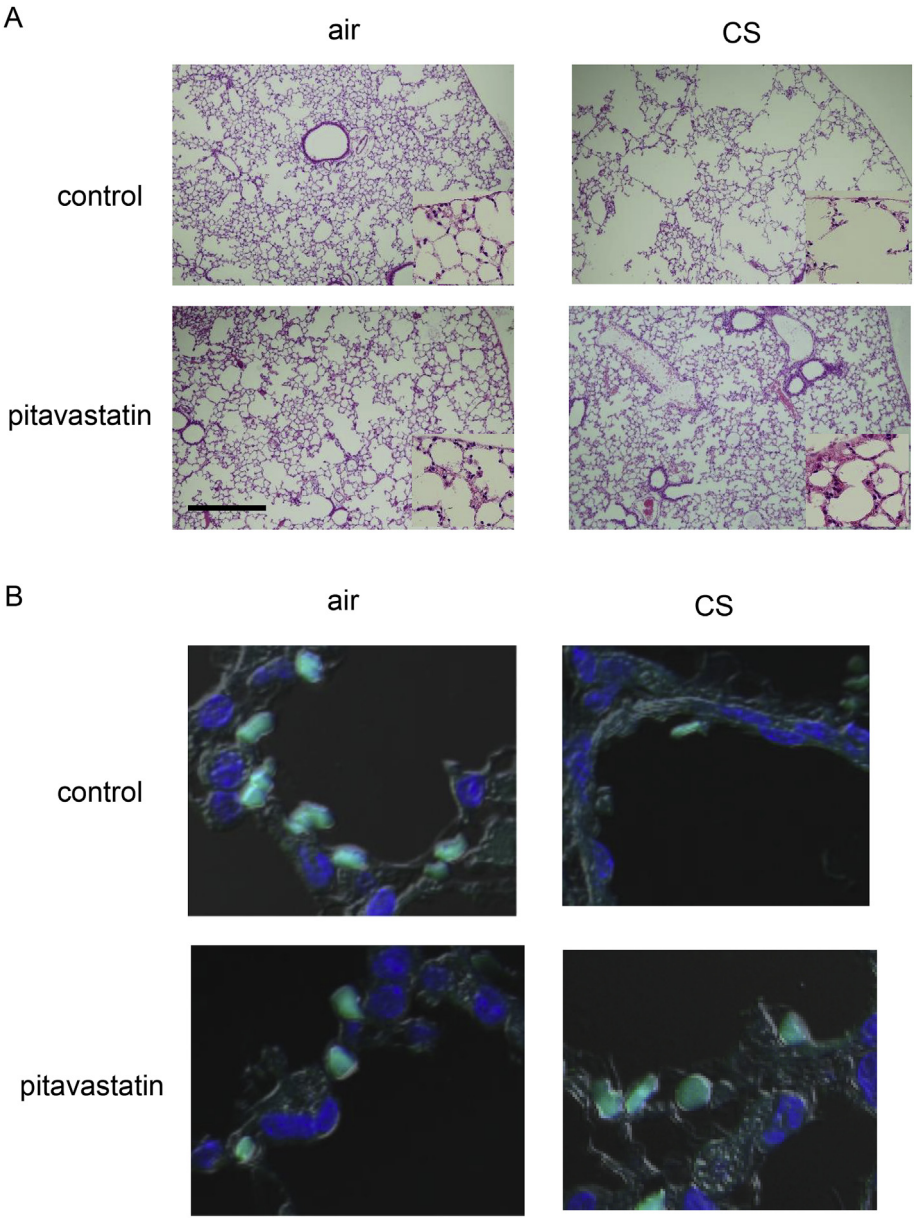

Fig. 1. Alveolar histology in *Nrf2*$^{-/-}$ mice. *Nrf2*$^{-/-}$ mice with air and cigarette smoke (CS) exposure were randomly pretreated with pitavastatin or 1% DMSO as a solvent for four weeks. The lungs were then removed and stained with HE (original magnification x 40) (A). Alveolar enlargement was observed in the CS-exposed *Nrf2*$^{-/-}$ mice pretreated without pitavastatin. Pretreatment with pitavastatin did not affect lung construction in the air-exposed *Nrf2*$^{-/-}$ mice; however, this treatment reduced air space enlargement in the lungs of CS-exposed *Nrf2*$^{-/-}$ mice (shown in the inserts). Scale bar: 500 μm. Immunohistochemistry was used to detect AmotL1 protein expression. Pretreatment with pitavastatin enhanced the expression of AmotL1 in the lungs of the CS-exposed *Nrf2*$^{-/-}$ mice. An overlay of green fluorescence and differential interference contrast images is shown. (Blue: DAPI staining. Green: AmotL1) (B). These photomicrographs are representative of the results obtained in a minimum of seven experiments evaluating the alveolar histology of the *Nrf2*$^{-/-}$ mice.

the study. We confirmed the development of emphysema in the *Nrf2*$^{-/-}$ mice after up to four weeks of CS exposure by using HE-stained lung sections (Fig. 1a). No pathological findings were observed in the *Nrf2*$^{-/-}$ mice before or after the four weeks of air exposure. However, after four weeks of CS exposure, the *Nrf2*$^{-/-}$ mice showed airspace enlargement and alveolar wall obstruction. To examine whether emphysema could be affected by pitavastatin *in vivo*, the CS-exposed *Nrf2*$^{-/-}$ mice were nasally administered pitavastatin 20 ng of pitavastatin. Pitavastatin pretreatment did not affect lung development in the control mice; however, it did reduce the air space enlargement in the lungs of the CS-exposed *Nrf2*$^{-/-}$ mice (Fig. 1a). To further investigate the role of pitavastatin in the TJs of emphysematous lungs, we performed immunohistochemistry to detect AmotL1, which forms the TJs of endothelial cells. Confocal immunofluorescence images of AmotL1 are shown in Fig. 1b. Pretreatment of pitavastatin enhanced the expression of AmotL1 in the emphysematous lungs of the CS-exposed *Nrf2*$^{-/-}$ mice. These results were consistent with the hypothesis that statins are involved in preventing the progression of emphysema by increasing the expression of the TJ protein, AmotL1.

3.2. Effects of pitavastatin on LKB1 and AMPK phosphorylation in HLMVECs

Many studies have shown that LKB1/AMPK signaling plays an important role in maintaining endothelial functions. AMPK activation also has beneficial effects on anti-atherogenesis [6,18]. To determine whether LKB1/AMPK activation is directly associated with the effects of pitavastatin, we performed immunoblotting studies to examine LKB1/AMPK activation. To avoid cell–cell and intracellular tensions [19], HLMVECs at subconfluency were exposed to pitavastatin (1–1000 nM) [20]. A morphological degeneration of HLMVEC, such as cell rounding and detachment, was induced by pitavastatin at a concentration of 1000 nM for 1 h. LKB1 and AMPK activation by pitavastatin was concentration-dependent until a peak was reached at 100 nM (data not shown). Time-course experiments performed with 100 nM pitavastatin showed a significant induction of pLKB1 after 30 min, which continued to increase for at least 90 min (Fig. 2a). In contrast, increased pAMPK expression was detected after 60 min of stimulation, peaked at 60 min, and remained elevated for at least 120 min (Fig. 2b).

3.3. Role of LKB1/AMPK activation in AmotL1 expression driven by pitavastatin

To further investigate the link between LKB1 and AMPK expression in response to pitavastatin treatment, we assessed the effects of shRNA targeting LKB1 and AMPK on AmotL1 expression. The silencing of LKB1 was associated with a decrease in both basal and pitavastatin-induced AMPK activation (Fig. 3). Conversely, the silencing of AMPK did not affect basal or pitavastatin-induced pLKB1 expression (Fig. 4), suggesting that shRNA-mediated LKB1 knockdown completely blocked these responses. Time-course experiments were performed with 100 nM pitavastatin showed a significant induction of AmotL1 after 24 h (data not shown). Pitavastatin-induced AmotL1 expression was significantly decreased after 24 h in the presence of silenced LKB1 or AMPK (Figs. 3 and 4).

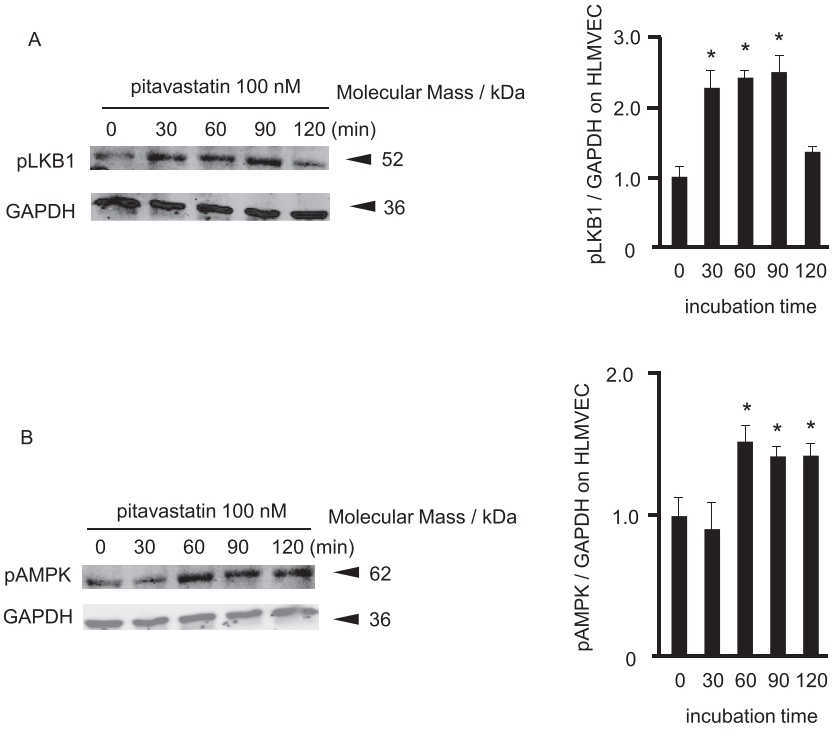

Fig. 2. Time course of pLKB1 and pAMPK induction by pitavastatin. Human lung microvascular endothelial cells (HLMVECs) were treated with 100 nM pitavastatin, and the lysates were subjected to immunoblotting. Representative immunoblotting (left) (A) and GAPDH-normalized band intensity quantification for pLKB1 expression in the HLMVECs after exposure to pitavastatin (100 nM) for the indicated time. Densitometry was significantly increased at 30 min after pitavastatin treatment and was sustained at 90 min. The basal expression in non-treated cells was set to 1.0. The whiskers indicate the standard errors of the mean. *$P < 0.05$ compared with the means of the expression when treated without pitavastatin treatment (right) (A). A representative immunoblot (left) (B) and GAPDH-normalized band intensity quantification for pAMPK are shown. Intensity levels were significantly increased at 60 min, and remained increased until 120 min following the addition of pitavastatin. The basal expression in non-treated cells was set to 1.0. The whiskers indicate the standard errors of the mean. *$P < 0.05$ compared with the means of the expression when treated without pitavastatin (right) (B).

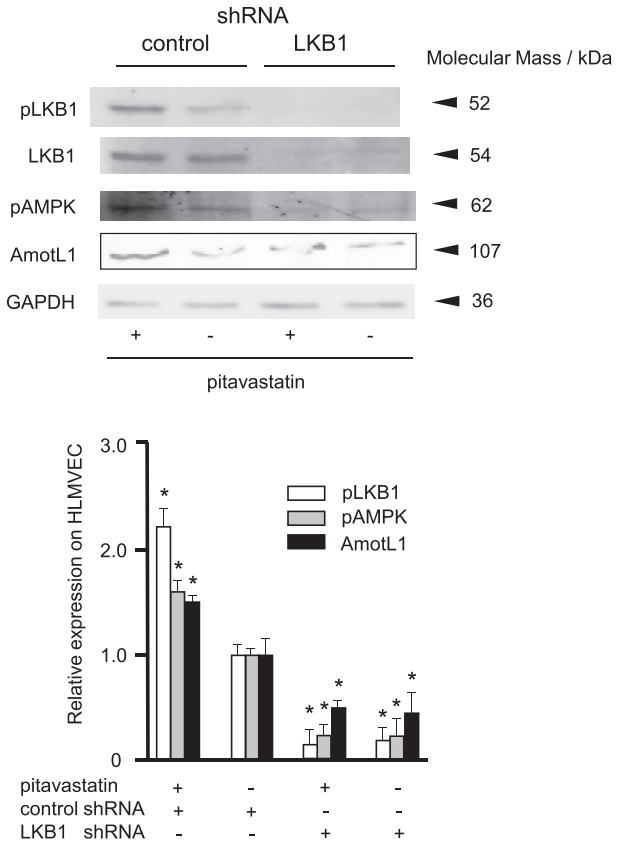

Fig. 3. Effects of shRNA-mediated LKB1 knockdown on pitavastatin-modulated AmotL1 expression and protein phosphorylation in the HLMVECs. The HLMVECs were treated with 100 nM pitavastatin for 24 h to examine the expression of AmotL1 and for 1 h to examine pLKB1, LKB1, and pAMPK. Using immunoblotting, cells were harvested, and the cell lysates were analyzed with antibodies directed against pLKB1, LKB1, pAMPK, AmotL1, and GAPDH. Pooled data and the quantification of immunoblot signals using infrared imaging for each antibody are shown. A representative experiment (upper panel) was repeated five times and yielded equivalent results. The basal expression in control shRNA-transfected and non-pitavastatin-treated cells was set to 1.0. The whiskers indicate the standard errors of the mean. $^{*}P < 0.05$ compared with the means of the basal expression (lower panel).

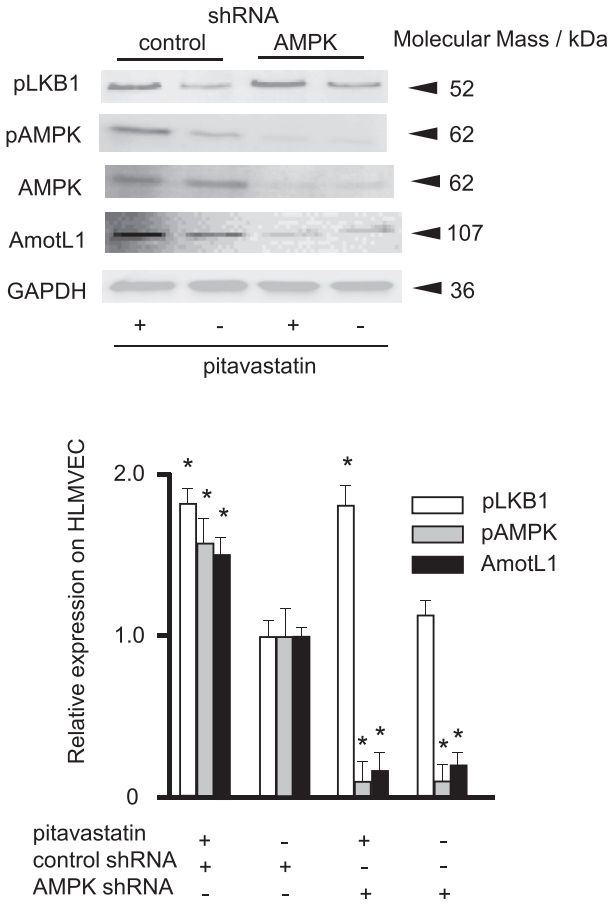

Fig. 4. Effects of shRNA-mediated AMPK knockdown on pitavastatin-modulated AmotL1 expression and protein phosphorylation in the HLMVECs. The HLMVECs were treated with 100 nM pitavastatin for 24 h to examine the expression of AmotL1, and for 1 h to examine pAMPK, AMPK, and pLKB1. Using immunoblotting, cells were harvested and the cell lysates were analyzed with antibodies directed against pAMPK, AMPK, pLKB1, AmotL1, and GAPDH. Pooled data and quantification of the immunoblot signals using infrared imaging for each antibody are shown. A representative experiment (upper panel) was repeated five times and yielded equivalent results. The basal expression in control shRNA-transfected and non-pitavastatin-treated cells was set to 1.0. The whiskers indicate the standard errors of the mean. $^{*}P < 0.05$ compared with the means of the basal expression (lower panel).

3.4. Effects of pitavastatin on HLMVECs TJs in the presence of IFN-γ

We previously characterized AmotL1 expression in emphysema *in vivo* and *in vitro*. Pretreatment of HLMVECs with IFN-γ diminished the expression of AmotL1 by blocking the degradation of IκB. In the present study, we examined the expression of AmotL1, pLKB1/pAMPK, the TJ protein ZO-1, and claudin-5 in HLMVECs while in the presence or absence of pitavastatin to elucidate the effect of IFN-γ and pitavastatin on endothelial cell TJs. Subconfluent cells were exposed for 90 min or 72 h to vehicle, 100 nM pitavastatin or 100 ng/mL of IFN-γ, or to a mixture of these two agents in order to detect the expression of intracellular signals and tight junction proteins. Unexpectedly, IFN-γ suppressed the expression of all TJ proteins, including AmotL1, ZO-1, and claudin-5; however, this treatment did not affect the expression of pLKB1/pAMPK. AmotL1 and claudin-5 were upregulated by pitavastatin regardless of IFN-γ treatment. Pitavastatin did not increase the expression of ZO-1; however, this treatment sustained the expression of ZO-1 despite the presence of IFN-γ (Fig. 5a). Given that IFN-γ attenuated the expression of AmotL1, ZO-1, and claudin-5, we next clarified the effects of IFN-γ and pitavastatin on the configuration of the TJ proteins of cultured HLMVECs. IFN-γ treatment for 72 h led to a decrease in the expression of TJ proteins surrounding the cells, and pitavastatin recovered the expression of these proteins (Fig. 5b).

3.5. Evaluation of IFN-γ-induced hyperpermeability of TJs and prevention by pitavastatin

Given that IFN-γ attenuated the expression of AmotL1, ZO-1, and claudin-5, we next investigated whether IFN-γ altered the hyperpermeability of TJs and the impact of pitavastatin on this process. IFN-γ increased the permeability of HLMVEC monolayers, while pitavastatin suppressed the IFN-γ-induced attenuation of cell permeability without any change in the expression of TJ proteins (Fig. 6). IFN-γ also induced a significant change in the permeability of both dextran (10 molecular mass/kDa) and F(ab')2 (140 molecular mass/kDa) at baseline; however, pitavastatin attenuated this permeability independent of molecular mass.

4. Discussion

In this study, we investigated the role of pitavastatin in IFN-γ-mediated responses, which include exacerbation in patients with COPD. Several new findings have emerged from this study. First, we

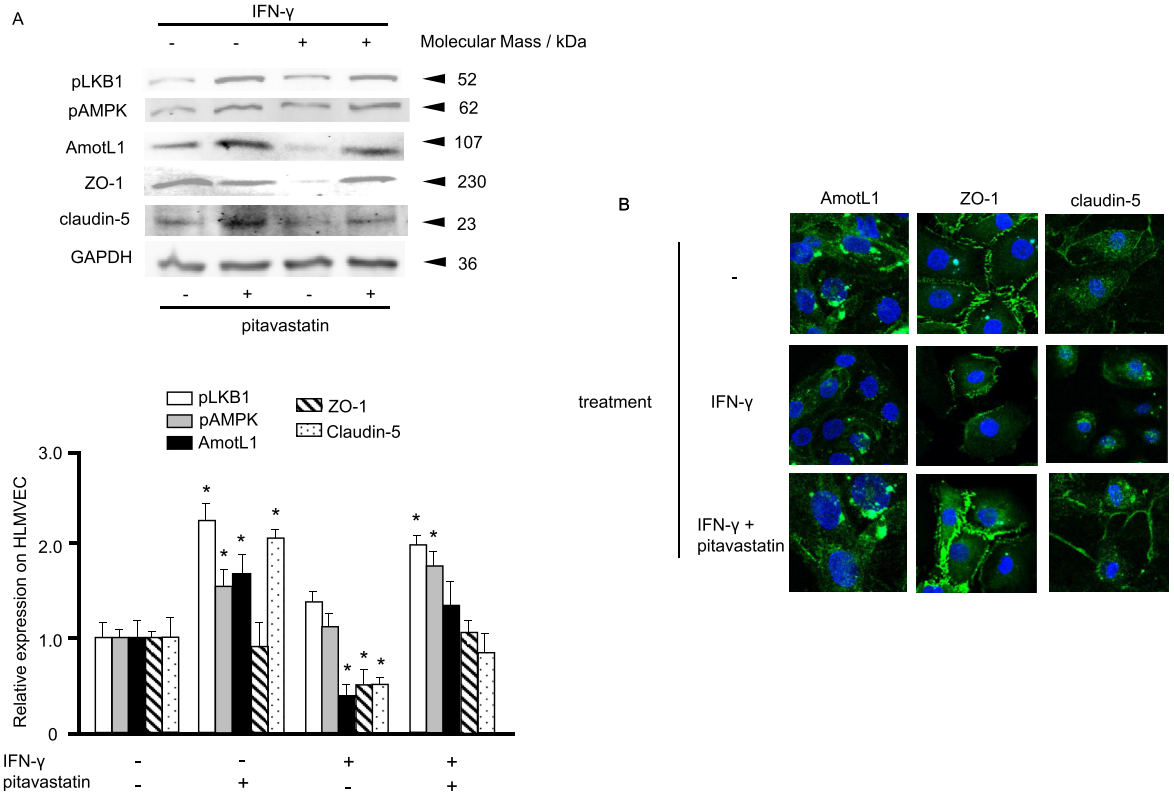

Fig. 5. Effects of IFN-γ on the pitavastatin-modulated expression of TJ proteins and the phosphorylation of signaling proteins in the HLMVECs, as well as the configuration of the TJ proteins in the HLMVECs. The HLMVECs were treated with IFN-γ in the presence of 100 nM pitavastatin for 24 h to examine the expression of AmotL1, ZO-1, and claudin-5, and for 1 h to examine pLKB1 and pAMPK. Cells were subjected to immunocytochemistry and harvested, and cell lysates were then analyzed with antibodies directed against pLKB1, pAMPK, AmotL1, ZO-1, claudin-5, and GAPDH. Pooled data and quantification of the immunoblot signals using infrared imaging for each antibody are shown. A representative experiment (upper panel) (A) was repeated five times and yielded equivalent results. The basal expression of non-IFN-γ- and pitavastatin-treated cells was set to 1.0. The whiskers indicate the standard errors of the mean. $^*P < 0.05$ compared with the means of the basal expression (lower panel) (A). The cells were also stained using immunofluorescence with antibodies against AmotL1, ZO-1, and claudin-5. A representative experiment was repeated five times and yielded equivalent results. IFN-γ-treated HLMVECs showed a decreased expression of the TJ proteins, and pretreatment with pitavastatin increased the expression of these proteins surrounding the HLMVECs. AmotL1 specifically localized around the nuclei of the HLMVECs (B).

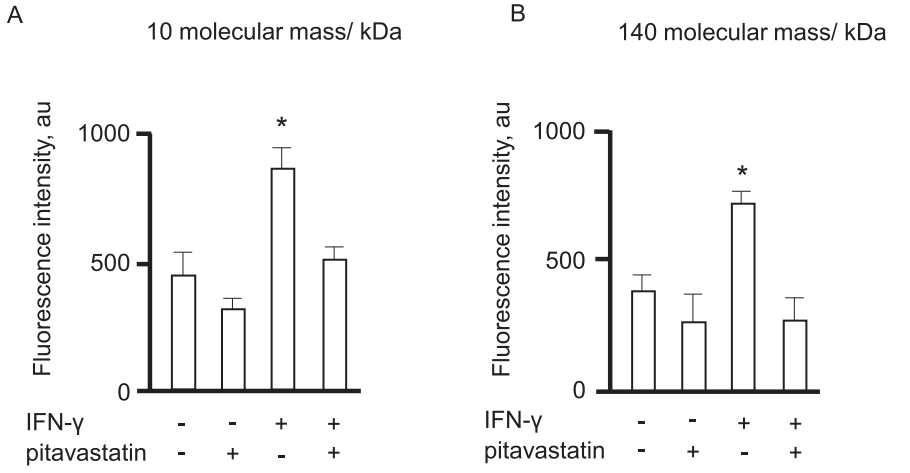

Fig. 6. Effects of IFN-γ and pitavastatin on the permeability of the HLMVECs. The HLMVECs were grown in transwell inserts and pretreated with pitavastatin. Pitavastatin prevented IFN-γ-induced enhanced permeability. FITC-conjugated dextran (10 molecular mass/kDa) (A) and F $(ab')_2$ (140 molecular mass/kDa) monolayer flux was measured 1 h after 100 ng/mL of IFN-γ stimulation. The whiskers indicate the standard errors of the mean. $^*P < 0.05$ compared with the means of the basal fluorescence intensity of non-IFN-γ- and pitavastatin-treated cells (B).

demonstrated that pitavastatin prevented decreased AmotL1 expression in the lungs of CS-exposed mice with emphysema. Second, we found that pitavastatin induced AmotL1 expression *via* LKB1/AMPK activation in HLMVECs. Third, we determined that IFN-γ decreased the expression of the TJ proteins AmotL1, ZO-1, and claudin-5, leading to increased hyperpermeability. Finally, pitavastatin suppressed IFN-γ-induced TJ disruption and consequent hyperpermeability in HLMVECs.

Since it is widely accepted that TJs play central roles in regulating the paracellular permeability of endothelial cells, the structure of endothelial TJs has been extensively examined [21–24], primarily for ZO-1 [25] and claudin-5 [26], which are relevant to myocardial ischemia and heart failure, respectively. AmotL1, a coiled coil, PDZ-binding and glutamine-rich domain-containing protein [27,28], has been characterized to function as a key regulator of endothelial cell migration and cell–cell TJ stability [13]. Previously, for the protein ZO-1, simvastatin-upregulated expression was specifically linked to the proper functioning of TJs and enhanced resistance to paracellular transport in endothelial monolayers *via* the suppression of RhoA/Rho-associated coiled coil–forming kinase (ROCK)1 signaling in glomerular endothelial cells in diabetic nephropathy [29]. In our present study, the LKB1/AMPK pathway was activated by pitavastatin to induce AmotL1 expression. Importantly, pitavastatin enhanced LKB1 and AMPK phosphorylation in endothelial cells; however, inhibition of LKB1 with pharmacological agents (LKB1 shRNA) abolished AMPK activation stimulated by pitavastatin. These results suggest that LKB1 is an upstream activator of AMPK, and that pitavastatin regulates the expression of AmotL1 by activating LKB1/AMPK signaling, resulting in an increase of NO bioavailability. In contrast, statin can inhibit the RhoA/ROCK1 pathway, which regulates blood flow indirectly through its negative effects on eNOS expression and activity [30]. ROCK has also been implicated in the expression of several genes pertinent to the process of leukocyte recruitment and vascular function, including monocyte chemoattractant protein-1, plasminogen activator inhibitor-1, and osteopontin [31]. In brief, a reduction in the leukocyte infiltration of the target organs is one of the most consistent outcomes of the effects of statin.

COPD exacerbations aggravate pulmonary emphysema and are often triggered by bacterial or viral infections. In particular, interactions between viruses, bacteria, and the host play a role in the pathogenesis of COPD. Moreover, the levels of type I cytokines, such as IFN-γ, are increased in COPD patients during virus-triggered exacerbations. Accordingly, $CD8^+$ lymphocyte infiltration is a prominent feature of the pathology of COPD, and the number of these cells correlates with tissue destruction and airflow limitation in this disorder [32–37]. Previous data have demonstrated that IFN-γ can cause COPD-relevant pulmonary structure remodeling through alterations in the protease/antiprotease balance [38]. Surprisingly, almost nothing is known about the mechanisms by which COPD inflammation causes TJ alterations. We demonstrated that IFN-γ-mediated changes in the TJ molecules of lung tissues could induce pulmonary emphysema. Cheng et al. studied patients with congestive heart failure secondary to non-ischemic disease, none of whom received statin therapy, and found increased serum levels of IFN-γ, which were derived from peripheral blood mononuclear cells when compared with controls [39]. However, the role of IFN-γ in the pathogenesis of cardiomyocyte function is limited and contradictory. Administration of atorvastatin at 10 mg/day for two weeks reduced serum IFN-γ levels, providing yet another potential mechanism through which statins are effective [39]. Additional investigation is required to define the conditions under which IFN-γ induces endothelial TJ protein suppression and how cardiovascular diseases affect coexisting COPD.

An increase in the albumin concentration in the sputum and airway epithelial lining fluid of COPD patients has been previously reported [40,41]. Moreover, increased microalbuminuria, plasma von Willebrand factor, and fibrinogen concentrations have been shown to be associated with COPD exacerbation [42]. These findings may suggest that airway microvascular permeability is increased in the airways of patients with COPD and disease exacerbation. In a murine model of acute lung injury, increased vascular permeability and disruption of vascular integrity occurred during the pathogenesis of lung injury, and statins conferred protection associated with TJ proteins, specifically claudin-5 [43]. Importantly, one of the vital mechanisms responsible for the increased permeability of endothelial cells could be damaging to endothelial TJs [44,45], which provide paracellular permeability to solutes and large molecules in the form of a structural barrier [46]. The expression of structural membrane proteins, such as ZO-1 [47], claudin-5 [48], and AmotL1, is connected to TJ permeability. NO signaling, which plays an important role in blood vasculature expansion [49], has been shown to be integral in regulating vascular permeability and the proliferation of vascular endothelial cells. Since IFN-γ has been demonstrated to induce NO expression in airways as a response to bacteria and bacterial products [50,51], increased vascular permeability might be, at least in part, an effect of NO in patients with COPD exacerbation. However, the effects of NO on TJ proteins and its regulation of vascular permeability in lung endothelial cells have not yet been fully elucidated. It is likely that patients with COPD, who were former or current smokers, incurred a dysfunction in their endothelial cells, thereby resulting in a lack of increased NO expression, which regulates vascular pumping. Indeed, increased permeability appears to be dependent on the dysfunction of TJ proteins. Clearly, these speculations need to be confirmed in future experimental studies.

4.1. Conclusions

The present study shows that AmotL1 is a key regulator of the collective activity of endothelial function related to angiogenesis and TJs. Thus, this protein could serve as a major target for the modification of endothelial cells in many immunological conditions. From a pharmaceutical perspective, statin-maintaining TJ proteins, such as AmotL1, may be a focus of intervention strategies for the treatment and prevention of COPD exacerbations.

Declaration of interest statement

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