Hepatocyte Growth Factor Protects Endothelial Barrier Against Oxidative Stress and Mitochondria-dependent Apoptosis

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Research

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Abstract

Background: Pulmonary microvascular endothelial cells(PMVECs) were incomplex and 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) could decrease endothelial apoptosis. Nevertheless, the mechanism by which HGF-suppressed oxidative stress contributes to endothelial mitochondria-dependent apoptosis is poorly understood.

Methods: In our current study, we introduced lipopolysaccharide(LPS)-induced PMEVCs with HGF treatment. To investigate the effects of mTOR/STAT-3 pathway in endothelial oxidative stress and mitochondria-dependent apoptosis, mammalian TOR (mTOR) inhibitor rapamycin and signal transducer and activator of transcription 3 (STAT-3) inhibitor S3I-201 were respectively used to inhibit mTOR/STAT-3 signaling. Moreover, lentivirus vector-mediated HGF, mTORC1(raptor) and mTORC2(rictor) gene knockdown modification were introduced to evaluate mTORC1 and mTORC2 pathway. Calcium measurement, ROS production, mitochondrial membrane potential and protein complex I expression, cell proliferation, apoptosis and endothelial junction protein were detected to evaluate HGF effects.

Results: Our study demonstrated that HGF protected endothelium via the suppression of ROS production and intracellular calcium uptake, which leading to increased mitochondrial membrane potential (JC-1 and mitochondria tracker green detection)and specific proteins(complex I), decreased endothelial apoptosis specific protein(Caspase-3), raised anti-apoptosis mRNA level(Bcl-2 and Bcl-xL), and increased endothelial junction proteins (VE-cadherin and occludin). Reversely, mTOR inhibitor rapamycin and STAT-3 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.

Conclusion: In all, these reveal that mTOR/STAT-3 signaling mediate the HGF suppression effects to oxidative level, mitochondria-dependent apoptosis and endothelial junction protein in LPS-stimulated PMVECs, which contributing to the endothelial survival and barrier integrity.

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 hepatocyte growth factor (HGF) 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 regulation of endothelial recovery.

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 mitochondrial entry, calcium can enhance the tricarboxylic acid cycle dehydrogenases activity and raise the mitochondrial complex I substrate, NADH, to stimulate 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 with decreased mitochondrial membrane potential and destroyed mitochondrial membrane protein. When mitochondria are damaged, mitochondria-dependent apoptotic pathway was activated and cause 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), DEPTOR, mLST8 and PRAS40, and regulates cell growth through the 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 Akt and PKC 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 down-regulate mitochondrial biogenesis and respiration [9] .

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

Here, we investigated whether mTOR/STAT-3 signaling acted in HGF protective effects against oxidative stress and mitochondria-dependent apoptosis in lipopolysaccharide (LPS)-induced endothelial barrier dysfunction. The effects of recombinant murine HGF on endothelial cell barrier dysfunction stimulated by gram-negative bacterial pathogen lipopolysaccharide (LPS) in vitro were introduced. mTOR inhibitor rapamycin and STAT-3 inhibitor S3I-201 were respectively used to abort HGF-suppression effects of oxidative stress and mitochondria-dependent apoptosis.

Materials And Methods

Cell culture and reagents

PMVECs were obtained from Shanghai Zhen Biotechnology Company Limited (Shanghai, China) and surface marker CD31 was identified by flow cytometer. PMVECs were cultured in endothelial growth medium (Wisent, Nanjing, China) and humidified 5% CO2 incubator at 37 ℃. Culture media were changed every 2-3 days according to cell growth status. We used gram-negative bacterial pathogen lipopolysaccharide (LPS, 100ng/ml, Sigma, USA) to stimulate PMVECs in vitro for mimic ARDS environment. And recombinant murine HGF (20ng/ml, ProSpec, Israel) were introduced to explore detailed mechanisms. PBS was applied as negative control and mTOR inhibitor rapamycin (100 nmol/l, Selleck, USA) or STAT-3 inhibitor S3I-201 (100 nmol/l, Selleck) was used to inhibit the activation of mTOR or STAT-3 pathway in LPS-induced PMVECs with HGF treatment.

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 EGFP was acted as a negative control. Passages less than 6 cells were used and the lentivirus was packaged in 293T cells (Cyagen Biosciences) with the aid of three pachaging plasmids to obtain a higher titer of lentivirus. Then we harvested PMVECs carrying EGFP (shRNA-control) and both the target gene (shRaptor, shRictor). Transfection efficiency were detected by real-time quantitative polymerase chain reaction and expression efficiency were tested by fluorescence microscope.

Calcium measurement

Fluo-4 AM is fluorescence probe usually used to detect cellular calcium concentration. Fluo-4 can combine with calcium leading to strong fluorescence. PMVECs were loaded with 2 umol/l Fluo-4 AM(Beyotime Biotechnology，China) in extracellular medium for 30 min at 37 ℃. Fluorescein intensity was record with flow cytometry (ACEA NovoCyte, China) after 1min of baseline recording. PMVECs were stimulated with LPS or HGF and fluorescein intensity were acquired after 1 min of basing recording. Moreover, 200 seconds 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 fluorescent. PMVECs were incubated with DCFH-DA(10 umol/l) in serum-free culture medium for 20min at 37 ℃ 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 488 nm wave-length. Drugs were loaded with 30min-2h respectively and data were collected and analyzed with using NovoExpress (ACEA NovoCyte).

JC-1 assay

JC - 1 is a ideal fluorescent probe used in the detection of mitochondrial membrane potential. Early apoptosis is characterized by mitochondrial membrane potential drop and fluorescent from red(aggregates) to green(monomer) fluorescence by JC - 1 shift can be easily detected as apoptosis index of an early detection. PMVECs were resuspended and centrifuged to collect and then incubated with JC-1 working solution(Beyotime Biotechnology，China) for 20min at 37 ℃ in the dark. PMVECs were washed by JC-1 dye buffer (1X, Beyotime Biotechnology) and centrifuged at 600g, 4 ℃, 4min. Finally, cells were incubated in 0.5ml 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 of cell culture and incubated with mitochondria tracker green for 30min at 37 ℃ in the dark. Then PMVECs were centrifuged and resuspended with 37 ℃ fresh cell culture. Subsequent observations were made with 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) 1 beta subcomplex subunit 8 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 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 incubation them with mitochondrial complex I activity assay buffer and tested with microplate reader(Infinite M200 Pro, Tecan, Switzerland). Data of baseline and 2 min treatment OD valve were recorded and analysis for mitochondrial complex I activity(nmol/min/10^4 cell).

Annexin V-PE/7-AAD stained flow cytometry

Cell apoptosis were 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 per 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 analysed by 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.

CCK8 assays

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

Western blot (WB) analysis

Cell lysates were collected in RIPA buffer supplemented with a protease and phosphatase inhibitor and phenylmethanesulfonyl fluoride (Beyotime), and then the reaction mixtures were cleared by centrifugation (12000 g for 30 min at 4 °C). Protein were separated with SDS-PAGE condensed electrophoresis (Beyotime) and transferred to immune-Blot PVDF membranes (Beyotime). Membranes were blocked with 5% BSA(Beyotime) in Tris-buffered saline with 0.1% Tween 20 for 1h at room temperature and incubated overnight at 4 ℃ with the following commercially available primary antibodies against Complex I subunit NDUFB8(Abcam; 1:1000), Cleaved-Caspase-3(1:1000;Cell Signaling), Caspase-3(1:1000;Cell Signaling), 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 were extracted with TriPure Isolation Reagent (Roch, Switzerland), and reverse transcriptase was applied (Thermo Fisher Scientific, USA) for cDNA synthesis. Real-time quantitative PCR 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';Bcl-xL, sense 5'-GCTTCACATAACCCCAGG -3' and antisense 5'- GCAATCCGACTCACCAAT -3'. Results were calculated using the $2^{-\triangle\triangle CT}$ method with β-actin as a control.

Statistical analyses

The statistical significance of the data was performed using Graphpad Prism 5 and SPSS 19.0. Data were conducted by Tukey's multiple comparison test, one-way analysis of variance and Student's t-test. $p < 0.05$ was regarded as significant statistic differences.

Results

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

First, we investigated the protective effects of HGF on LPS-induced PMVECs survival and integrity. LPS (100ng/ml)-induced PMVECs were treated with or without HGF(20ng/ml) for 0-24h. PMVECs apoptosis

and proliferation were respectively tested by Annexin V-PE/7-AAD staining with flow cytometry and CCK-8 kit. Endothelial tight junction protein ZO-1 was tested by immunofluorescence. It revealed that LPS-induced PMVECs early apoptosis ratio with HGF treatment(1h, 2h, 4h) were gradually decreasing in flow cytometry analysis(Fig. 1A and 1B). Reversely, cell proliferation of LPS-induced PMVECs treated with HGF treatment (4h, 12h and 24h) were gradually rising(Fig. 1C). Immunofluorescence analysis showed that endothelial tight junction protein ZO-1 in LPS-induced PMVECs with 24h HGF treatment were unregulated compared with LPS alone (Fig. 1D). Collectively, these data demonstrated that HGF could suppresses LPS-induced PMVECs injury via decreasing apoptosis and rising the endothelial tight junction protein ZO-1 integrity.

HGF exposure decreases cytosolic calcium levels in PMVECs via mTOR/STAT-3 pathway.

As described above, HGF could decrease PMVECs 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 20s with flow cytometry. It showed that fluo-4 fluoresence change of cytosolic calcium in LPS induced PMVECs were decreased after HGF treatment(Fig. 2A), implying that HGF exposure down-regulated intracellular calcium overload. However, the detailed mechanism of these phenomenon was not certain. Rapamycin and S3I-201 were respectively used to inhibit mTOR and STAT-3 signaling. mTOR and STAT-3 inhibitors could reverse HGF suppress effects of calcium uptake in 200s treatment tested by flow cytometry(Fig. 2B and 2C). Notably, HGF played a protective endothelial role by decreasing cytosolic calcium levels and mTOR/STAT-3 contributed to it.

mTOR/STAT-3 pathway upregulates cellular ROS production triggered by oxidative stress in LPS-induced PMVECs with HGF treatment.

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 were gradually decreasing with prolong HGF treatment(30, 60, 90 and 120min) detected by flow cytometry. To assess the contribution signaling of cellular ROS production, rapamycin and S3I-201 were respectively used to inhibit mTOR and STAT-3 signaling. Intracellular ROS were detected by high fluorescent DCF in flow cytometry. In 30min drug treatment, mTOR inhibitor rapamycin reversed HGF suppression effects of ROS production(Fig. 3C and 3D). Similarly, STAT-3 inhibitor S3I-201 gave the same effects(Fig. 3C and 3D). Taken together, these suggested that mTOR/STAT-3 pathway contributed to HGF exposure attenuated cellular ROS production in LPS-induced PMVECs.

mTOR/STAT-3 pathway contributes 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 STAT-3 pathway were mediated in mitochondrial membrane potential of LPS-induced PMVECs. We used LPS-induced PMVECs treated with or without HGF for 6h. mTOR and STAT-3 signaling inhibitiors rapamycin and S3I-201 were respectively used. Mitochondrial membrane loss was detected by JC-1 form change in flow cytometry and mitochondria tracker green was used for a mitochondria marker of live cells. Flow cytometry analysis showed that inhibition mTOR and STAT-3 gave change of red fluorescence to green fluorescence of JC-1 in LPS-induced PMVECs with HGF treatment, implying to increased green fluorescence and declined mitochondrial membrane potential compared with LPS and HGF treatment(Fig. 4A and 4B). And HGF treatment decreased LPS-induced mitochondrial membrane loss in PMVECs(Fig. 4A and 4B). Furthermore, HGF added mitochondria tracker green marked live cells than LPS alone and rapamycin /S3I-201 treatment reversed the results(Fig. 4C). These gave the results that HGF decreased LPS-induced mitochondrial membrane loss in PMVECs via mTOR/STAT-3 pathway.

mTOR/STAT-3 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 regulation of energy and ROS production and complexes I (NADH-dehydrogenase) is an important component. In this study, we detected complexes I protein level by western blot and activity by complex I activity assay kit. First, we established LPS-induced PMVECs treated with or without HGF for 24h. Then rapamycin and S3I-201 were respectively used to inhibit mTOR and STAT-3 pathway. WB analysis of specific mitochondrial protein complex I expression in LPS-induced PMVECs were ascended by HGF treatment and mTOR/STAT-3 pathway inhibited HGF increasing effects. Interestingly, activity analysis of specific mitochondrial protein complex I in LPS-induced PMVECs were also raised with HGF treatment via mTOR/STAT-3 pathway. In brief, specific mitochondrial protein expression and activity in LPS-induced PMVECs were raised with HGF treatment via mTOR/STAT-3 pathway.

HGF via mTOR/STAT-3 signaling decreases LPS-induced PMVECs apoptosis.

Caspase, which is a family of cysteine proteases, are the central regulators of apoptosis. Caspase-3 can be activated to execute apoptosis by cleaving targeted cellular proteins. 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 pro-apoptotic proteins. We used PMVECs with LPS and HGF treatment for 24h. And rapamycin and S3I-201 were respectively used to be mTOR and STAT-3 inhibitors. PMVECs apoptosis relevant mRNA expression and protein were respectively tested by RT-qPCR and WB analysis. In the figure (Fig. 6), WB analysis showed that mTOR and STAT-3 inhibitors increased LPS-induced PMVECs apoptosis protein Caspase-3 and Cleaved-Caspase-3 with HGF treatment(Fig. 6A). RT-qPCR analysis (Fig. 6B and 6C) showed that anti-apoptosis Bcl-2 and Bcl-xL mRNA relative expression were raised by HGF treatment and mTOR/STAT-3 inhibitors prevented it. Importantly,

mTOR/STAT-3 signaling was mediated by decreasing LPS-induced PMVECs apoptosis with HGF treatment.

Endothelial adherent proteins are altered in LPS-induced PMVECs by HGF treatment and mTOR/STAT-3 inhibitors reversed it.

VE-cadherin and occludin are respectively critical endothelial adherent and tight junction protein which protect against injury from various stimulations. Here, we used LPS-induced PMVECs treated with or without HGF for 24h. Rapamycin and S3I-201 were respectively used to inhibit mTOR and STAT-3 signaling. WB analysis were detected to evaluate adherent proteins expression level. It showed that LPS injured VE-cadherin and occludin could be raised by HGF treatment. In contrast, their expression levels were down-regulated by mTOR inhibitor rapamycin or STAT-3 inhibitor S3I-201. It implied that injured adherent protein VE-cadherin and tight junction occludin made loss of endothelial complex.

Both mTORC1 and mTORC2 mediates HGF protective effects against LPS-induced PMVECs mitochondria damage and apoptosis.

mTOR have two distinct multiprotein complexes, mTORC1(Raptor) and mTORC2(Rictor), that regulate different effects in cell activity. In order to explore the detailed mechanisms of them, we used lentiviral mediated Raptor and Rictor knockdown in PMVECs. We used PMVECs with LPS and HGF treatment for 24h and detected mitochondria damage and apoptosis respectively evaluated by Complex I activity and RT-qPCR analysis. In the figure (Fig. 8A), Complex I activity showed that raptor and rictor knockdown decreased mitochondria complex I activity in LPS-induced PMVECs damage in 24h. RT-qPCR analysis (Fig. 8B and 8C) showed that anti-apoptosis Bcl-2 and Bcl-xL mRNA relative expression were raised by HGF treatment and knockdown of mTORC1 and mTORC2 prevented the effect in 24h. Collectively, mTORC1 and mTORC2 signaling affected HGF protective effects in LPS-induced PMVECs mitochondria damage and apoptosis.

Discussion

Previous studies have demonstrated that HGF could decrease endothelial apoptosis[3, 4]. Researches point toward mitochondrial oxidative damage and apoptosis as important contributors to a number of pathological conditions associated with endothelial injury[19, 20]. 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 oxidative level in PMVECs, leading to the suppression of intracellular calcium mobilization and subsequent generation of ROS, which corrects with mitochondrial damage and endothelial impaired induced apoptosis and integrity, and mTOR/STAT-3 contributed to it.

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 interdependent with mitochondrial function[21]. Intracellular calcium overload could

consume large amounts of ATP 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 circle. 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 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 more than 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 cam impair 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 STAT-3 can also enhance interference of ROS production and lead mitochondria damage and calcium influx[24]. Because of the unknown 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 mediated by mTOR/STAT-3 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/STAT-3 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 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 regulation of energy production. Respiratory chain enzymes of complexes I (NADH-dehydrogenase) is the major source of ROS production[27]. mTOR and STAT-3 pathway mediated damaged mitochondria with varied stimulations and mTOR/STAT-3 played a major role in coupling mitochondrial functions and translation[9, 18]. Based on these findings, we found 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 was resulted in raised expression of complex I (NADH dehydrogenase) subunit NDUFB8. Similar findings of decreased activity of mitochondrial respiratory complex I in endothelial injury and it reversely improved after HGF treatment. mTOR/STAT-3 inhibited HGF protective effects. Furthermore, lentiviral experiment demonstrated that mTORC1 and mTORC2 have the same effects in HGF protective against LPS-induced mitochondria damage.

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. Apoptosis of various tissues activates the caspase family. Caspases play the central role in the transduction of apoptotic signals and caspase-3 is a key executor of apoptosis. Caspase-3 can be activated in the apoptotic cell both by mitochondrial pathways[28, 29]. Following caspase-3 activation 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 are 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 proteins heterodimers, and the normal mitochondrial membrane state under stress. STAT-3 is in a down target of mTOR and decrease endothelial apoptosis with decreasing apoptosis genes and proteins. In liver injury mouse model, STAT-3 could enhance Bcl-2 and Bcl-xL level involved in liver development and regeneration[30]. Hence, we investigated the caspase-3 and cleaved-caspase-3 protein and BCL-2/BCL-XL mRNA level in LPS-induced PMVECs with HGF treatment. Interestingly, HGF protective effects of decreasing caspase-3 and raising Bcl-2/Bcl-xL were inhibited by mTOR inhibitor or STAT-3 inhibitor S3I-201.Both mTORC1 and mTORC2 could contributed to Bcl-2/Bcl-xL mRNA level in LPS-induced endothelial apoptosis with HGF treatment. Collectively, mTOR/STAT-3 pathway mediated HGF anti-apoptotic effects in endothelium.

Integrity of intercellular junctions is a major determinant of endothelial permeability and the VE-cadherin-based adherent and occludin-based tight junction are thought to be particularly important[31, 32]. VE-cadherin is required for maintaining adherent endothelial barrier and blockage VE-cadherin with antibodies increased monolayer permeability[31]. Occludin is an important protein in tight junction function. Disruption of VE-cadherin and occludin regulation are important aspects of a number of diseases. In this experiment, we found that endothelial cells function with mitochondrial injury and apoptosis were also changed with decreased VE-cadherin-based adherent and occludin-based tight junction and it was related with mTOR/STAT-3 pathway.

Conclusion

In summary, our data suggests the protective effects of HGF to endothelial 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 oxidative level, mitochondrial damage, apoptosis and endothelial barrier integrity in LPS-induced PMVECs.

Abbreviations

ARDS Acute respiratory distress syndrome

CCK-8 Cell Counting Kit-8

HGF Hepatocyte growth factor

LPS Lipopolysaccharide

mTOR Mammalian TOR

PMVEC Pulmonary microvascular endothelial cell

RT-qPCR Real-time quantitative polymerase chain reaction

STAT3 Signal transducer and activator of transcription 3

WB Western blot

Declarations

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None

Ethics approval and consent to participate

This study was approved by the Local Ethics Committee for the Care and Use of Laboratory Animals of Southeast University

Consent for publication

All authors provided consent for publication.

Availability of data and materials

The data used to support the findings of this study are included within the article.

Competing interests

The author declare that there are no conflict of interests.

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Author Contributions

Conceptualization, YY, SSM and FMG; Methodology, SSM and FPX; Software, SSM and JYX; Validation, SSM, XWZ And FPX; Formal Analysis, MX and MYG; Investigation, FMG; Resources, SSM; Data Curation, SSM; Writing – Original Draft Preparation, SSM, FPX and JYX; Writing – Review & Editing, YZH; Visualization, FMG; Supervision, HBQ and YY; Project Administration, YY; Funding Acquisition, YY, HBQ, FMG,YZH, SSM and XWZ.

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Figures

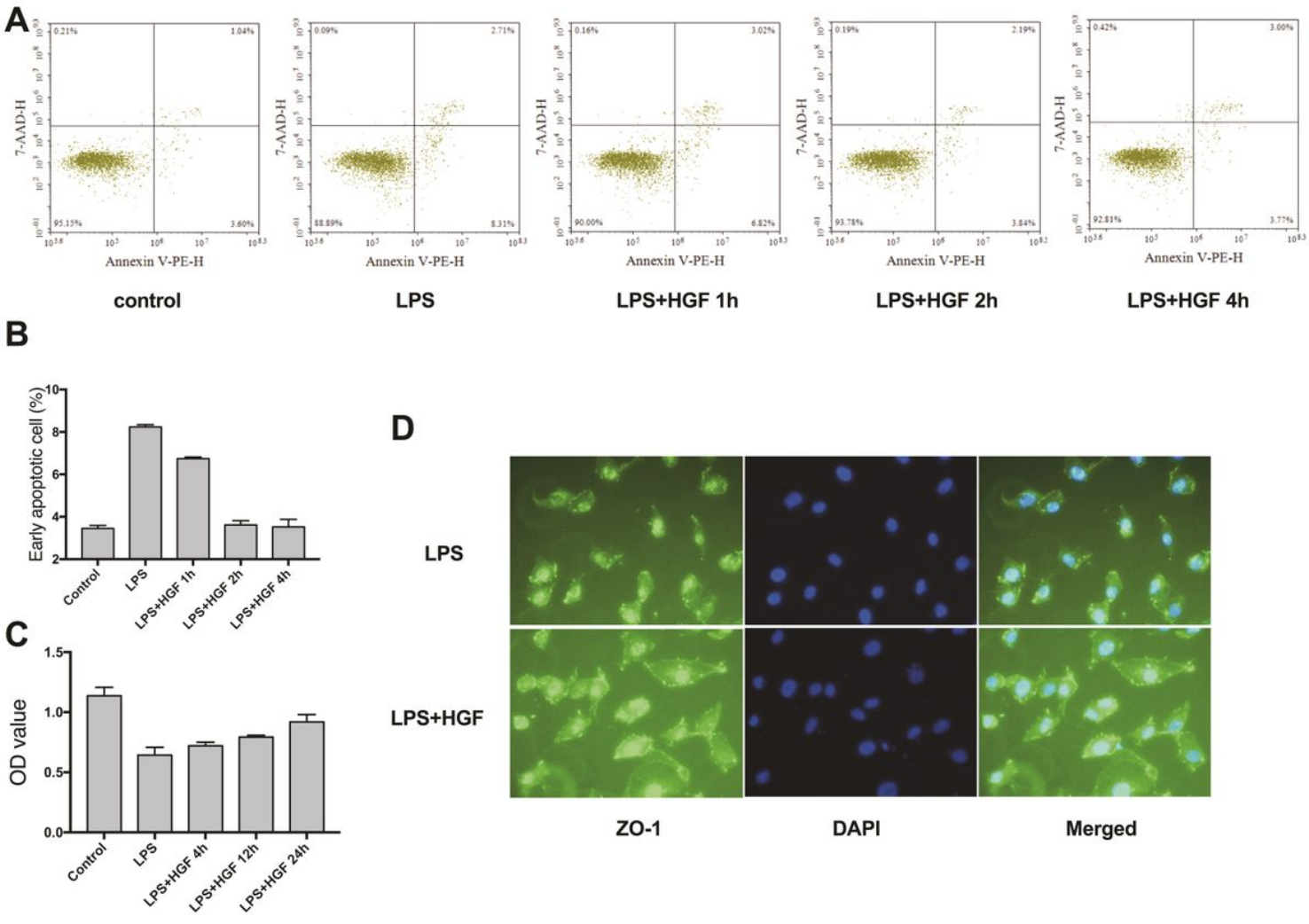

Figure 1

HGF treatment suppressed LPS-induced PMVECs injury. LPS treatment of PMVECs were treated with or without HGF for 0-24h. PMVECs apoptosis was tested by Annexin V-PE/7-AAD staining with flow cytometry. PMVECs proliferation was tested by CCK-8 kit. Endothelial tight junction protein ZO-1 was

tested by immunofluorescence. A: Flow cytometry analysis of LPS-induced PMVECs apoptosis with HGF treatment(0-4h). B: Early apoptosis ratio of LPS-induced PMVECs apoptosis with HGF treatment in flow cytometry analysis (0-4h). C: Cell proliferation of LPS-induced PMVECs survival with HGF treatment (0-24h). D: Endothelial tight junction protein ZO-1 of LPS-induced PMVECs with 24h HGF treatment(400×). Results are mean ± SD(n=3).

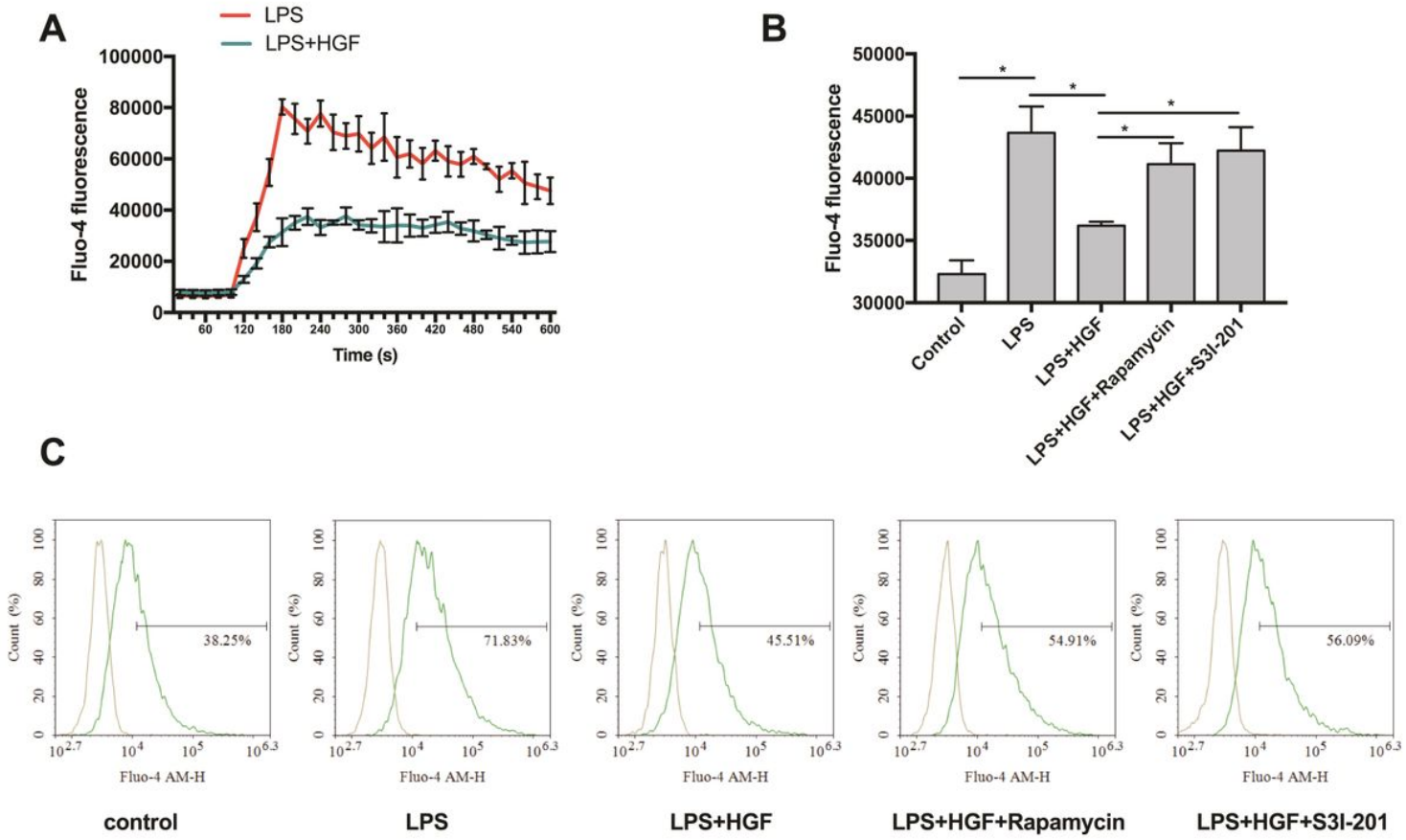

Figure 2

HGF exposure decreased cytosolic calcium levels in PMVECs via mTOR/STAT-3 pathway. LPS treatment of PMVECs were treated with or without HGF. Rapamycin and S3I-201were respectively used to inhibit mTOR and STAT-3 signaling. Fluorescence probe Fluo-4 AM was applied to detect cellular calcium concentration every 20s with flow cytometry. A: Fluo-4 fluoresence change of cytosolic calcium in LPS induced PMVECs with HGF treatment. B: Relative cytosolic calcium expressions of HGF on LPS-induced PMVECs with mTOR/STAT-3 pathway tested by flow cytometry(200s). C: The cytosolic calcium effects of HGF on LPS-induced PMVECs with mTOR/STAT-3 pathway tested by flow cytometry(200s). Results are mean ± SD(n=3). * $p<0.05$.

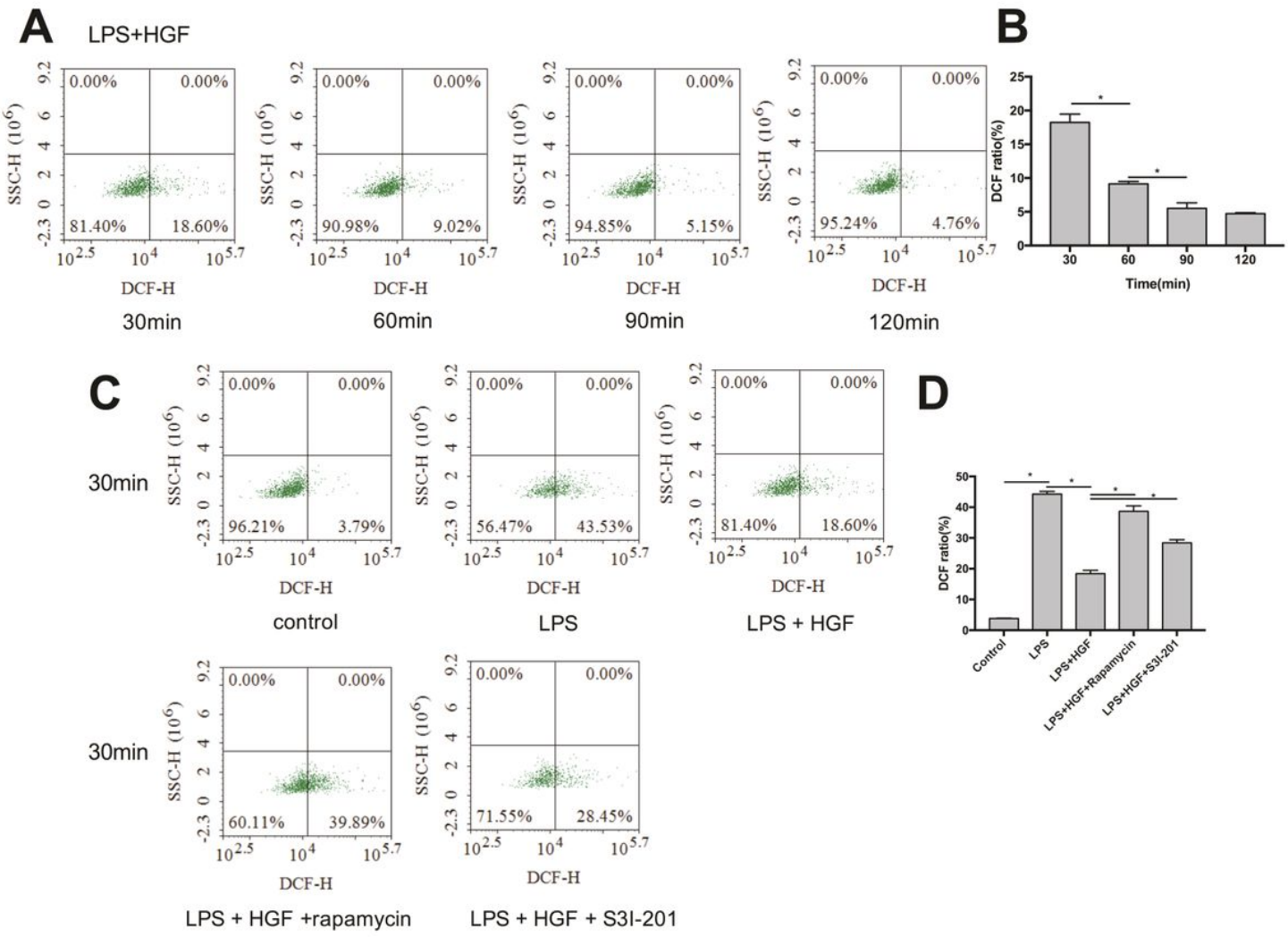

Figure 3

HGF exposure attenuated LPS-induced calcium increase triggering cellular ROS production in PMVECs via mTOR/STAT-3 pathway. LPS treatment of PMVECs were treated with or without HGF. Rapamycin and S3I-201 were respectively used to inhibit mTOR and STAT-3 signaling. Intracellular ROS were detected by high fluorescent DCF in flow cytometry. A: DCF change of cellular ROS production in LPS-induced PMVECs with HGF treatment test by flow cytometry for varying time points(30-120min). B: DCF ratio differences in LPS-induced PMVECs with HGF treatment test by flow cytometry for varying time points(30-120min). C: DCF change of cellular ROS production in LPS-induced PMVECs with HGF treatment via mTOR/STAT-3 pathway test by flow cytometry(30min). D：DCF ratio differences in LPS-induced PMVECs with HGF treatment via mTOR/STAT-3 pathway test by flow cytometry(30min). Results are mean ± SD(n=3). * $p<0.05$.

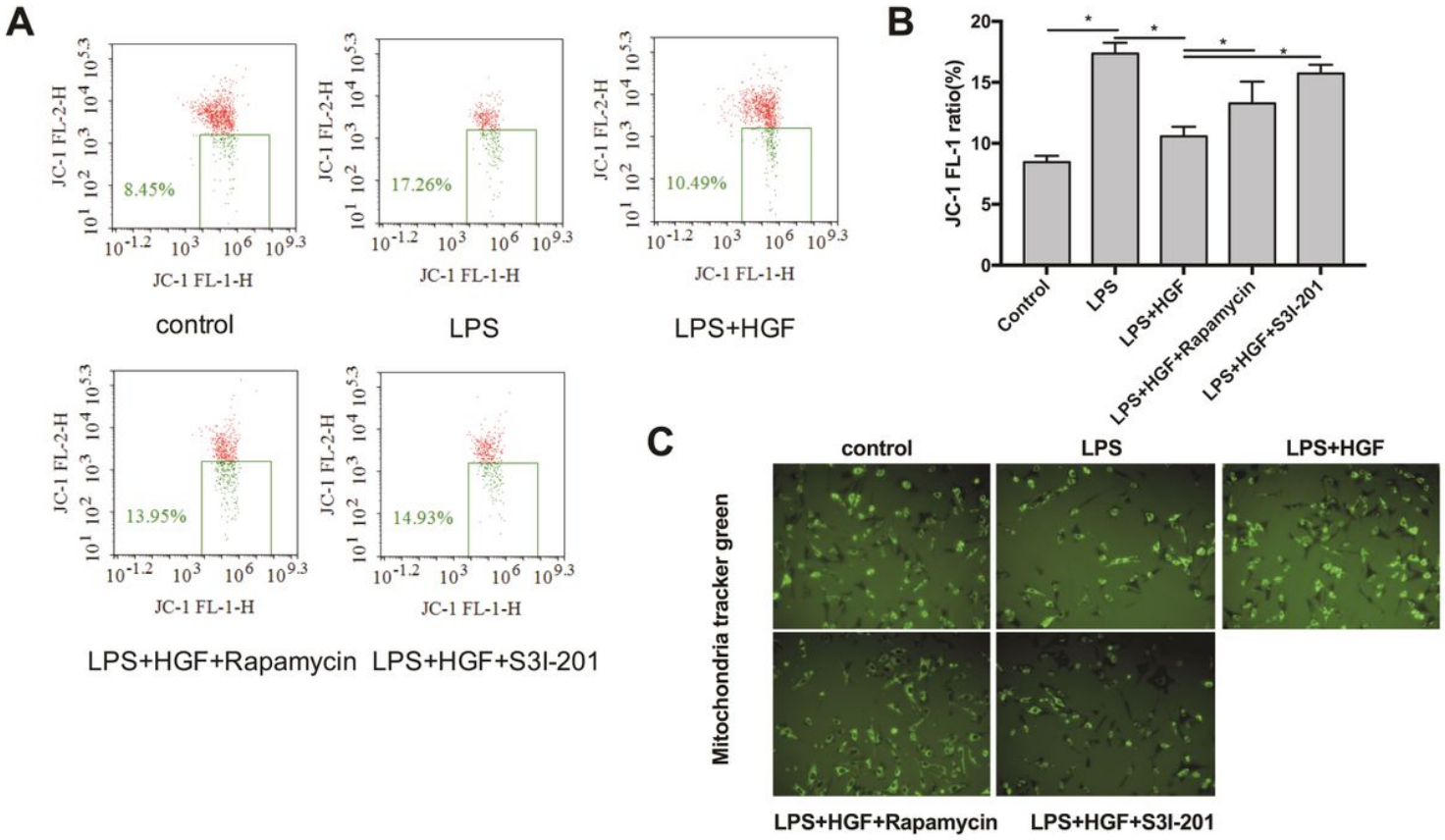

Figure 4

HGF decreased LPS-induced mitochondrial membrane loss in PMVECs via mTOR/STAT-3 pathway. LPS treatment of PMVECs were treated with or without HGF for 6h. Rapamycin and S3I-201 were respectively used to inhibit mTOR and STAT-3 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/STAT-3 pathway(12h). 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/STAT-3 pathway(12h). C: Representative photomicrographs of mitochondria tracker green(24h). Results are mean ± SD(n=3). * $p<0.05$.

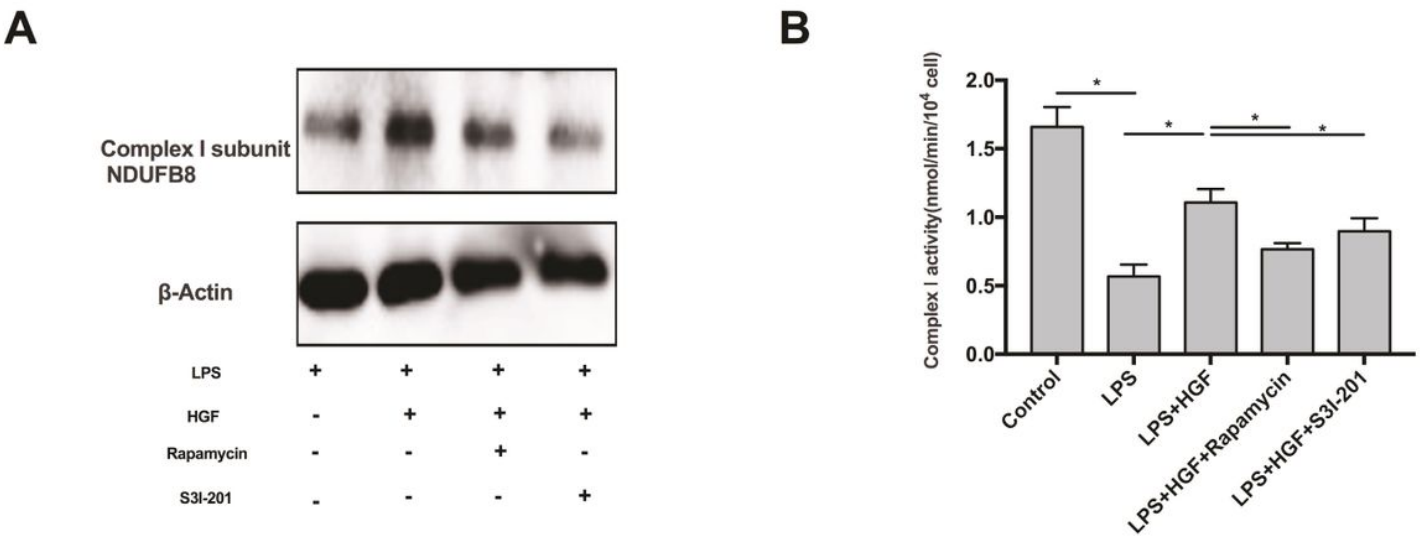

Figure 5

Changes in expression of specific mitochondrial protein and activity in LPS-induced PMVECs with HGF treatment in response to mTOR/STAT-3 inhibitors. LPS treatment of PMVECs were treated with or without HGF for 24h. Rapamycin and S3I-201 were respectively used to inhibit mTOR and STAT-3 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/STAT-3 pathway(24h). B: Activity analysis of specific mitochondrial protein complex I in LPS-induced PMVECs with HGF treatment via mTOR/STAT-3 pathway(24h). Results are mean ± SD(n=3). * p<0.05.

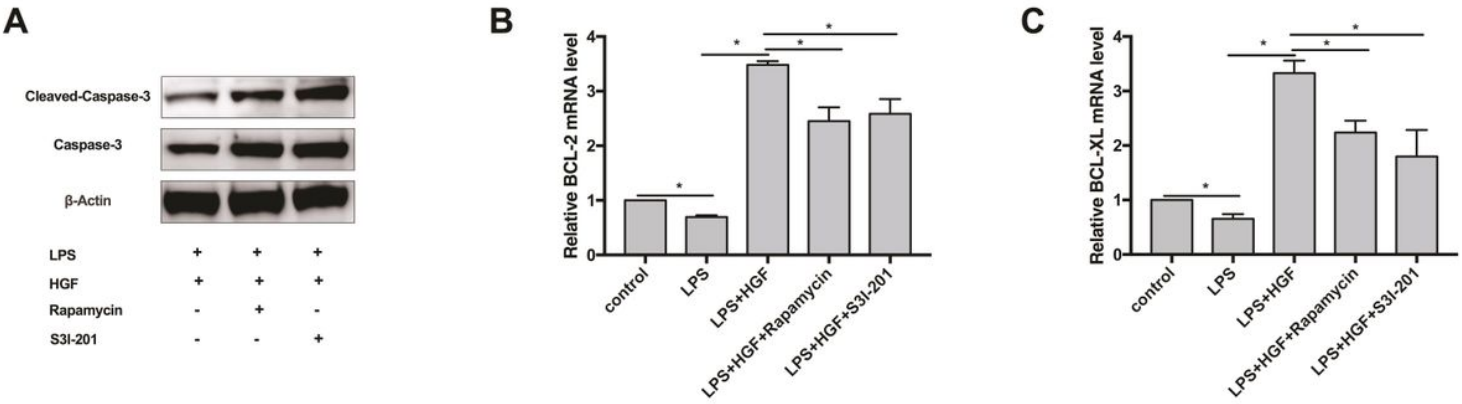

Figure 6

Change expression of LPS-induced PMVECs apoptosis relevant mRNAs and proteins with HGF treatment via mTOR/STAT-3 signaling. LPS treatment of PMVECs were treated with or without HGF for 24h. Rapamycin and S3I-201 were respectively used to inhibit mTOR and STAT-3 signaling. PMVECs apoptosis

relevant mRNA expression and protein were respectively tested by RT-qPCR and WB analysis. A: WB analysis of LPS-induced PMVECs apoptosis protein Caspase-3 and Cleaved-Caspase-3 with HGF treatment via mTOR/STAT-3 pathway(24h). B: RT-qPCR analysis of Bcl-2 mRNA relative expression in LPS-induced PMVECs apoptosis with HGF treatment via mTOR/STAT-3 pathway(24h). C: RT-qPCR analysis of Bcl-xL mRNA relative expression in LPS-induced PMVECs apoptosis with HGF treatment via mTOR/STAT-3 pathway(24h). Results are mean ± SD(n=3). * $p<0.05$.

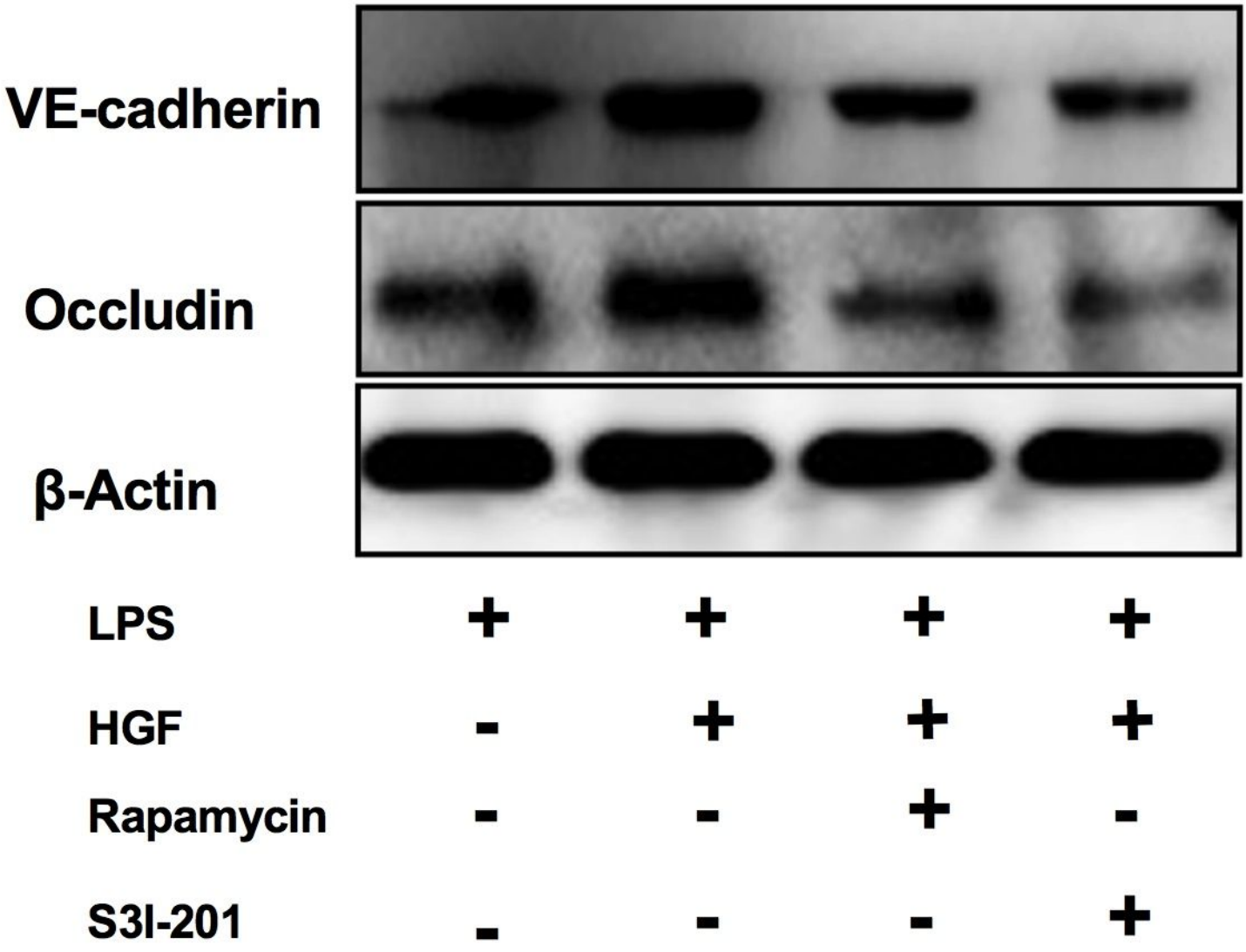

Figure 7

Changes in expression of endothelial adherent protein in LPS-induced PMVECs with HGF treatment in response to mTOR/STAT-3 inhibitors. LPS treatment of PMVECs were treated with or without HGF for 24h. Rapamycin and S3I-201 were respectively used to inhibit mTOR and STAT-3 signaling. Endothelial adherent protein VE-cadherin and tight junction protein Occludin were tested by WB analysis with 24h treatment.

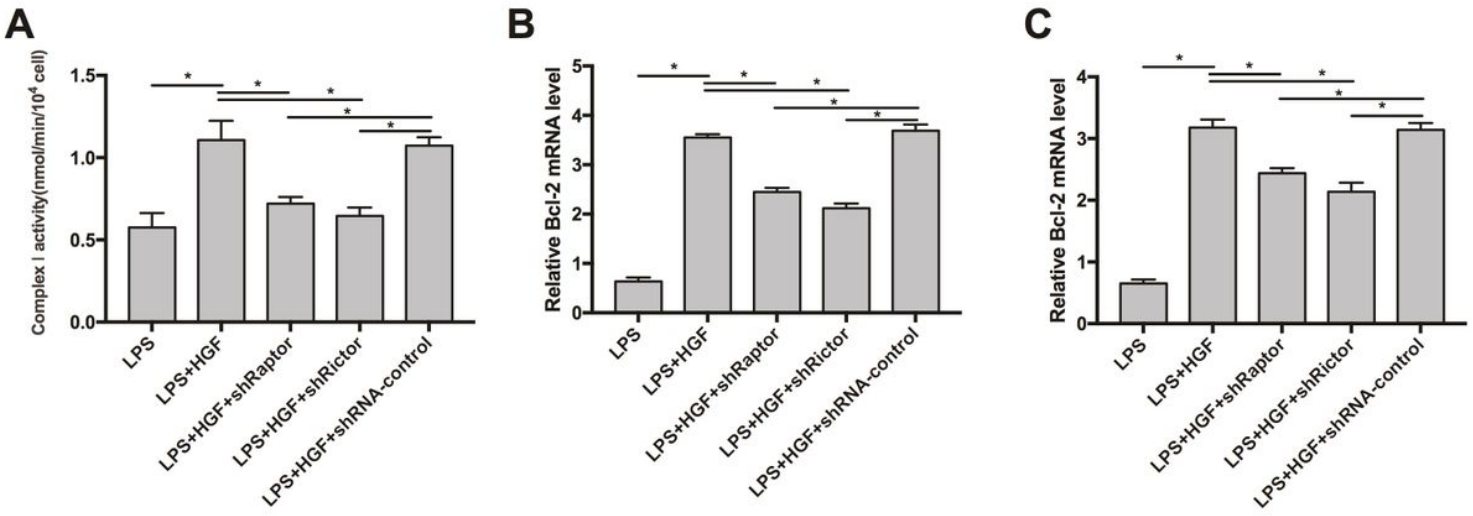

Figure 8

Protective effects of HGF via mTORC1 and mTORC2 in LPS-induced PMVECs 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 were treated with or without HGF for 24h. PMVECs 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 (24h). B: RT-qPCR analysis of Bcl-2 mRNA relative expression with HGF treatment in LPS-induced PMVECs apoptosis via mTORC1 and mTORC2 signaling (24h). C: RT-qPCR analysis of Bcl-xL mRNA relative expression with HGF treatment in LPS-induced PMVECs apoptosis via mTORC1 and mTORC2 signaling (24h). Results are mean ± SD(n=3). * $p<0.05$.