Protective Effects of Sphingosine 1-phosphate and FTY720 on Rat Pulmonary Microvascular Endothelial Cells Stimulated by TNF_{α}*

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Abstract: Objective In this study, the effects of sphingosine 1-phosphate (S1P) and its analogue FTY720 on lactate dehydrogenase (LDH), prostacyclin and PGE_2 release, monolayer paracellular permeability, and F-actin structural changes of cultured rat pulmonary microvascular endothelial cells stimulated by TNF_{α} were examined. **Methods** Rat pulmonary microvascular endothelial cells(rPMECs) were isolated, cultured and identified by immunofluorescent stain of von Willebrand-associatedan-antigen (vWF). Incubation with TNF_{α} at a dose of 150 ng/ml for 12 hours induced a significant increase in paracellular permeability determined by measurement of a paracellular solute flux, 3-kDa FD-3. **Results** PGE_2, $PGF_{2\alpha}$ and LDH productions by cell cultures exposed to TNF_{α} for 12 hours increased significantly compared with control cells. Also, the 12-hour exposure to TNF_{α} resulted in reorganization and retraction of the rat pulmonary microvascular endothelial cells that was visible both by phase contrast microscopy and cytoskeletal F-actin stain. Paracellular permeability of the monolayers, PGE_2, $PGF_{2\alpha}$ and LDH productions were significantly reduced following pretreatment with S1P or FTY-720. Moreover, S1P or FTY-720 pretreatment prevented the changes of morphologic and cytoskeletal F-actin induced by TNF_{α}. **Conclusion** S1P and FTY-720 can inhibited the LDH, $PGF_{2\alpha}$ and PGE_2 release by strengthening the cytoskeleton and protecting the barrier function, and may represent a novel therapeutic method for pulmonary vascular barrier damage.

Key words: pulmonary microvascular endothelial cell; TNF_{α}; sphingosine 1-phosphate; FTY720; endothelial barrier; F-actin

INTRODUCTION

The pulmonary microvascular endothelial cell is a primary target of inflammatory cytokines during acute pulmonary injury (ALI). Responses of endothelial cells to TNF_{α} include up-regulation of adhesion molecules such as ICAM-1, cytoskeletal changes, and permeability increases[1-2]. It is reported that TNF_{α} can induce signaling events in lung endothelial cells (ECs), resulting in cytoskeletal changes and increases in EC permeability[3]. Also, it has been demonstrated that TNF_{α} induced an increase in permeability in vivo, in the isolated lungs and in pulmonary microvascular ECs in vitro[4-5].

Sphingosine 1-phosphate (S1P), a biological active lipid, has been proved to be a potent barrier-enhancing agent through Edg receptor ligation and Rac GTPase-dependent cortical actin rearrangement[6-7]. Schaphorst, et al were the first to demonstrate that S1P can enhance pulmonary vascular barrier function in vitro. Through signaling

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initiated by ligation of G-protein coupled endothelial cell surface receptors, S1P induces cortical rearrangement of the cytoskeleton, stabilizing and enhancing intercellular and cell-matrix adherence[8]. They also demonstrated attenuation of inflammatory lung injury induced by intratraceal LPS in spontaneously ventilating C57BL/6 mice treated with parenteral administration of S1P or its structural analog FTY720[9-13].

In the present study, we examined the effects of S1P and its structural analogue FTY720 on the paracellular permeability of the monolayers, lactate dehydrogenase (LDH), PGE_2 and $PGF_{2α}$ release, and F-actin structural changes of cultured rat pulmonary microvascular endothelial cells stimulated by $TNF_α$. We concluded that S1P and FTY-720 have the protective effects on rPMECs by strengthening the cortical actin rearrangement, protecting the barrier function, and may represent a novel therapeutic method for pulmonary vascular barrier damage.

MATERIALS AND METHODS

1. Isolation and Culture of Rat Pulmonary Microvascular Endothelial Cells

Primary rat pulmonary microvascular endothelial cells cultures were prepared from male Sprague-Dawley rats weighing 65 g to 85g, using a modification of the technique described previously by Magee, et al[14]. All rats were sacrificed by cervical dislocation. The fresh lungs isolated from the sacrificed rats were washed 3 times with 50ml basal medium. The outer edges of the lung lobes, which did not contain large blood vessels, were cut off (by this procedure, the majority of larger vessel types can be excluded) and finely minced in basal medium using iris scissors. The minced lung tissues were suspended in 50 ml of basal medium and centrifuged at 250 g for two minutes and the fragments were collected on 40-nylon mesh. With constant gentle mechanical agitation, the tissue was digested with 0.6% collagenase in M199 at 37°C for 20 min and then incubated with 2.1% dispase in M199 at 37°C for 30 min. The suspension was mixed in M199 with 5% FBS. After centrifugation at 600 g for 10 min, the resulting tissue pellet was resuspended in M199 and filtered through 100-mesh. The microvessels were collected by centrifugation at 600 g for 10 min and resuspended in M199. The suspension was layered on a Percoll gradient formed by centrifugation of 50% Percoll at 26,000 g at 4°C for 60 min and was then centrifuged at 600 g for 10 min. After Percoll gradient centrifugation, three layers were observed. The endothelial cell aggregates formed a band around the middle third of the gradient, and the entire middle layer was collected from gradients. The cells were resuspended in M199 and collected by centrifugation at 600 g for 10 min. The cell suspensions were seeded onto collagencoated 225 cm^2 tissue culture flasks. Cells were allowed to attach and grow to monolayers at 37°C in a humidified atmosphere of 5% CO_2-95% air. The culture medium (DMEM/F-12 containing 14 mM sodium bicarbonate, 20 ng/ml of EGF, 50g/ml of gentamicin- amphotericin B solution, 10 U/ml of heparin, 5% FBS, and 5% HS) was changed every 3 days. Subculture was performed when the cells reached confluence (after 6-7 days). Cells were trypsinized at a 1:3 ratio after reaching confluence using 0.025% trypsin in Hanks' balanced salt solution containing 0.02% EDTA. Secondary subcultured cells were grown on collagen-coated 25 cm^2 tissue culture flasks.

2. Identification of The Endothelial Origin of Isolated Cells

Endothelial cells in culture were identified by their characteristic cellular morphology and the presence of von Willebrand-associated antigen (vWF). Briefly, cells grown on culture plates were routinely rinsed twice with PBS and fixed with 10% formalin for 25 min. Then permeabilized with 0.2% Triton X-100 in PBS for 25 min and washed three times with PBS. Incubation with antibody to vWF (H-300, Santa Cruz) diluted 1:200 with blocking solution was performed for 3 hours at 37℃ and then incubated with a secondary fluorescent antibody, goat

anti-rabbit IgG-FITC (sc-2021, Santa Cruz) for 40 min at room temperature. After washing with PBS the cells were mounted in 50% glycerol buffer and were observed under a fluorescence microscope Leica.

3. TNFα, S1P and FTY720 Treatment and Paracellular Permeability Assays

Cells were routinely passaged when they reached 80% confluence. Briefly, cells from each culture plate were detached with 0.1% trypsin+0.02%EDTA, resuspended in fresh culture medium supplemented with 15% (vol/vol) fetal bovine serum, 100 U/mL of penicillin, 10 μg/mL of streptomycin and were plated at 5×10^6 cells on 24 mm Transwell filters (Costar, MA). The transepithelial electrical resistance (TER) of cells grown on filters was measured with an epithelial voltohmmeter (World Precision Instruments). Cells were used only if their TER was >1000 $\Omega.cm^2$. Cells with stable TER readings>1000 $\Omega.cm^2$ were pretreated 60 min with culture medium alone or culture medium containing S1P (100 nM) or FTY720 (100 μmol/L) basolaterally. Following the pretreatment period, TNF_α was added basolaterally to monolayers to give a final concentration of 150 ng/mL and the cells were then incubated at 37℃ for 12 hours and then basolateral samples (200 μl) of the culture medium were collected. Then, the monolayers were washed in HBSS/10Mm HEPES (HBSS+) and equilibrated at 37℃ for 10min on an orbital shaker. Monolayers were loaded apically with 1mg/ml FD-3 (Molecular Probes, OR). Basolateral samples were taken at 0 and 120 min, and fluorescence intensity was analyzed on a fluorescent plate reader(Fluoroskan ascent FL, Fhermo Electron Corporation). FD-3 concentrations transported into the basolateral compartment were extrapolated from a standard curve and expressed as $\mu m.cm^{-2}.h^{-1}$ FD-3 transported.

4. F-actin Visualization by Immunofluorescent Microscopy

Fluorescence analysis was applied for F-actin visualization and to study organization of microfilaments. Treated and control cells growing on 6-well plates were rinsed with ice-cold PBS buffer, and fixed with 4% formaldehyde in 0.01 mol/L PBS for 20 min at room temperature and then permeabilized with 0.2% Triton X-100 in PBS for 25 min. F-actin microfilaments were visualized with TRITC-conjugated phalloidin (Sigma, USA) staining for 30 min. After washing with PBS the cells were mounted in 50% glycerol buffer and were observed under a fluorescence microscope Leica.

5. Prostaglandin and LDH Measurements

The media were collected and centrifuged at 12,000 g for 2min at 4℃ to pellet any particulates. The supernatants were collected and kept at -70℃ for PGE_2, $PGF_{2\alpha}$ and LDH examination. The concentrations of PGE_2 and $PGF_{2\alpha}$ were determined using enzyme-linked immunoassay kits (Cayman) according to the manufacture's instruction. LDH release was determined using a cytotoxicity detection LDH kit (Nanjing Jiancheng Biological Product, China) according to the manufacturer instructions. In brief, 25 μL of culture supernatants were mixed with 75 μL of the LDH substrate mixture in a 96-well plate. After incubation for 1 h at room temperature, the reaction was stopped by adding 100 μL of 1 M HCl, and the absorbance was read at 570 nm. Values of LDH level were expressed as units per liter. The cell pellet were rinsed twice with cold PBS, harvested in a hypotonic buffer (50mM Tris, 0.1mM EGTA, pH 7.4), and incubated for 5 min on ice. Total cellular protein of cell lysates was determined by the Bradford method and results of PGE_2, $PGF_{2\alpha}$ were normalized by the cellular protein content.

6. Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis was carried out using SPSS.11.0 software. Student's test was used to analyze the differences between two groups. When comparisons between multiple groups were carried out, one-way analysis of variance was employed. Statistical significance was considered at $p<0.05$.

RESULTS

1. Characterization of Pulmonary Microvascular Endothelial Cells

As shown in Fig. 1A, Fig. 1B, von Willebrand-associated antigen fluorescent stain, the characteristics of endothelial origin cells were positive in average 92% cells of high power field under fluorescence microscope. These can demonstrate that the purity of rat pulmonary microvascular endothelial cells is high. Fig. 1A is the same high power field as Fig. 1B, but is under the light microscope.

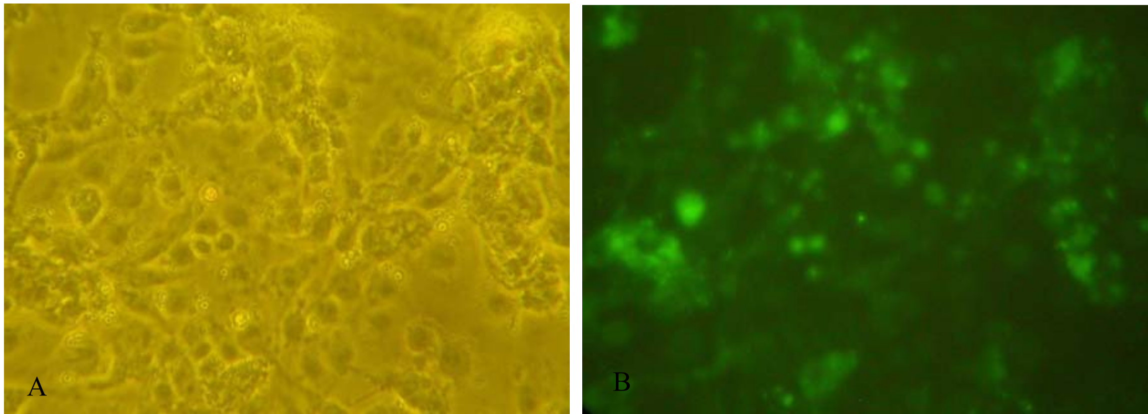
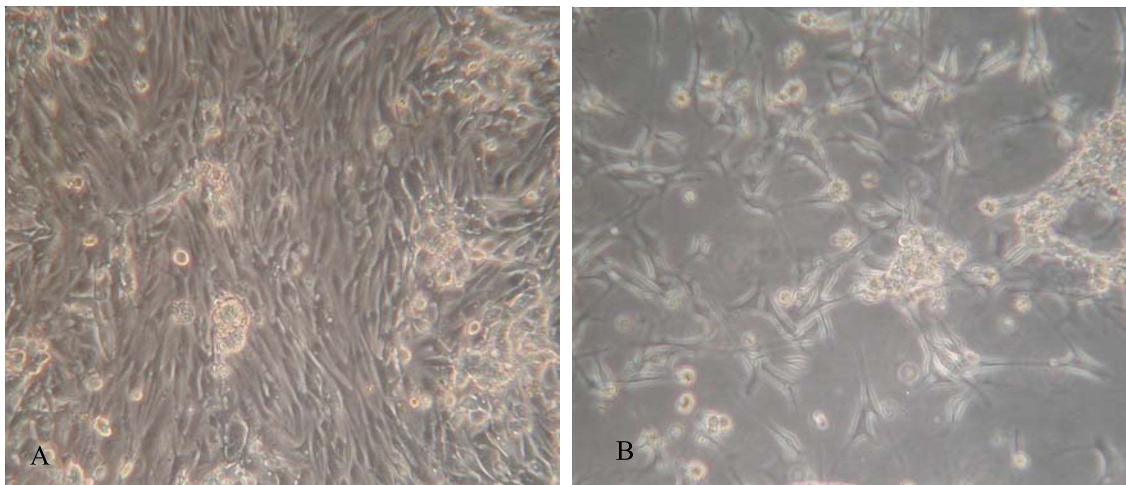


Fig. 1 Fluorescent positive staining for the von Willebrand factor antigen specific for endothelial cells (x100)

Notes: A. The same high power field as B, but is under the light contrast microscope;
B. The vWf fluorescent staining is almost positive in 92% cells under fluorescence microscope.

2. Cell Morphological Changes

The general morphological characteristics of the control cells are "cobblestone-like" appearance (Fig. 2A). After a 12 h of exposure to TNF_{α}(150 ng/mL), changes in morphology of the cells were apparent. Generally, the cells retracted towards the nucleus, shrank, forming a triangular cell body. These processes formed extensively and some of the cell bodies became refractile. At the same time, many cells fell off and died (Fig. 2B). In S1P or FTY720 pretreatedt cells, the morphological changes are mild, the cellular morphology is nearly normal and no died cells can be observed (Fig. 2C, Fig. 2D).



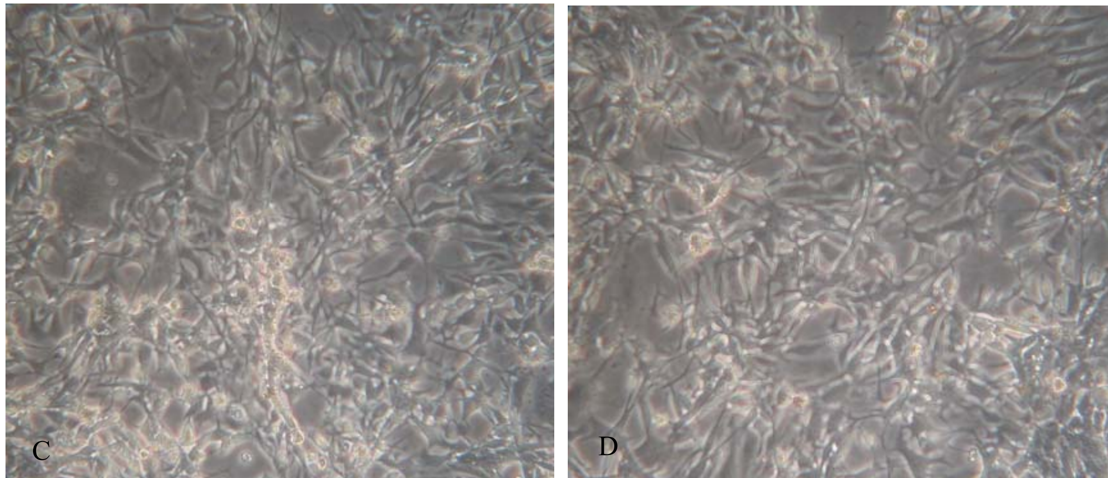


Fig. 2 The morphological photographs of pulmonary microvascular endothelial cells(x40)

Notes: A. rPMECscultured in the medium without TNF_α, S1P or FTY720 (control group); B. rPMECs treated with TNF_α 150 ng/mL in the medium (TNF_α-treated group); C. rPMECs pretreated with S1P cultured in the medium withTNF_α 150 ng/mL (S1P group); D. rPMECs pretreated with FTY720 cultured in the medium with TNF_α 150 ng/mL (FTY720 group). The time of TNF_α treatment was 12 h.

3. F-actin Cytoskeletal Changes in rPMECs

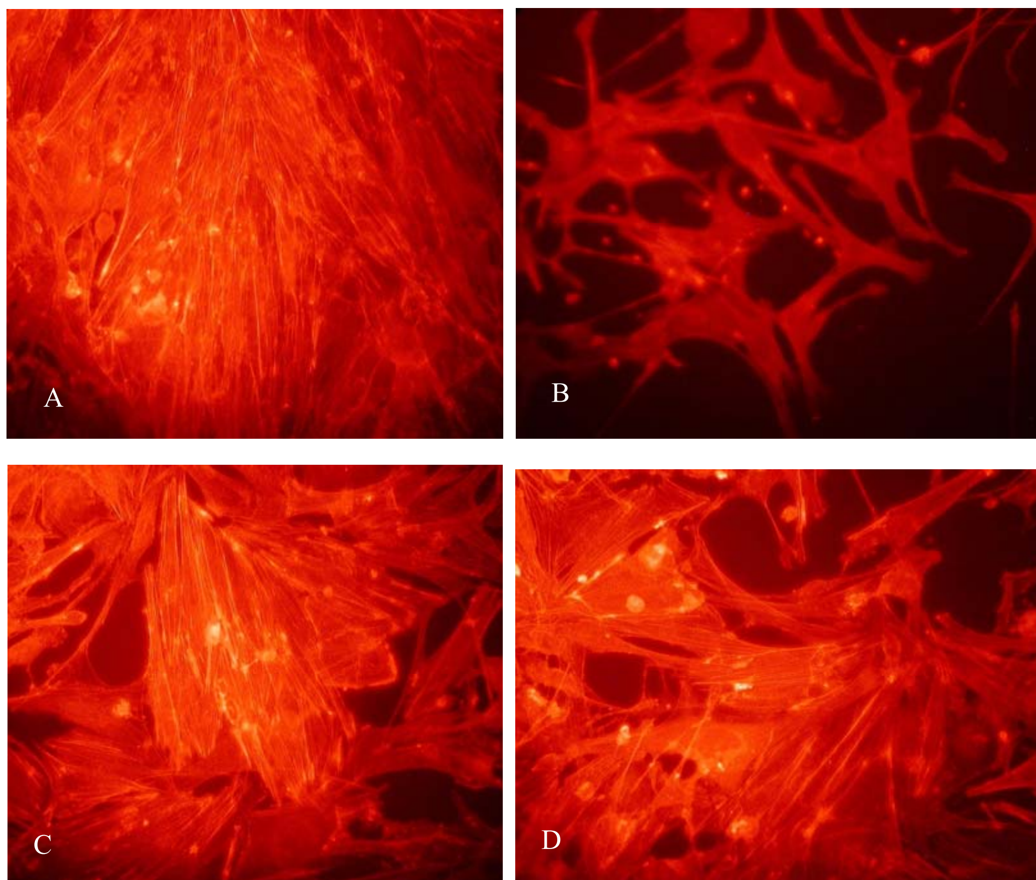


Fig. 3 F-actin staining of rPMECs monolayers using TRITC-phallalloidin treated with medium alone (control), TNF_α (150ng/ml), and S1P(100nM) + TNF_α or FTY720(100 μmol/L)+TNF_α for 12 hours. Images were captured under x400 magnification A. Control group; B. TNF_α treated group; C. S1P pretreated group; D. FTY720 pretreated group

It is known that TNF_{α} can cause changes in the cytoskeleton that alter the permeability of the endothelial barrier. Using TRITC-conjugated phalloidin, which binds to filamentous actin, we assessed the distribution of actin in control, TNF_{α} treated and S1P or FTY720 pretreated cells. The results indicate that after 12 hour exposure to TNF_{α}, actin had reorganized into prominent stress fibers, typically arranged in a parallel pattern along the longitudinal axis of the cell. The cells retracted and shrank leaving intercellular gaps. In S1P or FTY720 pretreated cells, the actin distribution changes are mild, and the intercellular gaps nearly absence (Fig. 3).

4. Paracellular Permeability Assays

Since it has been reported that TNF_{α} has demonstrated effluence on endothelial barrier function in diverse endothelial cell types[15-18], we analyzed its effects on the rPMECs. Confluent rPMECs grown on permeable supports were incubated with TNF_{α} and /or with S1P or FTY720 and paracellular permeability was determined by measurement of a paracellular solute flux, 3-kDa FD-3 as described previously[19]. This method is regarded as a sensitive parameter reflecting the paracellular permeability. In control monolayers, the flux of FD-3 was maintained at a very low value ($6.8\pm1.3\mu M.cm^{-2}.h^{-1}$). Basolateral incubation with TNF_{α} for 12 h induced a significant increase in paracellular flux of FD-3. The S1P or FTY720 pretreatment prevented the increases of paracellular flux of FD-3 remarkably compared with TNF_{α} treated cells (Fig. 4).

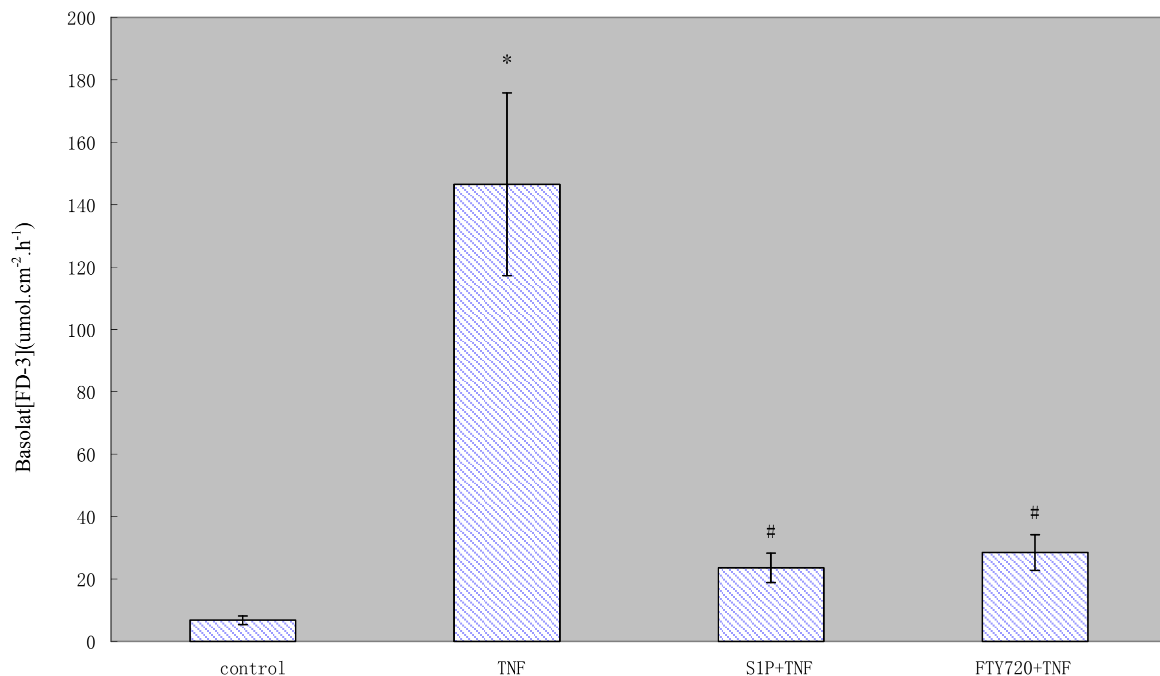


Fig. 4 Measurement of paracellular flux of FD-3 in rPMEC monolayers

Note: **P*<0.001, TNF_{α} treated vs control cells; #*P*<0.001, S1P or FTY-720 pretreated vs TNF_{α} treated cells.

5. Measurements of Prostaglandin and LDH

As shown in Table 1, exposure to TNF_{α} for 12 h induced a significant increase in PGE_2, $PGF_{2\alpha}$ and LDH release, the latter reflecting the cytotoxicity of TNF_{α} on rPMECs. The PGE_2, $PGF_{2\alpha}$ and LDH release in S1P or FTY720 pretreatment cells were reduced significantly compared with TNF_{α} treated cells.

Table 1 The concentrations of PGE_2, $PGF_{2\alpha}$ and LDH in rPMEC monolayers ($\bar{x}$ ±*SEM)*

	n	PGE_2(pg/μg protein)	$PGF_{2\alpha}$(pg/μg protein)	LDH(U/L)
Control	8	5.6 ± 0.9	1.5 ± 0.3	10.5 ± 2.3
TNF_{α}	8	22.3 ± 5.4*	9.7 ± 1.5*	85.6 ± 10.2*
S1P+TNF_{α}	8	10.5 ± 1.8$^{\#}$	3.2 ± 0.5$^{\#}$	19.3 ± 3.6$^{\#}$
FTY-720+TNF_{α}	8	11.8 ± 2.7$^{\#}$	2.9 ± 0.4$^{\#}$	21.7 ± 4.7$^{\#}$

Note: * $P<0.001$, TNF_{α} treated vs control cells; $^{\#}$ $P<0.001$, S1P or FTY-720 pretreated vs TNF_{α} treated cells.

DISCUSSION

Pulmonary microvascular endothelium is a functionally dynamic tissue that forms a semipermeable barrier between the vascular compartment and the parenchymal interstitum. It plays a key role in regulating lung function, both in health and in state of pathology, governing vascular permeability and inflammatory cell recruitment to the lung. Because of its enormous surface area, the lung vasculature is a particularly sensitive to barrier dysregulation with increases in permeability. Disruption of the integrity of the endothelial cellular barrier occurs via ultrastructural changes characterized by cytoskeletal rearrangement and the generation of tensile force within the cell resulting in cellular contraction, the interruption of intercellular adhesion complexes, and the creation of gaps between neighboring cells, which allow the exudation of fluid, macromolecules, and leukocytes into the interstitum and ultimately into the alveolar space, the pathological hallmark of acute lung injury (ALI)[4,20-21].

Tumor necrosis factor-alpha (TNF_{α}) is a member of the trimeric cytokine family, which has diverse bioregulatory activities engaged in inflammation/immunity responses, cell proliferation/differentiation, and apoptosis. Abundant evidence suggests that tumor necrosis factor (TNF_{α}) plays a key role in acute pulmonary injury and sepsis-associated multi-organ failure caused by different pathological reasons both in animal models and human subjects[22]. The structural changes and increased microvascular permeability in animals after a single infusion of TNF_{α} suggests that the hemodynamic and structural changes that occur in the lung.

Previous studies have demonstrated that TNF_{α} induces signaling events in lung ECs, resulting in cytoskeletal changes and increases in EC permeability. TNF_{α} induces an increase in permeability in vivo, in the isolated lungs, and in pulmonary microvascular ECs in vitro[4-5]. TNF_{α} also induces cytoskeletal changes within ECs, and the stabilization of F-actin using phalloidin prevents TNF_{α} induced decreases in endothelial barrier functions. The actin cytoskeletal changes likely occur through the modulation of actin-binding proteins by TNF_{α} induced signaling mechanisms that include activation of p38 MAPK, protein kinase C (PKC) isoforms, and Rho family GTPases, although the downstream targets of these signaling pathways leading to cytoskeletal changes remain to be clearly defined .

In vitro, confluent endothelial cell monolayers are widely used as a model for the investigation of permeability changes induced by TNF_{α}. While the cellular mechanisms by which TNF_{α} alters permeability have been investigated intensively in various microvascular endothelial cells lines, For example, Serrano et al. have shown that TNF_{α} enhances intercellular adhesion molecule-1 (ICAM-1), E-selectin, and vascular cell adhesion molecule-1 (VCAM-1) expression in human aortic endothelial cells monolayers, and Ferro et al. have shown that a 4 h incubation of TNF_{α} reduces pulmonary arterial endothelial monolayer selectivity by an nitric oxide-dependent mechanism.

In the present study, we examined the effects of S1P and its structural analog FTY720 on lactate

dehydrogenase (LDH), $PGF_{2\alpha}$ and PGE_2 release and F-actin structural changes of cultured rat pulmonary microvascular endothelial cells stimulated by TNF_{α}.

S1P is a sphingolipid metabolite produced via phosphorylation of sphingosine by sphingosine kinase[23] and was demonstrated recently to possess the capacity to enhance pulmonary vascular barrier function both in vitro and in vivo. For example, S1P can produce a rapid, sustained and dose-dependent increase in transendothelial monolayer electrical resistance across both human and bovine pulmonary artery and lung microvascular endothelial. In vivo, treatment with parenteral administration of S1P attenuated the inflammatory lung injury induced by intratracheal LPS in spontaneously ventilating C57BL/6 mice. Moreover, intravenous S1P also reduced the increased lung edema formation observed in a murine model of LPS-induced ALI[8,24].

FTY720 is a derivative of ISP-1(myrion), a fungal metabolite of the Chinese herb Iscaria sinclarill as well as a structural analogue of sphingosine. It is a novel immune modulator that prolongs allograft tansplant survival in numerous models by inhibiting lymphocyte emigration from lymphoid organs and it is currently in phase Ⅲ trials.

Our data shows that the cells exposed to TNF_{α} underwent retraction, increased paracellular permeability, and released larger amounts of PGE_2, $PGF_{2\alpha}$ and LDH compared with control cells. These results are similar to the previous studies. Meanwhile, PGE_2, $PGF_{2\alpha}$ and LDH productions were significantly reduced following pretreatment with S1P or FTY-720. Also, the increased paracellular permeability was prevented. Moreover, S1P or FTY-720 pretreatment prevented the changes of morphologic and cytoskeletal F-actin induced by TNF_{α}. To date, it is demonstrated that the mechanism responsible for the endothelial barrier protection is mediated by Edg-1 and Edg-3 receptors that are expressed on endothelial cells. These receptors couple to G1α-receptors and signal through RhoA, Src and Rac 1 to induce actin filament rearrangement[7].

In summary, this study provides further evidence that S1P and FTY-720 have the protective effects on rat pulmonary microvascular endothelial barrier destroyed by TNF_{α} through strengthening the cortical actin rearrangement and may represent a novel therapeutic method for pulmonary vascular barrier damage.

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