Introduction

Based on the function of mammalian target of rapamycin (mTOR) signaling in physiological and pathological processes in the cell, pharmacological inhibitors are often used as disease interventions. Rapamycin and its analogues (such as everolimus and temsirolimus) selectively inhibit mTOR by binding to FK506-binding protein 12 (FKBP12) and have been widely used in clinical studies. In addition, a newer generation of selective ATP-competitive inhibitor of mTOR, dactolisib (NVP-BEZ235), has been approved for clinical cancer treatment. Over the past few years, adverse effects such as pneumonitis have been reported in most large clinical trials of everolimus or temsirolimus. However, the incidence and risk of pneumonitis has not been reported in dactolisib clinical trials and case reports. The exact mechanisms underlying these two generations of mTOR inhibitor-induced lung toxicities remain unclear.

Although a few studies have investigated the mechanism by which mTOR inhibitors lead to lung injury, the results are poorly defined and controversial. Our group has recently published a paper showing that rapamycin aggravated lipopolysaccharide (LPS)-induced pulmonary endothelial cell (EC) hyperpermeability and lung injury in mice. The endothelial cells play a critical role in maintaining the integrity of the lung capillary-alveolar barrier, which is critically

Impact Statement

Lung injury is a frequent side-effect of everolimus, a first-generation mTOR inhibitor used in clinical therapies. Endothelial cell barrier dysfunction plays a major role in the pathogenesis of the lung injury, but previous studies have ignored its biological function in mTOR inhibitors-induced lung injury. Therefore, we used mouse model with lung injury and endothelial cell monolayers hyperpermeability in vitro induced by everolimus as the research model and examined dasatinib alters pulmonary endothelial permeability in a Ca2+-dependent manner. We observed that everolimus promotes endothelial hyperpermeability through MLC phosphorylation-mediated EC contraction by PKCα and MLCK activation, which may be dependent on an endothelial intracellular [Ca2+] leakage. Our results elucidated a molecular mechanism that may contribute to everolimus-induced endothelial barrier dysfunction and help to ameliorate the management of mTOR inhibitor-associated side effects.

Abstract

The mammalian target of rapamycin (mTOR) inhibitors, everolimus (but not dactolisib), is frequently associated with lung injury in clinical therapies. However, the underlying mechanisms remain unclear. Endothelial cell barrier dysfunction plays a major role in the pathogenesis of the lung injury. This study hypothesizes that everolimus increases pulmonary endothelial permeability, which leads to lung injury. We tested the effects of everolimus on human pulmonary microvascular endothelial cell (HPMEC) permeability and a mouse model of intraperitoneal injection of everolimus was established to investigate the effect of everolimus on pulmonary vascular permeability. Our data showed that everolimus increased human pulmonary microvascular endothelial cell (HPMEC) permeability which was associated with MLC phosphorylation and F-actin stress fiber formation. Furthermore, everolimus induced an increasing concentration of intracellular calcium Ca2+ leakage in HPMECs and this was normalized with ryanodine pretreatment. In addition, ryanodine decreased everolimus-induced phosphorylation of PKCα and MLC, and barrier disruption in HPMECs. Consistent with in vitro data, everolimus treatment caused a visible lung-vascular barrier dysfunction, including an increase in protein in BALF and lung capillary-endothelial permeability, which was significantly attenuated by pretreatment with an inhibitor of PKCα, MLCK, and ryanodine. This study shows that everolimus induced pulmonary endothelial hyper-permeability, at least partly, in an MLC phosphorylation-mediated EC contraction which is influenced in a Ca2+-dependent manner and can lead to lung injury through mTOR-independent mechanisms.

Keywords: Endothelial cell, everolimus, permeability, lung injury, myosin light chain, Ca2+
dependent on the stabilization of the F-actin cytoskeleton and the assembly of cell junctions. Activation of F-actin fiber formation and cytoskeletal contractility represents an important mechanism underlying the induction of intercellular gap formation and endothelial hyperpermeability. One of the determinant events in endothelial cell contraction is the phosphorylation of myosin light chain (MLC) at Ser19 and/or Thr18, which is mainly regulated by MLC kinase (MLCK) and PKCα. Intercellular Ca$^{2+}$ plays an important regulatory role in the disruption of endothelial barrier function by activation of PKCα or MLCK. Previous data has shown that displacement of FKBP12/12.6 from ryanodine receptors 2 (RyR2) induces an intracellular Ca$^{2+}$ leak, leading to the activation of PKCα in sirolimus-treated human aortic endothelial cells (HAECs).

In this study, we hypothesized that everolimus induces an intracellular calcium release and the activation of PKCα and MLCK, and that this has consequences for barrier dysfunction in human pulmonary microvascular endothelial cells (HPMECs). To test this, we compared the effects of everolimus and dactolisib on lung endothelial permeability using a mouse model to identify an MLC-dependent mechanism that was associated with an intracellular Ca$^{2+}$ release.

**Materials and methods**

**Chemicals and reagents**

Antibodies against p-MLC (3674), MLC (8505), and PKCα (2056) were purchased from Cell Signaling Technology (Beverly, MA, USA). MLCK Antibody (ab232949) was obtained from Abcam (Waltham, MA, USA). FITC-Phalloidin (P5282) and Evans blue (EB) were obtained from Sigma Aldrich (Saint Louis, MO, USA). Everolimus (HY-10218), ryanodine (HY-103306), dactolisib (HY-50673), GF109203X (HY-13867), and ML-7 (HY-15417) were provided by Med Chem Express (Newark, NJ, USA). The Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA).

**Cell culture and treatment**

HPMECs (ScienCell Research Laboratories, USA) were cultured in extracellular matrix (ScienCell, America) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, USA), at 37°C and 5% CO$_2$ incubator. For all experiments, cells were grown to 80% confluence and stimulated with PBS, everolimus, or dactolisib, with or without inhibitors (GFI09203X, ML-7, Ryanodine).

**Western blotting**

Western blotting was performed as described previously. The treated HPMEC lysates were collected and subjected to SDS-PAGE and immunoblotting. After being blocked with 5% bovine serum albumin (BSA), the blots were incubated overnight at 4°C with primary antibodies. The immunoreactive bands were then detected using ECL reagent (Pierce, 34078) and analyzed with Image J software (BIO RAD, Hercules, CA, USA).
Experimental animals

C57BL/6J (B6) male mice were purchased from Nanchang University and approval was granted by the Animal Studies Ethics Committee of Pingxiang people’s Hospital. The mice (aged 6–8 weeks, weighing 20–25 g) were randomly assigned into six groups: (1) control group (PBS), (2) everolimus, (3) dactolisib, (4) everolimus + Ryanodine, (5) everolimus + GF109203X (GF), (6) everolimus + ML-7. Groups 1–3 were treated daily with a single intraperitoneal (i.p.) injection of PBS, everolimus at 1 mg/kg/day, or dactolisib at 1 mg/kg/day, respectively, for 8 weeks. Based on the findings of previous studies, the inhibitor groups were pre-administered with GF (1 mg/kg/day), ML-7 (1 mg/kg/day), or ryanodine (2 mg/kg/day) for 2 days per week, 1 day prior to intraperitoneal injection with everolimus.

Detection of protein in bronchoalveolar lavage fluid (BALF)

All mice were anesthetized with isoflurane. After anesthesia, BALF was obtained, as per our previous description, and 2.0 mL of BALF was centrifuged at 3000 RPM for 10 min at 4°C. The concentration of total protein in the BALF was measured using BCA assay kits (Beyotime, Shanghai, China).

Evaluation of lung permeability

EB (5 mg/mL, dissolved in PBS) was injected intravenously into the tail vein, 30 min before exsanguination. The left lung was collected and weighed. Then, 0.5 mL of formamide (Sigma-Aldrich) was added and incubated at 60°C for 24 h. The intensity of EB in the lung tissue was determined by detecting absorbance at wavelength 610 nm. The extravasated EB concentration in the lung tissue was calculated against a standard curve (micrograms of EB dye per lung).

Statistical analysis

All data were analyzed by GraphPad Prism v6.0 software and SPSS statistics 17.0 (IBM Inc., Chicago, IL). The mean values of the groups were expressed as mean ± SD and were compared using one-way ANOVA followed by post hoc analysis. Results were considered statistically significant at p < 0.05.

Results

Everolimus induced lung endothelial hyperpermeability

Two generations of mTOR inhibitors, everolimus and dactolisib, were used in this study. TEER and the permeability of albumin (Pa) of the HPMEC monolayer were measured to evaluate the endothelial barrier function. As shown in Figure 1(A) and (B), 24 h treatment with 10 nM and 100 nM everolimus induced a decrease in TEER values (Figure 1(A)). Consistent with the TEER values, 10 nM, and 100 nM everolimus lead to an increase in Pa by 60% and 80%, respectively (Figure 1(B)). In contrast, dactolisib had no effect on the TEER and Pa values in the endothelial cells (Figure 1(A) and (B)). These data demonstrated that everolimus exhibits a dose-dependent hyperpermeability in HPMEC monolayers, while dactolisib does not.

Everolimus induced F-actin fiber formation and MLC phosphorylation in endothelial cells

Formation of actin stress fibers, which was mainly generated by MLC phosphorylation, was closely linked to increased endothelial permeability. An immunofluorescent assay, with FITC-phalloidin to label F-actin, was used to detect the formation of stress fibers and paracellular gaps in everolimus or dactolisib-treated HPMECs. Consistent with the results in
permeability detection, everolimus induced alterations in the distribution of F-actin stress fibers and lead to gap formation in the HPMECs (Figure 2(A) and (B)). In addition, we also observed a slight increase in MLC phosphorylation in everolimus-treated cells (Figure 2(C) and (D)). In contrast, dactolisib had no effect on the MLC phosphorylation, morphological changes, and gap formation on the monolayer of HPMECs (Figure 2(A) to (D)). These results suggest that everolimus- induced phosphorylation of MLC and F-actin formation are accompanied with increased cell contraction in HPMECs.

PKCα and MLCK are required for everolimus- induced MLC phosphorylation

PKCα and MLCK are the main regulators of MLC phosphorylation and have been reported to mediate cytoskeletal contraction regulation of endothelial permeability.12,20 Therefore, specific pharmacological inhibitors (ML-7 and GF109203X, GF) were used to examine the involvement of MLCK and PKCα in everolimus-induced endothelial hyperpermeability. As shown in Figure 3, everolimus-induced MLC phosphorylation was attenuated by GF (5 mM) and ML-7 (10 mM) in HPMECs (Figure 3(C) and (G)). Pretreatment with GF and ML-7 consistently abolished EC hyperpermeability responses to everolimus, which was evidenced by an increase in TEER (Figure 3(A)) and a decrease in the permeability coefficient for Pa (Figure 3(B)).

Moreover, we utilized siRNAs to reduce PKCα and MLCK expression in the everolimus- treated HPMECs. In accordance with the effects of pharmacological inhibition, knockdown of PKCα and MLCK significantly abolished everolimus-induced MLC phosphorylation (Figure 3(F)) and hyperpermeability (Figure 3(D) and (E)). These results indicate that both MLCK and PKCα contribute to MLC phosphorylation and EC contraction, which resulted in hyperpermeability in everolimus-induced EC.

Everolimus impairs endothelial cell barrier function via an increase in Ca2+ release

Intracellular calcium (Ca2+) binding to the CaM-binding domain of MLCK and PKCα resulted in activation of PKCα and MLCK.13,22,23 Previous studies have shown that everolimus can lead to intracellular calcium Ca2+ release via displacement of FKBP12.6 from the ryanodine receptor 2 (RyR2). We examined the intracellular (Ca2+) concentration in the HPMEC monolayers after treatment with everolimus or dactolisib. Compared with the control group, we observed a significant increase in intracellular calcium Ca2+ release after everolimus treatment for up to 24 h (Figure 4(A)). Contrastingly, dactolisib did not effect on Ca2+ release (Figure 4(A)). Moreover, pretreatment with ryanodine (a stabilizer of RyR2 channels) ameliorated the everolimus-induced increase in intracellular Ca2+ levels (Figure 4(B)) and cell barrier dysfunction, which was evidenced by a reduced the passage of

Figure 2. Everolimus induced endothelial cell F-actin fiber formation and MLC phosphorylation. (A) F-actin stress fiber formation was assessed via FITC–phalloidin staining, followed by the incubation of human pulmonary microvascular endothelial cells (HPMECs) with 100 nM everolimus or dactolisib for 24h. DAPI was used to stain the nuclei. Representative images are shown. Scale bar: 30 μm. (B) The quantification of the intercellular gap area in each group; n=8. (C and D) The levels of myosin light chain (MLC) phosphorylation were analyzed using western blotting. *p < 0.05; **p < 0.01; ***p < 0.001.
BSA-leakage (Figure 4(C)), and the alleviated drop of TEER (Figure 4(D)) in HPMECs. Moreover, the everolimus-induced phosphorylation of MLC was attenuated in the ryanodine pretreatment group (Figure 4(E) and (F)). Collectively, these data suggest that the disruption of calcium release through the RYR2 channel by everolimus induced an intracellular Ca\(^{2+}\) leakage and enhanced phosphorylation of PKC\(\alpha\) and MLC, resulting in an impaired lung endothelial barrier function.

**Everolimus increases lung capillary-alveolar permeability in vivo**

To determine whether the toxicity effects of 2 generations of mTOR inhibitors (everolimus and dactolisib) on the lung injury, we treated the male mice with PBS, everolimus, or dactolisib as per the previously described methods. Capillary-alveolar barrier injuries were attributable to lung injury. We examined the concentration of total protein in BALF and the amount of EB in the lungs to assess the lung vascular permeability. As shown Figure 5, we found that everolimus (EVE) significantly increased the amount of EB in the lungs (Figure 5(A)), and the total protein in BALF (Figure 5(B)) was increased compared to both the control and dactolisib groups. Compared to everolimus treatment alone, pretreatment with GF, ML-7, and ryanodine dramatically decreased the leakage of protein in BALF, as well as the accumulation of EB in the lungs, indicating an improved barrier function (Figure 5(A) and (B)). These data indicate that everolimus induces pulmonary vascular hyperpermeability and lung injury through an endothelial barrier dysfunction-dependent mechanism.
Figure 4. Everolimus impairs endothelial cell barrier function via an increase in Ca\(^{2+}\) release, whereas dactolisib does not. (A) Human pulmonary microvascular endothelial cells (HRGECs) were treated with 10 and 100 nM everolimus or dactolisib for 24 h and intracellular Ca\(^{2+}\) leak levels were measured via the fluorescent dye Fluo-3 AM (n = 8). (B) Two-hour pretreatment with the ryanodine receptor 2 channel (RYR2) stabilizer ryanodine (10 mM) blocked the EC intracellular calcium leak Ca\(^{2+}\), myosin light chain (MLC) phosphorylation (E and F), and hyperpermeability response (C and D) elicited by everolimus for 22 h (n = 8). *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 5. Chronic treatment with everolimus increases lung capillary-alveolar permeability in vivo. Mice (male) were treated with daily i.p. injections of vehicle (PBS), everolimus (EVE) at 1 mg/kg/day, or dactolisib at 1 mg/kg/day, for 8 weeks. In the inhibitor intervention in vivo experiments, mice were pretreated with GF, ML-7, and ryanodine, as described in Methods. (A) Quantification of vascular permeability, assessed by Evans blue accumulation in the lung tissue, and (B) total protein in BALF, measured by BCA protein assay kit. Data presented as mean ± SEM (n = 8). *p < 0.05; **p < 0.01; ***p < 0.001.
Discussion

While mTOR inhibitors are an effective method of systemic treatment for tumor and organ transplantations, their side effects, such as causing lung injury, have drawn extensive concerns. However, the underlying molecular mechanisms of everolimus on pulmonary toxicity remains largely unclear. Herein, we reported a novel role for everolimus in inducing endothelial cell barrier dysfunction and lung injury by promoting MLC phosphorylation and EC permeability, which was strongly dependent on the activation of PKCα and MLCK. Furthermore, we showed that the harmful effects of everolimus appeared to be mediated by destabilization of the intracellular Ca²⁺ release channel RyR2, which altered intracellular [Ca²⁺] stores. Moreover, we established the first animal model of everolimus-related lung injury, by treating mice with a daily regimen of everolimus. However, there was no damage of barrier function in the dactolisib-treated pulmonary endothelial cells and mice. Based on these data, we demonstrated that everolimus has the potential to induce endothelial barrier dysfunction, which can induce pulmonary vascular hyperpermeability and lung injury.

Previous studies have shown that lung injury is a frequent side-effect of mTOR inhibitors, such as everolimus and temsirolimus, that are used in clinical treatments. In vivo studies have shown that rapamycin augments lung injury induced by cigarette smoke in mice, and similar result was observed in temsirolimus-treated mice. Other studies have also demonstrated that rapamycin aggravates LPS-induced lung apoptosis by suppression of mTOR activation in mice. Clinically, serum Krebs von den Lugen, a biomarker of lung capillary-alveolar permeability injury, is elevated in patients with everolimus-induced lung disease. Consistent with these studies, our findings demonstrate that mice with a daily regimen of everolimus treatment have an altered endothelial barrier integrity, which results in increasing pulmonary vascular endothelial permeability and lung injury.

Pulmonary endothelial barrier dysfunction and plasma protein infiltration into the pulmonary alveoli occurs in the initial stages of the pathogenesis of pneumonitis. The hyperpermeability of pulmonary vasculature may lead to the leakage of plasma proteins within the alveolar lumen, which is a sign of pulmonary vascular endothelial injury. Previous studies have showed that rapamycin enhances LPS-induced pulmonary edema, an indication of disruption to the capillary-alveolar barrier. Furthermore, it has been reported that temsirolimus-induced lung injury can be induced following capillary-alveolar barrier injury. Consistent with previous studies, we highlighted a possible connection between everolimus-induced endothelial hyperpermeability and the development of lung injury through finding an increase in the concentrations of protein in BALF and EB in the lungs in mice treated with everolimus. This showed that there was detectable damage in the capillary-alveolar barrier. Furthermore, we performed in vitro studies using HPMECs and found that everolimus increased endothelial permeability by highlighting the alteration of TEER and Pa values. Contrastingly, dactolisib did not demonstrate this effect. Based on these data, our study demonstrated that everolimus has the potential to cause pulmonary endothelial injury, which could result in lung injury.

The critical role for MLC phosphorylation and F-actin stress fiber formation in EC contraction and barrier dysfunction has been well documented. Our data demonstrated that everolimus was associated with an increase in F-actin formation and endothelial hyperpermeability, whereas dactolisib did not demonstrate this effect. This was supported by a recent study which showed that dactolisib had no effect on HPMEC viability and LPS-induced lung injury in mice. Previous studies demonstrated that an upregulation in the activity of MLCK and PKCα resulted in increased MLC phosphorylation, leading to disruption of the vascular barrier integrity in a murine model of LPS-induced lung injury. The in vitro data showed that the increased activity in the MLCK/MLC pathway lead to paracellular gap formation and endothelial cell barrier disruption. Previous reports have shown that rapamycin induced PKCα phosphorylation in HUVECs. In this study, we noted that PKCα plays an essential role in everolimus-induced barrier disruption, through enhanced MLC phosphorylation and actin stress fiber formation. Furthermore, we also found that everolimus-induced MLC phosphorylation was dependent on the activation of both MLCK and PKCα, which was confirmed by the pretreatment inhibition or knockdown of MLCK or PKCα. Pretreatment with the inhibitor anti-MLCK and -PKCα in vivo prevents the everolimus-induced endothelial hyperpermeability and the development of lung injury. These results indicate that both PKCα and MLCK are responsible for the phosphorylation of MLC and the endothelial barrier dysfunction induced by everolimus.

Everolimus inhibits mTOR through binding to FKBP12, a key stabilizing component of RyR2 calcium release channels in various cell types. Dactolisib, which also named NVP-BEZ235, does not show this effect. Previous reports have shown that rapamycin induced Ca²⁺ release via RyR2 in endothelial cells. Studies have also demonstrated that low concentrations of Ca²⁺ induced PKCα and MLCK activation, which lead to phosphorylation of MLC. Herein, we found a different toxicity effect on HPMECs exposed to everolimus and dactolisib, which may have been associated with the Ca²⁺ leakage from the ryanodine receptors. Our data showed that everolimus induced an intracellular Ca²⁺ release in HPMECs, which was blocked by ryanodine. However, dactolisib, a selective ATP-competitive inhibitor of the mTOR that does not bind FKBP12/12.6, did not affect endothelial barrier function and intracellular Ca²⁺ leakage. Furthermore, we found that inhibition of RyR2 by ryanodine not only blocked intracellular Ca²⁺ leakage but also attenuated MLC phosphorylation and hyperpermeability in the everolimus-treated HPMECs. In addition, similar results were seen in vivo. Collectively, these results suggest that everolimus-induced leakage of calcium from RyR2
may enhance MLC phosphorylation, leading to impaired endothelial barrier function through mTOR-independent mechanisms.

Conclusions

In conclusion, our results demonstrate that everolimus promotes endothelial hyperpermeability, at least partly through MLC phosphorylation-mediated EC contraction by PKCu and MLCK activation, which may be dependent on an endothelial intracellular [Ca^{2+}] leakage. The effect was also observed in vivo, as mice treated with everolimus displayed pulmonary vascular leakage and lung injury. This study elucidated a molecular mechanism that may contribute to everolimus-induced endothelial barrier dysfunction and help to ameliorate the management of mTOR inhibitor-associated side effects.

AUTHORS’ CONTRIBUTIONS

All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript. Specific contribution; CJH and LXF designed and performed the experiments; Funding acquisition; LSH contributed reagents/materials/analysis tools; CXL designed the experiments and wrote the paper. All authors read and approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

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