Hepatocyte growth factor protects pulmonary endothelial barrier against oxidative stress and mitochondria-dependent apoptosis

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Abstract

Background: Pulmonary microvascular endothelial cells (PMVECs) were not complex, and the endothelial barrier was destroyed in the pathogenesis progress of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). Previous studies have demonstrated that hepatocyte growth factor (HGF), which was secreted by bone marrow mesenchymal stem cells, could decrease endothelial apoptosis. We investigated whether mTOR/STAT3 signaling acted in HGF protective effects against oxidative stress and mitochondria-dependent apoptosis in lipopolysaccharide (LPS)-induced endothelial barrier dysfunction and ALI mice.

Methods: In our current study, we introduced LPS-induced PMEVCs with HGF treatment. To investigate the effects of mammalian target of rapamycin (mTOR)/signal transducer and activator of transcription 3 (STAT3) pathway in endothelial oxidative stress and mitochondria-dependent apoptosis, mTOR inhibitor rapamycin and STAT3 inhibitor S3I-201 were, respectively, used to inhibit mTOR/STAT3 signaling. Moreover, lentivirus vector-mediated *mTORC1* (Raptor) and *mTORC2* (Rictor) gene knockdown modifications were introduced to evaluate *mTORC1* and *mTORC1* pathways. Calcium measurement, reactive oxygen species (ROS) production, mitochondrial membrane potential and protein, cell proliferation, apoptosis, and endothelial junction protein were detected to evaluate HGF effects. Moreover, we used the ALI mouse model to observe the mitochondria pathological changes with an electron microscope *in vivo*.

Results: Our study demonstrated that HGF protected the endothelium via the suppression of ROS production and intracellular calcium uptake, which lead to increased mitochondrial membrane potential (JC-1 and mitochondria tracker green detection) and specific proteins (complex I), raised anti-apoptosis Messenger Ribonucleic Acid level (B-cell lymphoma 2 and Bcl-xL), and increased endothelial junction proteins (VE-cadherin and occludin). Reversely, mTOR inhibitor rapamycin and STAT3 inhibitor S3I-201 could raise oxidative stress and mitochondria-dependent apoptosis even with HGF treatment in LPS-induced endothelial cells. Similarly, mTORC1 as well as mTORC2 have the same protective effects in mitochondria damage and apoptosis. In *in vivo* experiments of ALI mouse, HGF also increased mitochondria structural integrity via the mTOR/STAT3 pathway.

Conclusion: In all, these reveal that mTOR/STAT3 signaling mediates the HGF suppression effects to oxidative level, mitochondria-dependent apoptosis, and endothelial junction protein in ARDS, contributing to the pulmonary endothelial survival and barrier integrity.

Keywords: Hepatocyte growth factor; Acute respiratory distress syndrome; Endothelial barrier; mTOR/STAT3 pathway; Permeability

Introduction

Acute respiratory distress syndrome (ARDS) is a high mortality disease with dyspnea in critical care medicine. Recent studies have enumerated the deleterious effects of ARDS on endothelial barrier complex and survival.[1,2] We have previously demonstrated that the hepatocyte growth factor (HGF) secreted by bone marrow mesenchymal stem cells could attenuate the gram-negative bacterial pathogen lipopolysaccharide (LPS)-induced endothelial barrier apoptosis.[3,4] HGF is a critical proliferation factor and regulates varied cellular survival and angiogenesis. However, little is known about the direct effects of HGF on endothelial apoptosis and how it may lead to compromises in the regulation of endothelial recovery.

The mitochondrion is a double-membrane-bound organelle for cellular energy supplying, cell survival, and cellular differentiation. The stability of mitochondria is essential for reducing apoptosis and promoting cell growth. On

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mitochondrial entry, calcium can enhance the tricarboxylic acid cycle dehydrogenase activity and raise the mitochondrial complex I substrate, nicotinamide adenine dinucleotide (NADH), to stimulate reactive oxygen species (ROS) and energy. Mitochondrial oxidative stress is the imbalance of oxidative and antioxidant effects, producing a large amount of ROS, resulting in mitochondrial damage.[5,6] Damaged mitochondrion is characterized by decreased mitochondrial membrane potential and destroyed mitochondrial membrane protein. When mitochondria are damaged, the mitochondria-dependent apoptotic pathway was activated and causes cell dysfunction.

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that can regulate cellular proliferation and respond to stimuli including growth factors, hormones, nutrients, energy status, and oxygen levels.[7,8] Recent data demonstrated that mTOR acted as a major regulator of energy production in mitochondria.[7-9] mTOR can also eliminate mitochondria to inhibit autophagy.[10-13] Activated mTOR is well-positioned to play a central role in tumorigenesis and angiogenesis.[14] mTORC1 and mTORC2, which are two distinct multiprotein complexes of the mTOR protein kinase, regulate different branches of the mTOR network. mTORC1 includes mTOR, regulatory-associated protein of mTOR (Raptor), Dep domain containing mTOR interacting protein (DEPTOR), mLST8, and PRAS40 and regulates cell growth through phosphorylation of S6K1 and 4E-BP1. mTORC2 includes mTOR, rapamycin-insensitive companion of mTOR (Rictor), mSIN1, mLST8, DEPTOR, Protor, GβL, TTI1, and TEL2 and regulates actin cytoskeletal reorganization via prosurvival kinase protein kinase B (Akt) and protein kinase C by phosphorylating it on S473. In mammalian cells, mTORC1 stimulates the synthesis of mitochondrial ribosomal proteins and components of complex I and V.[9] Inhibition of mTOR signaling strongly downregulates mitochondrial biogenesis and respiration.[9]

Signal transducer and activator of transcription 3 (STAT3) is an important downstream target of mTOR. STAT3 mainly acts as a transcription factor associated with various physiological and pathological functions including angiogenesis.[15] STAT3 can serve as a sensor for various metabolic stressors including ROS and a link for regulating the activity of the electron transport chain and adenosine 5′-triphosphate production. STAT3 increases pivotal transcription factors in gene regulatory network to angiogenesis. STAT3 contributes to rapid cellular proliferation. STAT3 is a critical target in mitochondrial role,[16,17] oxidative stress,[18] and cytokines.

Here, we investigated whether mTOR/STAT3 signaling acted in HGF protective effects against oxidative stress and mitochondria-dependent apoptosis in LPS-induced endothelial barrier dysfunction and acute lung injury (ALI) mice. The effects of recombinant murine HGF on endothelial cell barrier dysfunction stimulated by gramnegative bacterial pathogen LPS *in vitro* and *in vivo* were introduced. mTOR inhibitor rapamycin and STAT3 inhibitor S3I-201 were, respectively, used to abort HGF suppression effects of oxidative stress and mitochondria-dependent apoptosis.

Methods

Ethics approval

This study was approved by the Local Ethics Committee for the Care and Use of Laboratory Animals of Southeast University (No. 20200226001).

Cell culture and reagents

Pulmonary microvascular endothelial cells (PMVECs) were obtained from Shanghai Zhen Biotechnology Company Limited (Shanghai, China), and surface marker CD31 was identified by a flow cytometer. PMVECs were cultured in endothelial growth medium (Wisent, Nanjing, China) and humidified in an incubator with 5% CO_2 at 37°C. Culture media were changed every 2 to 3 days according to cell growth status. We used gram-negative bacterial pathogen LPS (100 ng/mL, Sigma, USA) to stimulate PMVECs *in vitro* for mimicking an ARDS environment. And recombinant murine HGFs (20 ng/mL, ProSpec, Israel) were introduced to explore detailed mechanisms. PBS was applied as a negative control, and mTOR inhibitor rapamycin (100 nmol/L, Selleck, USA) or STAT3 inhibitor S3I-201 (100 nmol/L, Selleck) was used to inhibit the activation of mTOR or STAT3 pathway in LPS-induced PMVECs with HGF treatment.

Animal models

The experiment was endorsed by the Committee of Animal Care and Use of Southeast University. All experiments were performed in accordance with Chinese legislation regarding experiment animals. Male C57BL/6 mice (8 weeks) were purchased from the Animal Center of Yangzhou University (Yangzhou, China). Mice were randomly divided with 10 mice per group. To ALI, mice were intratracheally given 5 mg/kg LPS dissolved in 100 μL of PBS. Mice were sacrificed at 24 h after drug injections, and the lung lobes were collected for electron microscope observation.

Gene modification

We conducted lentivirus vector-mediated Raptor and Rictor knockdown in PMVECs. The Raptor and Rictor knockdown was conducted using lentivirus vector (Raptor-target-seq: CCTCATCGTCAAGTCCTTCAA; Rictor-target-seq: GCTGAGATTTCTTTCCATTCC). Knockdown specific for enhanced green fluorescent protein (EGFP) acted as a negative control. Passages < six cells were used, and the lentivirus was packaged in 293T cells (Cyagen Biosciences, Guangdong, China) with the aid of three packaging plasmids to obtain a higher titer of lentivirus. Then, we harvested PMVECs carrying EGFP (short hairpin RNA control) and both target genes (shRaptor, shRictor). Transfection efficiency was detected by real-time quantitative polymerase chain reaction, and expression efficiency was tested by a fluorescence microscope.

Calcium measurement

Fluo-4 AM is a fluorescence probe usually used to detect cellular calcium concentration. Fluo-4 can combine with

calcium leading to strong fluorescence. PMVECs were loaded with 2 μmol/L Fluo-4 AM (Beyotime Biotechnology, Shanghai, China) in the extracellular medium for 30 min at 37°C. Fluorescein intensity was recorded with flow cytometry (ACEA, NovoCyte, China) after 1 min of baseline recording. PMVECs were stimulated with LPS or HGF, and fluorescein intensity was acquired after 1 min of basing recording. Moreover, 200 s fluorescein intensity and Fluo-4 AM positive ratio cells were also recorded. Data were collected and analyzed by NovoExpress (ACEA NovoCyte).

Intracellular ROS detection

Intracellular ROS were measured by flow cytometry using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime Biotechnology), which can be oxidized by ROS to fluorescent dichlorodihydrofluorescein (DCF) with high fluorescence. PMVECs were incubated with DCFH-DA (10 μmol/L) in serum-free culture medium for 20 min at 37°C in the dark. At the incubation end, PMVECs were washed and resuspended in serum-free culture medium three times. ROS generation was monitored and detected using flow cytometry (ACEA NovoCyte) at an excitation of 488 nm wavelength. Drugs were loaded for 30 min to 2 h, and data were collected and analyzed using NovoExpress (ACEA NovoCyte).

JC-1 assay

JC-1 is an ideal fluorescent probe used in the detection of mitochondrial membrane potential. Early apoptosis is characterized by mitochondrial membrane potential drop, and fluorescence from red (aggregates) to green (monomer) by JC-1 shift can be easily detected as an apoptosis index of early detection. PMVECs were resuspended and centrifuged to collect and then incubated with JC-1 working solution (Beyotime Biotechnology, China) for 20 min at 37°C in the dark. PMVECs were washed by JC-1 dye buffer (Beyotime Biotechnology) and centrifuged at 600*g* and 4°C for 4 min. Finally, cells were incubated in 0.5 mL cold JC-1 dye buffer (1X, Beyotime Biotechnology) and transferred to a tube on ice for flow cytometry analysis by flow cytometry (ACEA NovoCyte). Analysis was carried out by NovoExpress software (ACEA NovoCyte).

Mitochondria tracker green detection

Mitochondria tracker green is a green fluorescent probe used in mitochondrial-specific fluorescent staining of living cells. PMVECs were removed from cell culture and incubated with mitochondria tracker green for 30 min at 37°C in the dark. Then, PMVECs were centrifuged and resuspended with 37°C fresh cell culture. Subsequent observations were made with a fluorescence microscope. At this time, the mitochondria of live cells could be dyed and observed with bright strong green fluorescence.

Mitochondrial complex I activity assay

NADH dehydrogenase (ubiquinone) 1β subcomplex subunit eight is an accessory subunit of the NADH dehydrogenase (ubiquinone) complex, which is located in the mitochondrial inner membrane. It is the largest of the five complexes of the electron transport chain and is also known as complex I. The rate of NADH oxidation is measured by a decrease in absorbance at 340 nm and is proportional to the activity of complex I. We tested mitochondrial complex I activity according to its operation manual of complex I activity kits (Solarbio, China). We extracted the cytoplasmic protein and further extracted the mitochondria. Then, incubate them with the mitochondrial complex I activity assay buffer and test with a microplate reader (Infinite M200 Pro, Tecan, Switzerland). Data of baseline and 2 min treatment optical density of the valve were recorded and analyzed for mitochondrial complex I activity ($nmol \cdot min^{-1} \cdot 10^{-4}$ cell).

Annexin V-PE/7-AAD stained flow cytometry

Cell apoptosis was detected by double staining with Annexin V-PE/7-AAD (BD Biosciences, USA). Briefly, cells were harvested and suspended in 1 × binding buffer at a concentration of 5×10^6 cells/mL. Then, the cells were incubated with AnnexinV-PE and 7-AAD for 15 min in dark at room temperature. Finally, the stained cells were immediately analyzed by a flow cytometer (ACEA NovoCyte) and analyzed with NovoExpress (ACEA NovoCyte). PE(−)7-AAD(−) referred to live cells; PE(+) 7-AAD(−) referred to early apoptotic cells, and PE(+)7-AAD(+) referred to late apoptotic and dead cells.

Cell counting Kit-8 assays

PMVECs stimulated with different treatments were seeded into 96-well plates. After stimulations, Cell Counting kit-8 (Beyotime) was added for 4 h, and absorbance was recorded with a 450-nm wavelength microplate reader (Infinite M200 Pro, Tecan).

Western blotting analysis

Cell lysates were collected in Radio Immunoprecipitation Assay buffer supplemented with a protease and phosphatase inhibitor and phenylmethanesulfonyl fluoride (Beyotime), and then, the reaction mixtures were cleared by centrifugation (12,000*g* for 30 min at 4°C). Proteins were separated with SDS-PAGE-condensed electrophoresis (Beyotime) and transferred to immun-Blot polyvinylidene difluoride Membrane (Beyotime). Membranes were blocked with 5% bovine serum albumin (Beyotime) in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature and incubated overnight at 4°C with the following commercially available primary antibodies against complex I subunit NDUFB8 (Abcam; 1:1000), VE-cadherin (1:1000; Cell Signaling), occludin (1:1000; Abcam), and β-actin (1:1000; Cell Signaling). Then, membranes were incubated with 1:3000 dilutions of peroxidase-conjugated secondary antibodies (Fcmacs) for 1 h at room temperature. In the final step, immune complexes were detected using the chemiluminescence imaging system (Bioshine ChemiQ 4800mini, Ouxiang, Shanghai, China).

Real-time quantitative PCR (RT-qPCR)

Total RNA from PMVECs was extracted with TriPure Isolation Reagent (Roch, Switzerland), and reverse

transcriptase was applied (Thermo Fisher Scientific, USA) for cDNA synthesis. RT-qPCR was performed for gene expression by using the Syber Green PCR Master Mix (Thermo Fisher Scientific) and Real-Time PCR System (Applied Biosystems, USA). The forward and reverse primers were designed by Primer Express Software (Vector NTI advance10) and listed as follows: β-actin, sense 5′-AGGTCTTTACGGATGTCAACG-3′, and antisense 5′-TCTTTTCCAGCCTTCCTTCTT-3′; Bcl-2, sense 5′-CTGGCATCTTCTCCTTCC-3′, and antisense 5′-AGTTCCTCCACCACCGT-3′; and Bcl-xL, sense 5′-GCTTCACATAACCCCAGG-3′, and antisense 5′-GCAATCCGACTCACCAAT-3′. Results were calculated using the $2^{-\Delta\Delta CT}$ method with β-actin as a control.

Statistical analyses

The statistical significance of the data was performed using GraphPad Prism 5 (GraphPad Software, CA, USA) and SPSS 19.0 (IBM Corporation, Armonk, NY USA). Data were conducted by Tukey multiple comparison test, oneway analysis of variance, and Student t test. $P < 0.05$ was regarded as significant statistical differences.

Results

HGF treatment suppresses LPS-induced PMVEC apoptosis and endothelial junction protein injury

First, we investigated the protective effects of HGF on LPS-induced PMVEC survival and integrity. LPS (100 ng/mL)-induced PMVECs were treated with or without HGF (20 ng/mL) for 0 to 24 h. PMVEC apoptosis and proliferation were, respectively, tested by Annexin V-PE/7-AAD staining with flow cytometry and CCK-8 kit. Endothelial tight junction protein zonula occludens-1 (ZO-1) was tested by immunofluorescence. It revealed that LPS-induced PMVEC early apoptosis ratio with HGF treatment (1,2, and 4 h) gradually decreased in flow cytometry analysis [Figure 1A and 1B]. Reversely, cell proliferation of LPS-induced PMVECs treated with HGF treatment (4, 12, and 24 h) gradually rised [Figure 1C]. Immunofluorescence analysis showed that endothelial tight junction protein ZO-1 in LPS-induced PMVECs with 24 h HGF treatment was unregulated compared with LPS alone [Figure 1D]. Collectively, these data demonstrated that HGF could suppress LPS-induced PMVEC injury via decreasing apoptosis and rising the endothelial tight junction protein ZO-1 integrity.

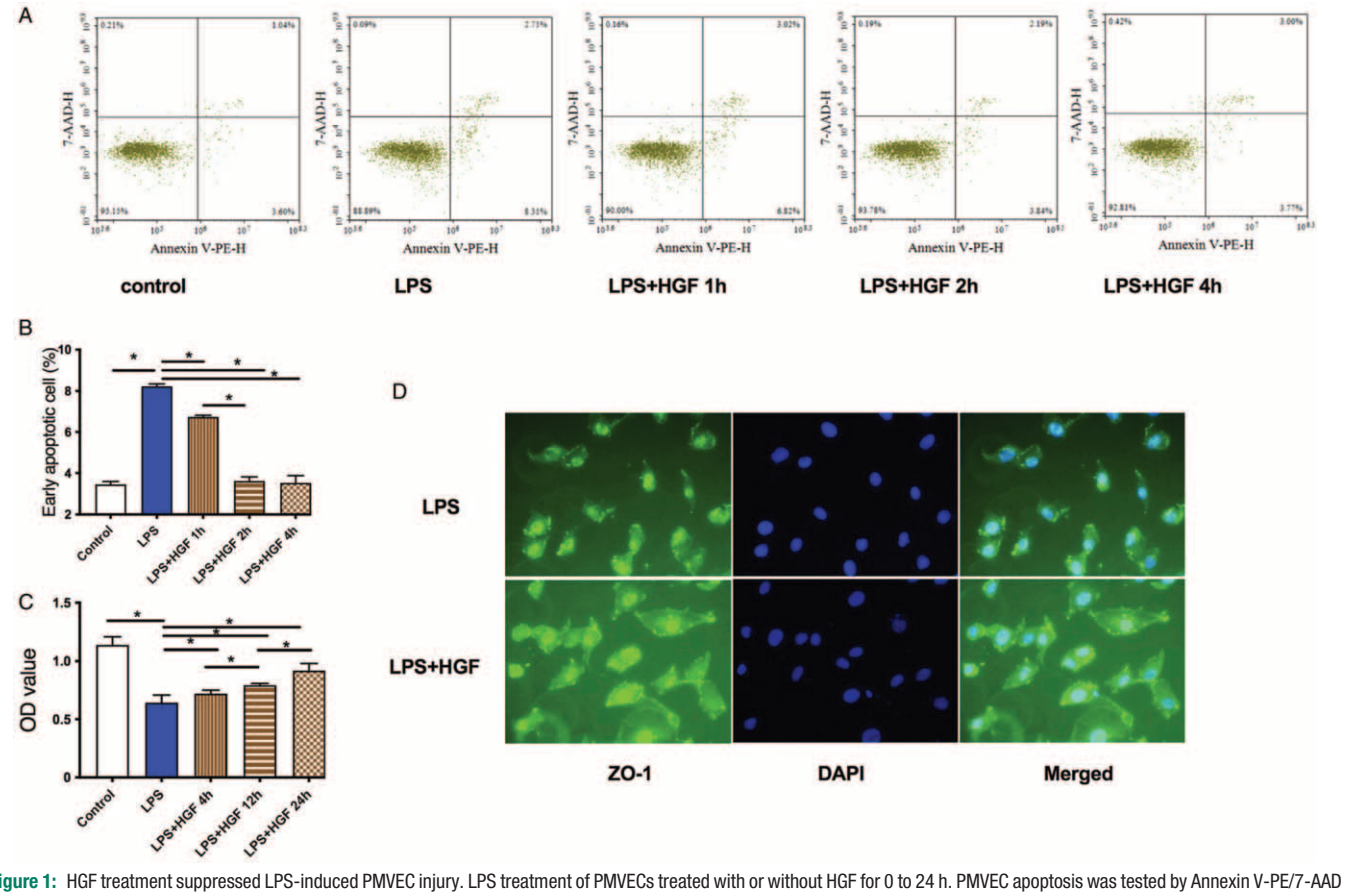

Figure 1: HGF treatment suppressed LPS-induced PMVEC injury. LPS treatment of PMVECs treated with or without HGF for 0 to 24 h. PMVEC apoptosis was tested by Annexin V-PE/7-AAD staining with flow cytometry. PMVEC proliferation was tested by the CCK-8 kit. Endothelial tight junction protein ZO-1 was tested by immunofluorescence. (A) Flow cytometry analysis of LPS-induced PMVEC apoptosis with HGF treatment (0-4 h). (B) Early apoptosis ratio of LPS-induced PMVEC apoptosis with HGF treatment in flow cytometry analysis (0–4 h). (C) Cell proliferation of LPS-induced PMVEC survival with HGF treatment (0–24 h). (D) Endothelial tight junction protein ZO-1 of LPS-induced PMVECs with 24 h HGF treatment (400 ×). Results are mean ± SD ($n = 3$). CCK-8: Cell counting kit-8; DAPI: 4',6-diamino-2-phenylindole; HGF: Hepatocyte growth factor; LPS: Lipopolysaccharide; PMVECs: Pulmonary microvascular endothelial cells.

HGF exposure decreases cytosolic calcium levels in PMVECs via mTOR/STAT3 pathway

As described above, HGF could decrease PMVEC apoptosis and promote survival. Intracellular calcium concentration was a critical danger factor for mitochondrial integrity and apoptosis inhibition. Hence, we used LPS-induced PMVECs treated with or without HGF. Fluorescence probe Fluo-4 AM was applied to detect cellular calcium concentration every 20 s with flow cytometry. It showed that fluo-4 fluorescence change of cytosolic calcium in LPS-induced PMVECs was decreased after HGF treatment [Figure 2A], implying that HGF exposure downregulated intracellular calcium overload. However, the detailed mechanism of this phenomenon was not certain. Rapamycin and S3I-201 were, respectively, used to inhibit mTOR and STAT3 signaling. mTOR and STAT3 inhibitors could reverse HGF suppression effects of calcium uptake in 200 s treatment tested by flow cytometry [Figure 2B and 2C]. Notably, HGF played a protective endothelial role by decreasing cytosolic calcium levels and mTOR/STAT3 contributed to it.

mTOR/STAT3 pathway contributes to HGF exposure attenuating cellular ROS production in LPS-induced PMVECs

Mitochondria injury is a target of oxidative stress, referring to mitochondrial energy disorders and ROS generation. LPS-stimulated PMVECs were treated with or without HGF. Intracellular ROS were detected by high fluorescent DCF in flow cytometry. As [Figure 3A and 3B] described, DCF change of cellular ROS production in LPS-induced PMVECs gradually decreased with prolonged HGF treatment (30, 60, 90, and 120 min) detected by flow cytometry. To assess the contribution signaling of cellular ROS production, rapamycin and S3I-201, were, respectively used to inhibit mTOR and STAT3 signaling. Intracellular ROS were detected by highly fluorescent DCF in flow cytometry. In 30 min drug treatment, mTOR inhibitor rapamycin reversed HGF suppression effects of ROS production [Figure 3C and 3D]. Similarly, STAT3 inhibitor S3I-201 showed the same effects [Figure 3C and 3D]. Taken together, these suggested that the mTOR/STAT3 pathway contributed to HGF exposure-attenuated cellular ROS production in LPS-induced PMVECs.

mTOR/STAT3 pathway contributes to the loss of mitochondrial membrane in LPS-induced PMVECs even with HGF treatment

Mitochondrial membrane potential represents primary indicators of membrane stability in mitochondria, and increased ROS have been demonstrated to directly facilitate mitochondrial dysfunction. Because mitochondrial membrane potential is critical for oxidative phosphorylation activity, we sought to assess whether mTOR and STAT3 pathways were mediated in mitochondrial

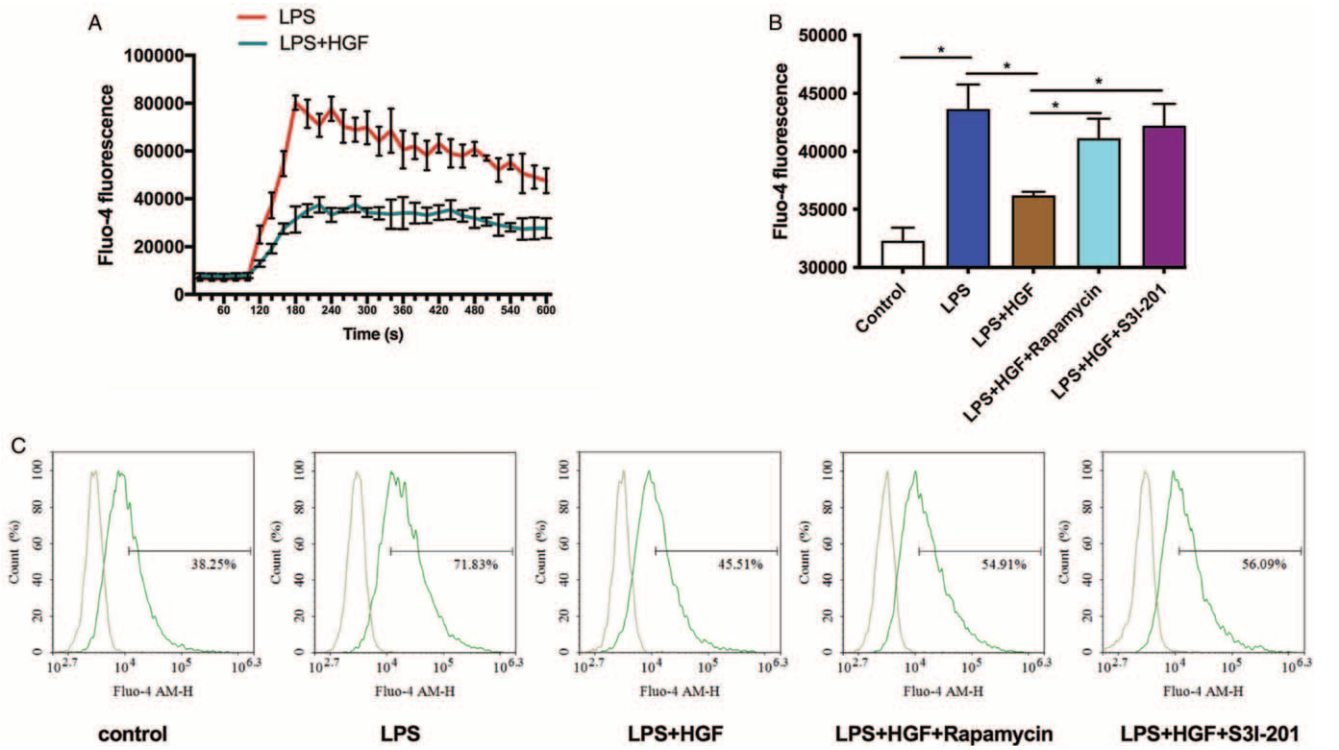

Figure 2: HGF exposure decreased cytosolic calcium levels In PMVECs via the mTOR/STAT3 pathway. LPS treatment of PMVECs treated with or without HGF. Rapamycin and S3I-201 were, respectively, used to inhibit mTOR and STAT3 signaling. Fluorescence probe Fluo-4 AM was applied to detect cellular calcium concentration every 20 s with flow cytometry. (A) Fluo-4 fluorescent change of cytosolic calcium in LPS-induced PMVECs with HGF treatment. (B) Relative cytosolic calcium expressions of HGF on LPS-induced PMVECs with mTOR/STAT3 pathway tested by flow cytometry (200 s). (C) The cytosolic calcium effects of HGF on LPS-induced PMVECs with mTOR/STAT3 pathway tested by flow cytometry (200 s). Results are mean ± SD ($n = 3$). $^{*}P < 0.05$. HGF: Hepatocyte growth factor; LPS: Lipopolysaccharide; mTOR: Mammalian target of rapamycin; PMVECs: Pulmonary microvascular endothelial cells; STAT3: Signal transducer and activator of transcription 3.

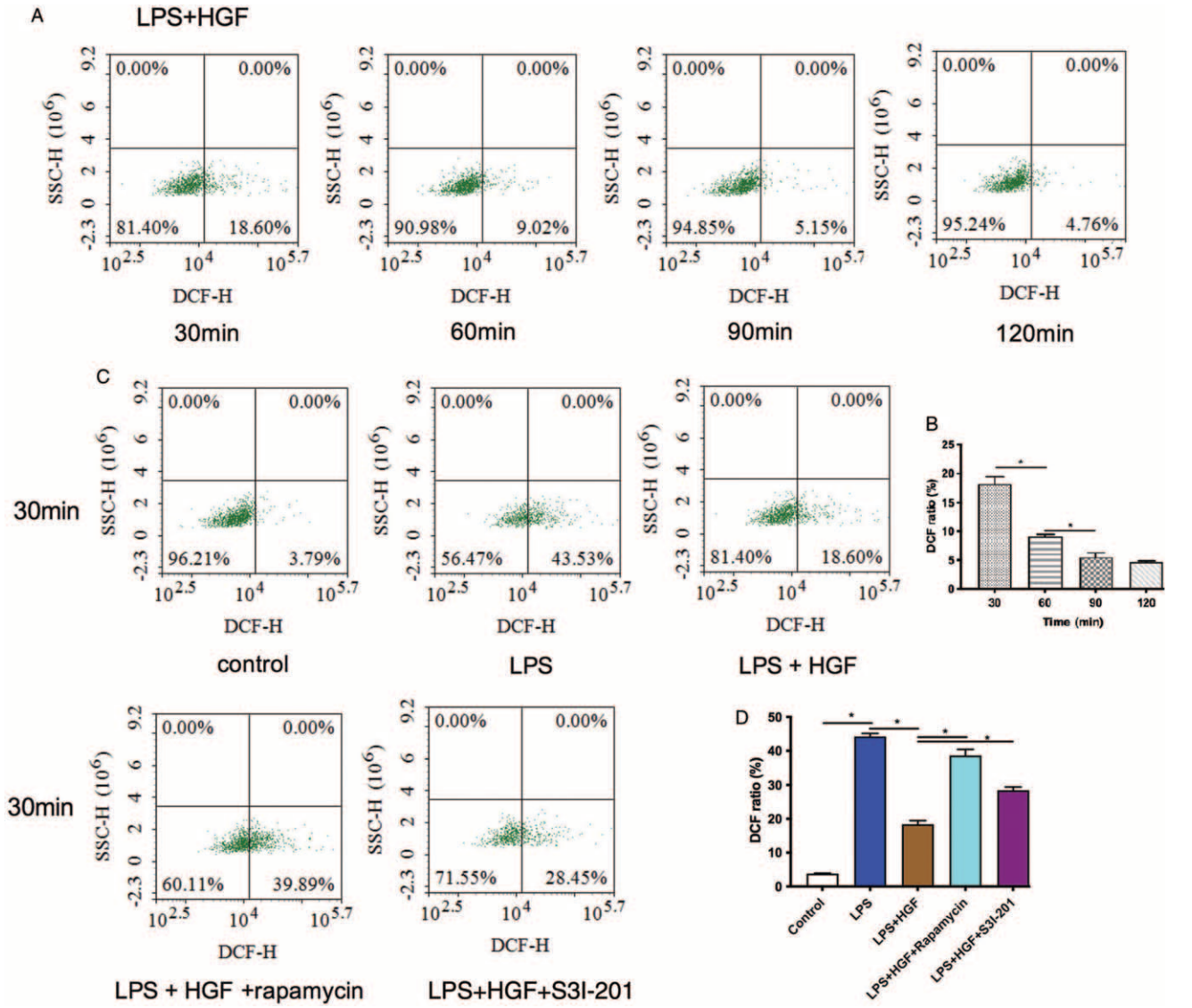

Figure 3: HGF exposure attenuated LPS-induced calcium increase triggering cellular ROS production in PMVECs via mTOR/STAT3 pathway. LPS treatment of PMVECs treated with or without HGF. Rapamycin and S3I-201 were, respectively, used to inhibit mTOR and STAT3 signaling. Intracellular ROS were detected by highly fluorescent DCF in flow cytometry. (A) DCF change of cellular ROS production in LPS-induced PMVECs with the HGF treatment test by flow cytometry for varying time points (30–120 min). (B) DCF ratio differences in LPS-induced PMVECs with HGF treatment test by flow cytometry for varying time points (30–120 min). (C) DCF change of cellular ROS production in LPS-induced PMVECs with HGF treatment via mTOR/STAT3 pathway test by flow cytometry (30 min). (D) DCF ratio differences in LPS-induced PMVECs with HGF treatment via mTOR/STAT3 pathway test by flow cytometry (30 min). Results are mean ± SD ($n = 3$). $^{*}P < 0.05$. DCF: Dichlorodihydrofluorescein; HGF: Hepatocyte growth factor; LPS: Lipopolysaccharide; mTOR: Mammalian target of rapamycin; PMVECs: Pulmonary microvascular endothelial cells; ROS: Reactive oxygen species; STAT3: Signal transducer and activator of transcription 3.

membrane potential of LPS-induced PMVECs. We used LPS-induced PMVECs treated with or without HGF for 6 h. mTOR and STAT3 signaling inhibitors rapamycin and S3I-201 were, respectively, used. Mitochondrial membrane loss was detected by JC-1 from a change in flow cytometry, and mitochondria tracker green was used for a mitochondria marker of live cells. Flow cytometry analysis showed that inhibition of mTOR and STAT3 changed red fluorescence to green fluorescence of JC-1 in LPS-induced PMVECs with HGF treatment, implying increased green fluorescence and declined mitochondrial membrane potential compared with LPS and HGF treatment [Figure 4A and 4B]. And HGF treatment decreased LPS-induced mitochondrial membrane loss in PMVECs [Figure 4A and 4B]. Furthermore, HGF-added mitochondria tracker green marked live cells than LPS alone and rapamycin S3I-201 treatment reversed the results [Figure 4C]. These gave the results that HGF decreased LPS-induced mitochondrial membrane loss in PMVECs via the mTOR/STAT3 pathway.

mTOR/STAT3 pathway improves specific mitochondrial protein expression and activity in LPS-induced PMVECs with HGF treatment

The mitochondria electron transport chain complexes play a pivotal role in the regulation of energy and ROS production, and complex I (NADH dehydrogenase) is an important component. In this study, we detected the complex I protein (NDUFB8) level by Western blotting (WB) and activity by complex I activity assay kit. First, we

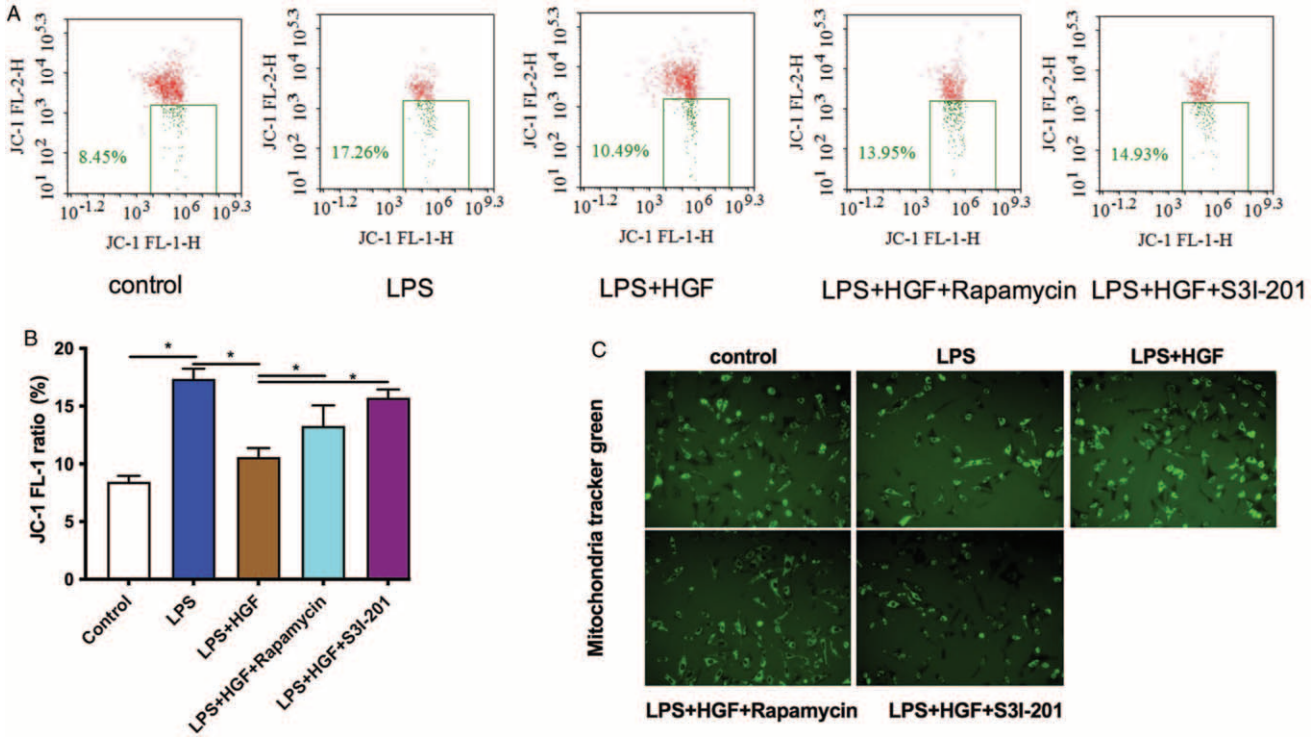

Figure 4: HGF decreased LPS-induced mitochondrial membrane loss in PMVECs via mTOR/STAT3 pathway. LPS treatment of PMVECs treated with or without HGF for 6 h. Rapamycin and S3I-201 were, respectively, used to inhibit mTOR and STAT3 signaling. Mitochondrial membrane loss was detected by JC-1 form change in flow cytometry. Mitochondria tracker green was a mitochondria marker of live cells. (A) The change of red fluorescence to green fluorescence of JC-1 in LPS-induced PMVECs with HGF treatment test by flow cytometry via mTOR/STAT3 pathway (12 h). (B) Quantitative analysis of red fluorescence changing to green fluorescence of JC-1 in LPS-induced PMVECs with HGF treatment test by flow cytometry via mTOR/STAT3 pathway (12 h). (C) Representative photomicrographs of mitochondria tracker green (24 h). Results are mean ± SD ($n = 3$). $^{*}P < 0.05$. HGF: Hepatocyte growth factor; LPS: Lipopolysaccharide; mTOR: Mammalian target of rapamycin; PMVECs: Pulmonary microvascular endothelial cells; STAT3: Signal transducer and activator of transcription 3.

established LPS-induced PMVECs treated with or without HGF for 24 h. Then, rapamycin and S3I-201 were, respectively, used to inhibit mTOR and STAT3 pathways. WB analysis [Figure 5A and 5B] of specific mitochondrial protein complex I expression in LPS-induced PMVECs was ascended by HGF treatment, and mTOR/STAT3 pathway inhibited HGF increasing effects. Interestingly, activity analysis of specific mitochondrial protein complex I in LPS-induced PMVECs was also done with HGF treatment via the mTOR/STAT3 pathway [Figure 5C]. In brief, specific mitochondrial protein expression and activity in LPS-induced PMVECs were raised with HGF treatment via the mTOR/STAT3 pathway.

HGF via mTOR/STAT3 signaling decreases LPS-induced PMVEC apoptosis

Bcl-2 (localized to the outer membrane of mitochondria) and Bcl-xL (localized to the transmembrane of mitochondria) act as an important role in promoting cellular survival and inhibiting the actions of proapoptotic proteins. We used PMVECs with LPS and HGF treatment for 24 h. And rapamycin and S3I-201 were, respectively, used to be mTOR and STAT3 inhibitors. PMVEC apoptosis-relevant mRNA expression was tested by RT-qPCR. RT-qPCR analysis [Figure 6] showed that anti-apoptosis Bcl-2 and Bcl-xL mRNA relative expressions were raised by HGF treatment and mTOR/STAT3 inhibitors prevented it. Importantly, mTOR/STAT3 signaling was mediated by decreasing LPS-induced PMVEC apoptosis with HGF treatment.

Endothelial adherent proteins were altered in LPS-induced PMVECs by HGF treatment and reversedby mTOR/STAT3 inhibitors

VE-cadherin and occludin are, respectively, critical endothelial adherent and tight junction proteins which protect against injury from various stimulations. Here, we used LPS-induced PMVECs treated with or without HGF for 24 h. Rapamycin and S3I-201 were, respectively, used to inhibit mTOR and STAT3 signaling. WB analysis was carried out to evaluate the adherent protein expression level [Figure 7]. It showed that LPS-injured VE-cadherin and occludin could be raised by HGF treatment. In contrast, their expression levels were downregulated by mTOR inhibitor rapamycin or STAT3 inhibitor S3I-201. It implied that injured adherent protein VE-cadherin and tight junction occludin made a loss of the endothelial complex.

Both mTORC1 and mTORC2 mediate HGF protective effects against LPS-induced PMVEC mitochondria damage and apoptosis

mTOR has two distinct multiprotein complexes, mTORC1 (Raptor) and mTORC2 (Rictor), that regulate different effects in cell activity. To explore the detailed mechanisms of them, we used lentiviral mediated Raptor and Rictor

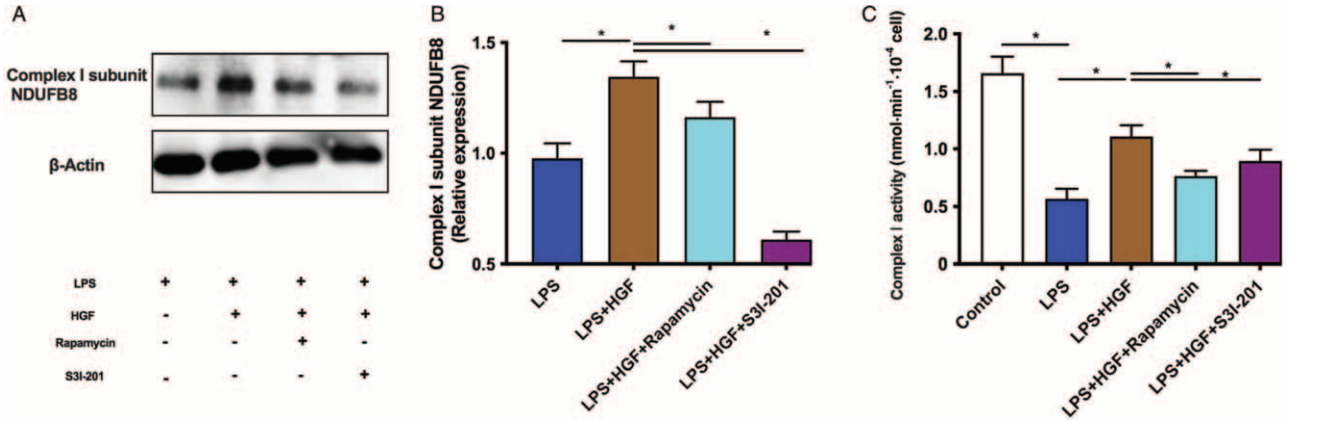

Figure 5: Changes in expression of specific mitochondrial protein and activity in LPS-induced PMVECs with HGF treatment in response to mTOR/STAT3 inhibitors. LPS treatment of PMVECs treated with or without HGF for 24 h. Rapamycin and S3I-201 were, respectively, used to inhibit mTOR and STAT3 signaling. Specific mitochondrial protein complex I, which was also the largest of the five complexes of the electron transport chain, was tested by WB analysis, and complex I activity was detected by complex I activity assay kit. (A) WB analysis of specific mitochondrial protein complex I expression in LPS-induced PMVECs with HGF treatment via mTOR/STAT3 pathway (24 h). (B) WB analysis of mitochondrial protein complex I expression relative expression in LPS-induced PMVECs with HGF treatment via mTOR/STAT3 pathway (24 h). (C) Activity analysis of specific mitochondrial protein complex I in LPS-induced PMVECs with HGF treatment via mTOR/STAT3 pathway (24 h). Results are mean ± SD ($n = 3$). $^{*}P < 0.05$. HGF: Hepatocyte growth factor; LPS: Lipopolysaccharide; mTOR: Mammalian target of rapamycin; PMVECs: Pulmonary microvascular endothelial cells; STAT3: Signal transducer and activator of transcription 3; WB: Western blotting.

knockdown in PMVECs. We used PMVECs with LPS and HGF treatment for 24 h and detected mitochondria damage and apoptosis, respectively, by complex I activity and RT-qPCR analysis. In the figure [Figure 8A], complex I activity showed that Raptor and Rictor knockdown decreased mitochondria complex I activity in LPS-induced PMVEC damage in 24 h. RT-qPCR analysis [Figure 8B and 8C] showed that anti-apoptosis Bcl-2 and Bcl-xL mRNA relative expressions were raised by HGF treatment, and knockdown of mTORC1 and mTORC2 prevented the effect in 24 h. Collectively, mTORC1 and mTORC2 signaling affected HGF protective effects in LPS-induced PMVEC mitochondria damage and apoptosis.

Inhibition of mTOR and STAT3 raises mitochondrion damage in ALI pulmonary endothelial barrier

We used LPS (5 mg/kg) intra-tracheal injection to mice for the ALI model. HGF (1 mg/kg) and inhibitors (4 mg/kg rapamycin and 5 mg/kg S3I-201) were injected into the tail vein 4 h after ALI challenge. Mice have experimented after injections for 24 h treatment. Lung tissues from ALI mice models were observed with electron microscopy. Electron microscopy observation [Figure 9] showed that mitochondrion structure integrity of pulmonary endothelium in ALI mice with HGF treatment increased compared with rapamycin or S3I-201 groups. Importantly, activation of mTOR and STAT3 signaling was critical for mitochondrion recovery in ALI.

Discussion

Previous studies have demonstrated that HGF could decrease endothelial apoptosis.[3,4] Research studies point toward mitochondrial oxidative damage and apoptosis as important contributors to several pathological conditions associated with endothelial injury.[19,20] The efficacy of bone marrow stem cells in endothelial barrier recovery was demonstrated by our previous experiments.[3,4] Nevertheless, little is known about the protective effects of HGF mediated on endothelium via oxidative express and mitochondria-dependent apoptosis as a potential mechanism. In the current study, we showed that HGF restrains the oxidative level, leading to the suppression of intracellular calcium mobilization and subsequent generation of ROS, which corrects mitochondrial damage and endothelially impaired induced apoptosis and integrity, and mTOR/STAT3 contributed to it in ARDS.

Calcium, a secondary messenger of intracellular signaling, is the key link coupling cellular and mitochondria energy production. Intracellular calcium balance maintains normal cellular physiological activities and is interdependent with mitochondrial function.[21] Intracellular calcium overload could consume large amounts of adenosine triphosphate leading to mitochondrial function dysfunction and oxidative phosphorylation disorder. In turn, increased damaged mitochondria permeability allow more calcium entry into the cells and form vicious circles. Oxidative stress refers to the imbalance of oxidative and anti-oxidative effects, which tends to be oxidized and produces large numbers of oxidative intermediates. Reactive oxygen, which is produced by oxidative stress, is considered to be a critical factor leading to cell senescence, endothelial injury, and diseases. The mitochondria are the main parts of ROS production. The activity of mitochondrial respiratory chain enzyme system and the production of ROS are far more than the sum of all other enzymes, and > 95% of the reactive oxygen in the cell comes from the mitochondria. As important secondary messages, ROS act as mediators of physiology[22]; however, ROS overproduction can impair the endothelial barrier. Reports identified that ROS-induced mTORC1 signaling was involved in autophagy, which is an integration node for cellular metabolism, protein synthesis, and cell survival.[23] And STAT3 can also enhance interference of ROS production and lead to mitochondria damage and calcium influx.[24] Because of the unknown

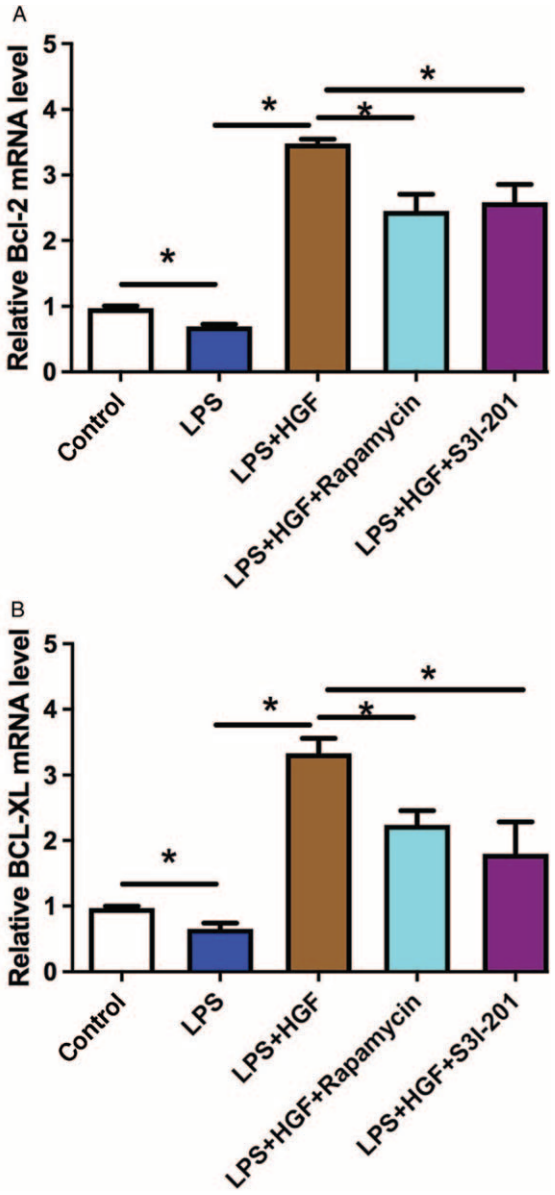

Figure 6: Change in expression of LPS-induced PMVEC apoptosis-relevant mRNAs and proteins with HGF treatment via mTOR/STAT3 signaling. LPS treatment of PMVECs treated with or without HGF for 24 h. Rapamycin and S3I-201 were, respectively, used to inhibit mTOR and STAT3 signaling. PMVEC apoptosis-relevant mRNA expression and protein were, respectively, tested by RT-qPCR and WB analysis. (A) RT-qPCR analysis of Bcl-2 mRNA relative expression in LPS-induced PMVEC apoptosis with HGF treatment via mTOR/STAT3 pathway (24 h). (B) RT-qPCR analysis of Bcl-xL mRNA relative expression in LPS-induced PMVEC apoptosis w ith HGF treatment via mTOR/STAT3 pathway (24 h). Results are mean ± SD ($n = 3$). $^{*}P < 0.05$. HGF: Hepatocyte growth factor; LPS; Lipopolysaccharide; mTOR: Mammalian target of rapamycin; PMVECs: Pulmonary microvascular endothelial cells; RT-qPCR: Real-time quantitative PCR; STAT3: Signal transducer and activator of transcription 3; WB: Western blotting.

suppression mechanisms of HGF on endothelium, we tested the level of intracellular calcium and ROS production, which in turn trigger mitochondrial oxidative damage and dysfunction. Our data indicated that HGF decreased cellular calcium entry which responded to a decrease in intercellular ROS levels which were mediated by mTOR/STAT3 signaling. Interestingly, we found that cytosolic calcium levels significantly decreased in response to mTOR or S3I-201 inhibition with HGF treatment. Mitochondria are damaged following with decreased mitochondrial membrane potential and mitochondrial membrane protein. The mitochondrial membrane potential assures major bioenergetic function of the mitochondrion, and mitochondrial membrane potential collapse contributes to the loss of cellular functions.[25,26] Common mitochondrial fluorescent probes include MitoTracker and JC-1, which have lipophilic and penetrating properties. To investigate the effects of HGF exposure and mTOR/STAT3 signaling on PMVECs, we measured mitochondrial membrane potential using two-mitochondrial probes, mitochondria tracker green, and a fluorescent cationic dye, JC-1, that effectively detect a change in membrane potential. The lipophilic dye JC-1 form aggregates in healthy cells, which stains the mitochondria red. In conditions where there is a decrease in mitochondrial membrane potential, the dye leaks from the mitochondria, remains in its monomeric form, and appears green. The electron transport chain complexes of the mitochondria play a pivotal role in the regulation of energy production. Respiratory chain enzymes of complexes I (NADH dehydrogenase) are the major source of ROS production.[27-29] mTOR and STAT3 pathway mediated damaged mitochondria with varied stimulations, and mTOR/STAT3 played a major role in coupling mitochondrial functions and translation.[9,18] Based on these findings, we found that HGF could also protect endothelium against injured mitochondria, rising mitochondria tracker green dye and JC-1 red ratio. HGF exposure of LPS-induced endothelial barrier resulted in raised expression of complex I (NADH dehydrogenase) subunit NDUFB8. Similar findings of decreased activity of mitochondrial respiratory complex I in endothelial injury were observed, and it reversely improved after HGF treatment. mTOR/STAT3 inhibited HGF protective effects. Furthermore, the lentiviral experiment demonstrated that mTORC1 and mTORC2 have the same effects in HGF protection against LPS-induced mitochondria damage. In *in vivo* experiments, we also used ALI models to observe the changes in mitochondrial structure. We further proved mTOR and STAT3 activation in HGF treatment to mitochondria recovery.

Apoptosis is a form of programmed cell death. Apoptotic proteins that target mitochondria may cause mitochondrial swelling, increase the permeability of the mitochondrial membrane, decrease mitochondrial membrane potential, and cause apoptotic effectors to leak out. The destruction of mitochondrial membrane potential is a landmark event in early apoptosis. In mammalian cells, a balance between proapoptotic (BAX, BID, BAK, or BAD) and anti-apoptotic (Bcl-xL and Bcl-2) members of the Bcl-2 family is established. The bcl-2 protein family is the main component that regulates mitochondrial permeability. Respectively, bcl-2 exerts its survival function by preventing mitochondrial cytochrome c release under different apoptotic stimulation and is related to the regulation of mitochondrial calcium homeostasis; Bcl-xL prevents apoptosis through the formation of apoptotic protein heterodimers and the normal mitochondrial membrane state under stress. STAT3 is in a down target of mTOR and decreases endothelial apoptosis with decreasing apoptosis genes and proteins. In the liver

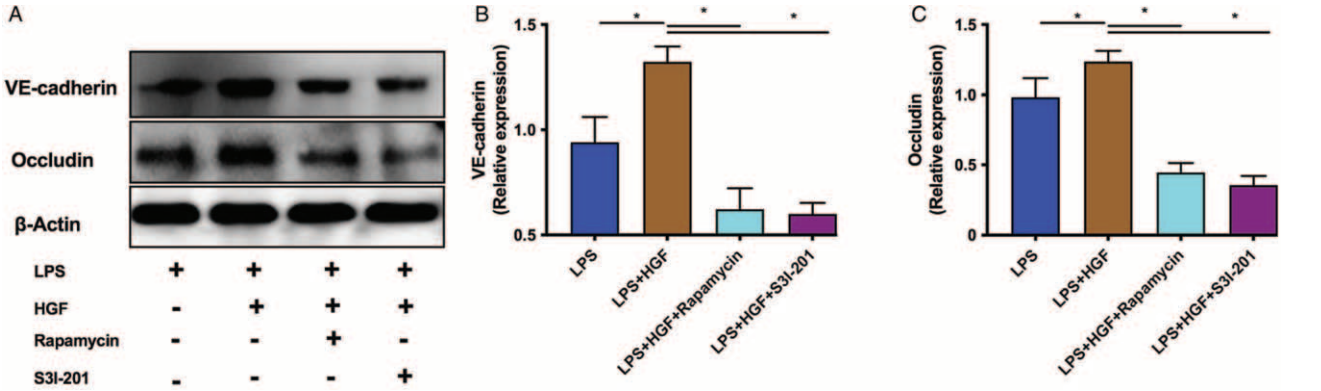

Figure 7: Changes in expression of endothelial adherent protein in LPS-induced PMVECs with HGF treatment in response to mTOR/STAT3 inhibitors. LPS treatment of PMVECs treated with or without HGF for 24 h. Rapamycin and S3I-201 were, respectively, used to inhibit mTOR and STAT3 signaling. Endothelial adherent protein VE-cadherin and tight junction protein occludin were tested by WB analysis with 24 h treatment. (A) WB analysis of VE-cadherin and occludin in LPS-induced PMVECs with HGF treatment via mTOR/STAT3 pathway (24 h). (B) WB analysis of VE-cadherin relative expression in LPS-induced PMVECs with HGF treatment via mTOR/STAT3 pathway (24 h). (C) WB analysis of occludin relative expression in LPS-induced PMVECs with HGF treatment via mTOR/STAT3 pathway (24 h). Results are mean ± SD ($n = 3$). $^{*}P < 0.05$. HGF: Hepatocyte growth factor; LPS: Lipopolysaccharide; mTOR: Mammalian target of rapamycin; PMVECs: Pulmonary microvascular endothelial cells; STAT3: Signal transducer and activator of transcription 3; WB: Western blotting.

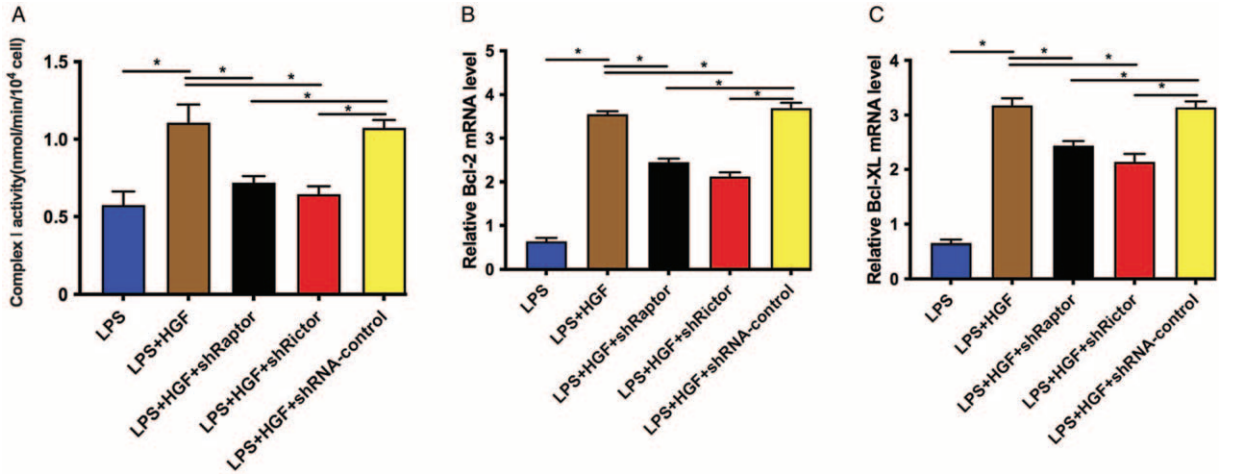

Figure 8: Protective effects of HGF via mTORC1 and mTORC2 in LPS-induced PMVEC mitochondria damage and apoptosis. Lentivirus vector-mediated Raptor and Rictor knockdown in PMVECs (shRaptor and shRictor as knockdown, shRNA control as negative control) were conducted. LPS treatment of PMVECs treated with or without HGF for 24 h. PMVEC mitochondria damage and apoptosis were, respectively, detected by complex I activity and RT-qPCR. (A) Activity analysis of specific mitochondrial protein complex I in LPS-induced PMVECs with HGF treatment via mTORC1 and mTORC2 signaling (24 h). (B) RT-qPCR analysis of Bcl-2 mRNA relative expression with HGF treatment in LPS-induced PMVEC apoptosis via mTORC1 and mTORC2 signaling (24 h). (C) RT-qPCR analysis of Bcl-xL mRNA relative expression with HGF treatment in LPS-induced PMVEC apoptosis via mTORC1 and mTORC2 signaling (24 h). Results are mean ± SD ($n = 3$). $^{*}P < 0.05$. HGF: Hepatocyte growth factor; LPS: Lipopolysaccharide; mTOR: Mammalian target of rapamycin; PMVECs: Pulmonary microvascular endothelial cells; RT-qPCR: Real-time quantitative PCR.

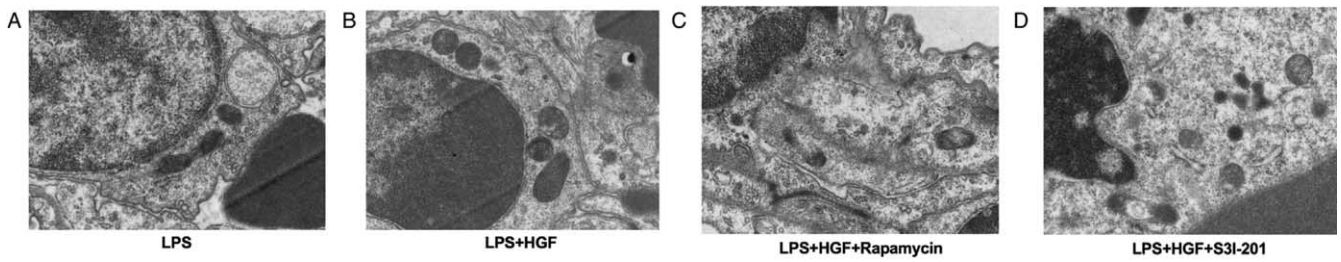

Figure 9: Mitochondria changes observed with electron microscopy for ALI mice pulmonary endothelial barrier with HGF treatment in response to mTOR/STAT3 inhibitors. LPS intratracheal injection to mice for ALI model. HGF and inhibitors (rapamycin and S3I-201) were injected into the tail vein 4 h after ALI challenge. Lung tissues from ALI mice models were observed with electron microscopy after injections of 24 h treatment (scale bar 2 μm). (A) Mitochondrion structure of the pulmonary endothelium in ALI mice. (B) Mitochondrion structure of pulmonary endothelium in ALI mice with HGF treatment. (C) Rapamycin effects on mitochondrion structure of pulmonary endothelium in ALI mice with HGF treatment. (D) S3I-201 effects on mitochondrion structure of pulmonary endothelium in ALI mice with HGF treatment. ALI: Acute lung injury; HGF: Hepatocyte growth factor; LPS: Lipopolysaccharide; mTOR: Mammalian target of rapamycin; STAT3: Signal transducer and activator of transcription 3.

injury mouse model, STAT3 could enhance Bcl-2 and Bcl-xL levels involved in liver development and regeneration.[30] Hence, we investigated the BCL-2/BCL-XL mRNA level in LPS-induced PMVECs with HGF treatment. Interestingly, HGF protective effects raising Bcl-2/Bcl-xL were inhibited by mTOR inhibitor or STAT3 inhibitor S3I-201. Both mTORC1 and mTORC2 could contribute to the Bcl-2/Bcl-xL mRNA level in LPS-induced endothelial apoptosis with HGF treatment. Collectively, mTOR/STAT3 pathway mediated HGF anti-apoptotic effects in the endothelium.

The integrity of intercellular junctions is a major determinant of endothelial permeability, and the VE-cadherin-based adherent and occludin-based tight junctions are thought to be particularly important.[31,32] VE-cadherin is required for maintaining adherent endothelial barrier and blockage of VE-cadherin with antibodies increasing monolayer permeability.[31] Occludin is an important protein in tight junction function. Disruption of VE-cadherin and occludin regulation are important aspects of several diseases. In this experiment, we found that endothelial cells function with mitochondrial injury, and apoptosis was also changed with decreased VE-cadherin-based adherent and occludin-based tight junction, and it was related with mTOR/STAT3 pathway. Furthermore, we applied ALI mouse experiments. It demonstrated that HGF also increased mitochondria structural integrity via the mTOR/STAT3 pathway.

In summary, our data suggest that the protective effects of HGF to endothelial barrier are evaluated in the suppression of ROS production and intracellular calcium uptake, which upregulate the mitochondrial membrane potential, specific complex I proteins, and endothelial junction proteins to avoid oxidative stress damage and mitochondria-dependent apoptosis. These reveal that HGF restrained the oxidative level, mitochondrial damage, apoptosis, and pulmonary endothelial barrier integrity in ARDS.

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Conflicts of interest

None.

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