Angiotensin II aggravates lipopolysaccharide induced human pulmonary microvascular endothelial cells permeability in high glucose status

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Abstract. Lung infection is one of the most common infections in diabetes mellitus and is characterized by increased pulmonary microvascular endothelial permeability. Local Angiotensin II (AngII) plays an important role in the pathogenesis of lung diseases. However, whether AngII can aggravate diabetic infectious lung injury is not clear. Therefore, we investigated the effects of AngII on the permeability of human pulmonary microvascular endothelial cells (HPMVECs) challenged by lipopolysaccharide (LPS) in high glucose states in vitro. HPMVECs were divided into five groups: a control group (CON), a high glucose group (HG), an LPS + high glucose group (LH), an LPS + high glucose + AngII group (LHA), and an LPS + high glucose + Losartan group (LHL). The HPMVECs permeability as well as the F-actin levels, cytoskeleton, apoptosis and TNF-α concentrations were evaluated. Compared to the CON group, the HG, LH and LHA groups had significantly higher cellular permeability, cellular apoptosis and TNF-α levels, with more extensive cytoskeletal damage and lower F-actin levels. Additionally, cells in the LHA group exhibited significantly elevated permeability, apoptosis and TNF-α concentrations, lower F-actin levels and more extensive cytoskeletal damage than either the LH or HG group. However, compared to the HG or LH group, the LHL group showed significantly lower cellular permeability, cell apoptosis, cytoskeletal damage and TNF-α concentrations and higher F-actin levels. This study suggests that in a diabetic infectious lung injury cellular model, AngII could aggravate the permeability of HPMVECs via F-actin dynamics and cell apoptosis. Furthermore, blocking the Angiotension II Type 1 Receptor could significantly alleviate the hyperpermeability of HPMVECs.

Key words: Diabetes mellitus, Cellular permeability, F-actin, Angiotensin II, Angiotension II type 1 receptor

DIABETES MELLITUS, a group of metabolic diseases primarily diagnosed by hyperglycemia, results in significant morbidity and mortality worldwide [1]. Due to an immune deficiency, patients with diabetes mellitus have increased susceptibility to infections and poor outcomes [2]. One study reported that in emergency departments in the USA, 10% of inpatients with diabetes were hospitalized due to infection, and diabetics were more than twice as likely to be admitted to hospital for infection management than patients without diabetes [3]. The results of a population-based study from Canada reported that nearly half of patients with diabetes in each cohort year suffered from infectious diseases, leading to hospital admission or physician claims [4]. Lung infections are one of the most common infections in diabetes mellitus patients and can lead to sepsis, acute lung injury (ALI), acute respiratory distress syndrome (ARDS) and even death [5, 6]. The pneumonia mortality has been reported to be as high as 32.1%, similar to that of sepsis (31.7%), among the UK population aged ≥65 years with diabetes [5].

The circulating renin-angiotensin system (RAS) is a critical component for regulating blood pressure, electrolytes and fluid homeostasis [7]. However, the local RAS in organs and tissues has the ability to promote cell growth, cell differentiation and inflammation, and angiotensin enzyme (ACE), Angiotensin II (AngII) and the
Angiotension II Type 1 Receptor (AT1R) represent the deleterious axis [8, 9]. The RAS might play an important role in the pathophysiology of lung injury [10]. In an animal model of lung injury induced by lipopolysaccharide (LPS), AngII was shown to be significantly increased in lung and blood plasma, which further promoted the inflammatory response by its direct binding to the AT1R [11]. Interestingly, the use of AT1R antagonists and ACE inhibitors obviously alleviated lung injury and decreased the level of inflammatory factors (NF-κB, TNF-α) in vivo [11-13].

Increased vascular endothelial permeability plays a critical role in the pathogenesis and disease progression for a range of infectious syndromes, including sepsis, ALI and ARDS [14]. Increased vascular endothelial permeability is a major hallmark of sepsis [15]. Multiple studies have shown that injury of lung vascular endothelial cells is the major cause of ALI/ARDS, and an increase in lung vascular permeability occurs primarily at the level of the lung microcirculation, which in turn results in the accumulation of protein-rich pulmonary edema [16]. The stability of the endothelial barrier formed by tight junctions and adherens junctions depends on their connection to the actin cytoskeleton [17]. F-actin is an important protein of cytoskeletal elements and is a major contributor to inflammation and LPS-induced endothelial barrier dysfunction [18, 19]. A decrease in F-actin levels can lead to increased human umbilical vein endothelial cellular permeability [20]. The other important factor involved in the increase in vascular endothelial permeability is the increase in endothelial cell apoptosis [21, 22]. Our previous study demonstrated that Losartan can attenuate microvascular permeability in mechanical ventilator-induced lung injury in a diabetic mouse model [23]. Studies have demonstrated that AngII can increase the permeability of endothelial cells of umbilical vein and kidney [24, 25]. AngII induces F-actin cytoskeletal reorganization and cell apoptosis in podocytes [26, 27]. However, whether AngII could affect the alterations of vascular endothelial cellular permeability in diabetic infectious lung injury and whether this effect occurs through F-actin and cell apoptosis remain unclear. Therefore, in the present study, we investigated the effects of AngII on the permeability, apoptosis, F-actin levels and rearrangement and secretion of TNF-α in human pulmonary microvascular endothelial cells (HPMVECs) induced by LPS in high glucose states. Furthermore, we assessed the effects of Losartan, a specific antagonist of the AT1R, on HPMVEC injury induced by LPS in high glucose states. In infectious diseases, due to the diversity of pathways activated by the pathogen or by the host cells themselves, pathogen destruction alone is not sufficient to reduce mortality or morbidity [21]. Inflammatory and endothelial barrier alterations must both be controlled. This study investigates the effects of AngII and its antagonist on pulmonary microvascular endothelium permeability induced by LPS in high glucose states and will provide new adjunctive strategies to be used in combination with therapies targeting pathogens, with the aim of protecting the endothelial barrier and improving the recovery of diabetic patients with pulmonary infection.

Materials and Methods

Cell culture

HPMVECs (American type culture collection, China agent-Shanghai Princeton Biotechnology Development Co., Ltd) were thawed and cultured in RPMI-1640 medium (Thermo-Fisher Biochemical Products, Beijing, China), supplemented with 1% penicillin-streptomycin (Gino Biological Medical Technology Co Ltd, China) and 10% fetal bovine serum (Thermo-Fisher Biochemical Products, Beijing, China) at 37°C in a 5% CO₂ incubator. The culture medium was changed every 48 h. HPMVECs were subcultured at 80% confluence. Then, HPMVECs were digested, collected and cultured in RPMI-1640 medium at 37°C in a 5% CO₂ incubator.

Experimental groups

The treatments were divided into five groups: the control group (CON), without any treatment; the high glucose group (HG), treated with a high concentration of glucose (33.3 mmol/L, Thermo-Fisher Biochemical Products, Beijing, China); the high glucose + LPS group (LH), treated with a high concentration of glucose (33.3 mmol/L) and LPS (100 ng/mL, Sigma Aldrich, Saint Louis, Missouri, USA); the high glucose + LPS + AngII group (LHA), treated with a high concentration of glucose (33.3 mmol/L), LPS (100 ng/mL, Sigma Aldrich, Saint Louis, Missouri, USA); and the high glucose + LPS + Losartan group (LHL), pretreated with Losartan (1 × 10⁻⁷ mol/L, Sigma Aldrich, Saint Louis, Missouri, USA) for 6 h prior to treatment with a high concentration of glucose (33.3 mmol/L) and LPS (100 ng/mL).
Evaluation of HPMVEC monolayer permeability

A monolayer cell culture model was established to detect the HPMVEC permeability coefficient using transendothelial $^{14}$C-bovine serum albumin ($^{14}$C-BSA) (1.1 pmol/0.5 mL, Sigma Aldrich, Saint Louis, Missouri, USA) flux. HPMVECs ($1 \times 10^5$) of all groups were cultured on a gelatin-coated polycarbonate membrane (diameter: 13 mm, pore size: 0.4 μm) at 37°C for 24 h. Then, a polystyrene 24-cell culture plate was inserted. The barrier function of each cell monolayer was measured by the equivalent penetration concentration of $^{14}$C-BSA tracer. The permeability coefficient of HPMVEC monolayers was determined by the penetration quantity of a $^{14}$C-BSA tracer for 1 h. Just the results that over 97% of the endothelial cell monolayer retained tracer were valid.

Evaluation of cellular F-actin levels

HPMVECs ($1 \times 10^5$/mL) with or without the treatments described above were cultured for 24 h. Then, HPMVECs were collected and washed in phosphate buffer saline (PBS) three times, followed by fixation in paraformaldehyde and permeabilization in 0.5% Triton X-100. HPMVECs were labeled with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma Aldrich, Saint Louis, Missouri, USA) for 40 min at 4°C in the dark. Data were acquired and analyzed with a multi-parameter flow cytometry (Beckman Coulter, USA) instrument using the CXP software.

Observation of the cellular F-actin cytoskeleton by confocal microscopy

HPMVECs ($1 \times 10^5$/mL) with or without the treatments described above were cultured for 24 h. Then, HPMVECs were collected and washed in PBS three times, followed by fixation in paraformaldehyde and permeabilization in 0.5% Triton X-100. HPMVECs were labeled with FITC conjugated phalloidin (Sigma Aldrich, Saint Louis, Missouri, USA) for 30 min at 37°C in the dark, followed by washing in PBS and labeling with DAPI (Sigma Aldrich, Saint Louis, Missouri, USA) for 15 min at 37°C in the dark. Changes in F-actin were observed under a confocal microscope.

Cell apoptosis analysis by flow cytometry

HPMVECs ($1 \times 10^5$/mL) with or without the treatments described above were cultured for 24 h. Then, HPMVECs were collected and washed with PBS, followed by labeling with FITC-conjugated Annexin-V and PI (Annexin-V kit, BD Biosciences, San Jose, California, USA) for 15 min at room temperature in the dark. The cells were then analyzed with a multi-parameter flow cytometry (Beckman Coulter, USA) instrument using the CXP software. This method identifies apoptotic cells as Annexin V$^+$/PI$^-$ and Annexin V$^+$/PI$^+$.

Measurement of TNF-α levels by ELISA

The levels of TNF-α in the supernatant of each group were measured using ELISA kits (Sigma Aldrich, Saint Louis, Missouri, USA) strictly according to the manufacturer’s instructions.

Statistical analysis

Statistical analyses were performed using SPSS software (version 17.0; Chicago, IL). All data are presented as the means ± standard deviation. One-way analysis of variance (ANOVA) was used for statistical analysis to compare values among all groups. A $p$ value < 0.05 was considered statistically significant.

Results

HPMVEC monolayer permeability

As shown in Table 1, the permeability of HPMVEC monolayers was measured by detecting the permeability coefficient. Compared to the CON group, the HG, LH and LHA groups all exhibited significant increases in their monolayer permeability ($5.0 \pm 0.5$ vs. $10.3 \pm 1.6$, $5.0 \pm 0.5$ vs. $22.8 \pm 2.6$ and $5.0 \pm 0.5$ vs. $32.9 \pm 2.8$ nmol/h, respectively; all $p < 0.01$). Notably, the permeability of the LH group was significantly increased compared to that of the HG group ($22.8 \pm 2.6$ vs. $10.3 \pm 1.6$ nmol/h, $p < 0.05$). AngII significantly exacerbated the hyperpermeability induced by high glucose alone or in

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<td>Permeability coefficient (nmol/h)</td>
<td>5.0 ± 0.5</td>
<td>10.3 ± 1.6*</td>
<td>22.8 ± 2.6**</td>
<td>32.9 ± 2.8***</td>
<td>5.5 ± 1.8***</td>
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* $p < 0.01$ vs. the group CON; ** $p < 0.05$ vs. the group HG; *** $p < 0.05$ vs. the group LH; **** $p < 0.01$ vs. the group LHA.
association with LPS (32.9 ± 2.8 vs. 10.3 ± 1.6, 32.9 ± 2.8 vs. 22.8 ± 2.6 nmol/h, respectively; all \( p < 0.05 \)). However, in the LHL group, Losartan significantly attenuated the hyperpermeability induced by high glucose alone or in association with LPS or LPS and AngII (5.5 ± 1.8 vs. 10.3 ± 1.6, 5.5 ± 1.8 vs. 22.8 ± 2.6, 5.5 ± 1.8 vs. 32.9 ± 2.8 nmol/h, respectively; all \( p < 0.05 \)).

**F-actin level**

As the reconstruction of the endothelial cytoskeleton is an important factor involved in the increase in vascular endothelial permeability, we examined whether AngII could promote cytoskeleton rearrangement. Therefore, we first determined the F-actin concentration in various conditions by flow cytometry. As shown in Fig. 1, compared to the CON group, the HG, LH and LHA groups exhibited significantly decreased concentrations of F-actin (151.12 ± 2.733 vs. 184.13 ± 3.62, 116.63 ± 5.423 vs. 184.13 ± 3.62, 34.12 ± 7.52 vs. 184.13 ± 3.62 AU, respectively; all \( p < 0.01 \)). AngII exacerbated the significant depolymerization of F-actin induced by high glucose alone or by the combined treatment of high glucose and LPS (34.12 ± 7.52 vs. 151.12 ± 2.733, 34.12 ± 7.52 vs. 116.63 ± 5.423 AU, respectively; all \( p < 0.01 \)). In contrast, in the LHL group, Losartan significantly attenuated the F-actin depolymerization induced by high glucose alone or in association with LPS or LPS and AngII (183.67 ± 3.22 vs. 151.12 ± 2.733, 183.67 ± 3.22 vs. 116.63 ± 5.423 and 183.67 ± 3.22 vs. 34.12 ± 7.52 AU, respectively; all \( p < 0.05 \)).

**Distribution and morphology of the F-actin cytoskeleton**

Next, we examined the cytoskeleton by confocal microscopy. The results are presented in Fig. 2. In the CON group, the F-actin cytoskeleton of HPMVECs stained as yellow-green and was regularly distributed along the longitudinal axis of the cell in a filamentous arrangement evenly distributed in the center and periphery of the cell, maintaining cell integrity. After treatment with high glucose, the regular arrangement of the F-actin cytoskeleton disappeared, and the cytoskeleton was disorganized; however, the whole cell structure was still visible. In the LH group, the F-actin cytoskeleton of the whole cell was disordered, and F-actin fracture and thickening were visible. Furthermore, the intermediate filaments and the middle net contracted into clusters. In the LHA group, the whole cytoskeleton was destroyed, and the cells were fuzzy. Additionally, F-actin was irregularly arranged around the cell and broken, and the central F-actin had completely disappeared. In the LHL group, the cytoskeleton was clear, and the distribution of F-actin was not significantly different from that in the CON group, suggesting that Losartan significantly attenuates the cytoskeleton destruction induced by high glucose levels and LPS.

**Apoptosis of HPMVECs**

Another important factor involved in the increase in vascular endothelial permeability is the increase of endothelial cell apoptosis, and we examined whether AngII can increase apoptosis of HPMVECs. Therefore, we analyzed cell apoptosis by flow cytometry. As shown in Fig. 3, compared to the CON group, the HG, LH and LHA groups all exhibited significantly increased cell apoptosis (14.98 ± 0.22% vs. 3.18 ± 0.37%, 20.86 ± 0.49% vs. 3.18 ± 0.37%, 32.63 ± 0.55% vs. 3.18 ± 0.37%, \( p < 0.01 \)). The percentage of apoptotic cells in the LH group was significantly increased compared to that in the HG group (20.86 ± 0.49% vs. 14.98 ± 0.22%, \( p < 0.01 \)). AngII significantly exacerbated the cell apoptosis induced by high glucose alone or in association with LPS (32.63 ± 0.55% vs. 14.98 ± 0.22%, 32.63 ± 0.55% vs. 20.86 ± 0.49%, \( p < 0.01 \)). However, in the LHL group, Losartan significantly...
attenuated the cell apoptosis induced by high glucose alone or in association with LPS or LPS and AngII (5.13 ± 0.26% vs. 14.98 ± 0.22%, 5.13 ± 0.26% vs. 20.86 ± 0.49%, 5.13 ± 0.26% vs. 32.63 ± 0.55%, p < 0.01).

**TNF-α production**

TNF-α, an important inflammatory cytokine, has been found to increase cell permeability, therefore, we measured its concentration in various conditions. The results are shown in Fig. 4. Compared to the CON group, the TNF-α level was significantly increased in the HG group (78.22 ± 5.25 vs. 39.10 ± 3.32 pg/mL; p < 0.01), LH group (112.26 ± 5.80 vs. 39.10 ± 3.32 pg/mL; p < 0.01) and LHA group (154.89 ± 7.70 vs. 39.10 ± 3.32 pg/mL; p < 0.01). AngII significantly exacerbated the increase in the TNF-α concentration induced by high glucose alone or in association with LPS (154.89 ± 7.70 vs. 78.22 ± 5.25, 154.89 ± 7.70 vs. 112.26 ± 5.80 pg/mL, respectively, p < 0.05). Treatment with Losartan resulted in decreased TNF-α levels in the LHL group compared to those in the HG, LH and LHA groups (all p < 0.05). No significant difference was observed in the concentration of TNF-α between the LHL and CON groups (39.43 ± 2.61 vs. 39.10 ± 3.32 pg/mL).

**Discussion**

Due to an immune deficiency, diabetes mellitus patients have greater susceptibility to infections and worse outcomes [2]. Lung infection is one of the most
common infections in patients with diabetes mellitus [5, 6]. It can progress to sepsis, ALI and even serious ARDS. A semi-permeable barrier, composed of endothelium, separates the blood stream from the underlying organs and tissues and controls the transport of fluids, solutes and cells across blood vessel walls [28]. Increased vascular endothelial permeability has been identified as a key process in the pathogenesis and disease progression of a range of infectious syndromes and organ dysfunctions [14]. Vascular permeability is mainly controlled by cell-cell junctions, including tight junctions and adherens junctions, and their stability depends on their connection to the actin cytoskeleton [17]. Therefore, actin dynamics are crucial for the regulation of endothelial barrier stability and vascular permeability. Actin has a polymeric (F-actin) and monomeric form (G-actin), and actin filament polymer is important for maintaining cell stability [17]. The other important factor involved in the increase in vascular endothelial permeability is the increase in endothelial cell apoptosis [21, 22]. TNF-α, an important inflammatory cytokine has been found to increase the cell permeability [17]. In the present study, we examined alterations in HPMVEC permeability, apoptosis, F-actin levels, cytoskeletal rearrangement and TNF-α levels to explain the changes in cell permeability, hallmark of cell injury and the underlying mechanism. We found that LPS combined with high glucose levels (LH group) resulted in increased cellular permeability and apoptosis, decreased F-actin levels, and increased secretion of TNF-α and cytoskeletal damage, compared to the HG group, suggesting that the generation of a cellular model of diabetic lung injury was successful.

Diabetes is strongly associated with systemic vascular disease, but the relationship with pulmonary vascular disease has often been disregarded [29]. The lungs display a complicated microcirculation network, and its structure and function can be altered in diabetes (pulmonary microangiopathy) [30]. In the present study, we demonstrated that high glucose levels resulted in significantly higher microvascular permeability than in the CON group, consistent with our previous in vivo results [23], and this effect was relevant to the decreased F-actin levels and cytoskeletal rearrangement and the increased cell apoptosis and TNF-α levels. Recently, using an animal model, Clemmer JS et al. reported that hyperglycemic or diabetic rats exhibited higher pulmonary microvascular permeability [31, 32]. However, they did not further investigate the modulation of the actin cytoskeleton or cell apoptosis [31, 32]. Studies on glomerular barrier permeability have shown that hyperglycemia can lead to F-actin cytoskeleton rearrangements and cell
apoptosis and then increase vascular or glomerular permeability, which may involve the activation of protein kinase C, the polyol pathway, and an increased release of reactive oxygen species (ROS) and cytokines [33]. In our study, pulmonary vascular permeability, cell apoptosis, cytoskeletal rearrangement and TNF-α levels were decreased, and the F-actin level was increased in the Losartan group compared to the high glucose group, suggesting that the AngII/AT1 signaling pathway might be involved in this process. Our previous results showed that the AngII and AT1 levels were significantly increased in diabetic mice relative to the control [23].

The local RAS in organs and tissues promotes cell growth, differentiation and inflammation, and ACE, AngII and AT1R represent the deleterious axis [8, 9]. Studies have shown that the RAS plays an important role in lung injury. In the animal model of LPS-induced lung injury, the AngII level was increased in the lung, which further promoted the inflammatory response by binding to the AT1R [11]. The use of an AT1R antagonist and ACE inhibitor can alleviate lung injury and attenuate the inflammatory response by inhibiting NF-κB-DNA-binding activity and the expression of TNF-α mRNA in vivo [11-13]. Zhang H et al. reported that AngII worsened LPS-induced lung injury and increased the permeability of rat pulmonary microvascular endothelial cells [34]. Recently, Wu Z et al. reported that AngII led to pulmonary microvascular endothelial cell barrier injury by promoting skeletal rearrangement and apoptosis of pulmonary microvascular endothelial cells [10]. Our previous study showed that Losartan could attenuate microvascular permeability in a mechanical ventilator-induced lung injury model in diabetic mice [23]. To our knowledge, this is the first regarding the effects of AngII on diabetic lung injury via measurement of cell permeability and its two major mechanisms, cytoskeletal recombination and endothelial apoptosis, using a cellular model. Our study found that AngII significantly aggravated cell permeability, accompanying decreased F-actin levels, increased cytoskeletal rearrangement, increased cell apoptosis and secretion of TNF-α in LPS-induced HPMVECs in a high glucose state. Losartan significantly attenuated its impact, suggesting that an AT1R antagonist can prevent HPMVEC injury caused by LPS in high glucose states.

Studies on diabetic nephropathy and cardiovascular complications suggest that AngII induces vascular complications in diabetes through the AGE-RAGE-oxidative stress axis [35, 36]. Hyperglycemia increases local RAS activity and up-regulates the expression of AT1R [35], consistent with our previous study in mice [23]. Meanwhile, AngII promotes ROS generation through the activation of NADPH oxidase [35]. Hyperglycemia leads to the formation and accumulation of AGE. AngII has been shown to potentiate the deleterious effects of AGEs on pericytes by inducing RAGE expression [36]. A direct interaction occurs between LPS and AngII. In animal models, local lung AngII was increased in response to LPS challenge, leading to the exacerbation of LPS-induced endothelium injury. An AT1R antagonist significantly reduced these changes [34]. Li HP et al. showed that LPS enhanced AT1R expression in HPMVECs, and LPS increased the binding of AT1R to AngII [37]. Therefore, it seems that AngII, high glucose and LPS could synergistically act and lead to HPMVEC injury. Recently, Wu Z et al. demonstrated that AngII promoted cytoskeletal rearrangement and apoptosis of PMECs via VE-cadherin and monocyte chemoattractant protein-1 [10, 38]. However, the mechanism of F-actin cytoskeletal alterations and cell apoptosis induced by AngII in diabetic infectious pulmonary vascular cells needs to be further elucidated.

In conclusion, our data demonstrated that pulmonary microvascular endothelial cell injury occurred in diabetes mellitus due to hyperglycemia, and AngII aggravated the permeability of HPMVECs challenged by LPS in high glucose states, via F-actin dynamics, and increased cell apoptosis. Losartan, an AT1R antagonist, alleviated the hyperpermeability of HPMVECs induced by high glucose combined with LPS. One limitation of this study should be taken into account: our study does not explain whether AngII can aggravate infectious lung injury in diabetic patients well-controlled. The role of ANGII in this part of the patients with pulmonary infection needs further study. This study provides theoretical evidence for the use of AngII antagonists in the treatment of diabetic pulmonary infection, with the aim of protecting the vascular endothelial barrier.

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Disclosure

The authors declare that they have no conflict of interest.
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