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Tongxinluo attenuates oxygen-glucose-serum deprivation/restoration-induced endothelial barrier breakdown via peroxisome proliferator activated receptor-α/angiopoietin-like 4 pathway in high glucose-incubated human cardiac microvascular endothelial cells

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Abstract

Background: Traditional Chinese medicine Tongxinluo (TXL) has been widely used to treat coronary artery disease in China, since it could reduce myocardial infarct size and ischemia/reperfusion injury in both non-diabetic and diabetic conditions. It has been shown that TXL could regulate peroxisome proliferator activated receptor-α (PPAR-α), a positive modulator of angiopoietin-like 4 (Angptl4), in diabetic rats. Endothelial junction substructure components, such as VE-cadherin, are involved in the protection of reperfusion injury. Thus, we hypothesized cell-intrinsic and endothelial-specific Angptl4 mediated the protection of TXL on endothelial barrier under high glucose condition against ischemia/reperfusion-injury via PPAR-α pathway.

Methods: Incubated with high glucose medium, the human cardiac microvascular endothelial cells (HCMECs) were then exposed to oxygen-glucose-serum deprivation (2 hours) and restoration (2 hours) stimulation, with or without TXL, insulin, or rhAngptl4 pretreatment.

Results: TXL, insulin, and rhAngptl4 had similar protective effects on the endothelial barrier. TXL treatment reversed the endothelial barrier breakdown in HCMECs significantly as identified by decreasing endothelial permeability, upregulating the expression of JAM-A, VE-cadherin, and integrin-α5 and increasing the membrane location of VE-cadherin and integrin-α5, and these effects of TXL were as effective as insulin and rhAngptl4. However, Angptl4 knock-down with small interfering RNA (siRNA) interference and PPAR-α inhibitor MK886 partially abrogated these beneficial effects of TXL. Western blotting also revealed that similar with insulin, TXL upregulated the expression of Angptl4 in HCMECs, which could be inhibited by Angptl4 siRNA or MK886 exposure. TXL treatment increased PPAR-α activity, which could be diminished by MK886 but not by Angptl4 siRNA.

Conclusion: These data suggest cell-intrinsic and endothelial-specific Angptl4 mediates the protection of TXL against endothelial barrier breakdown during oxygen-glucose-serum deprivation and restoration under high glucose condition partly via the PPAR-α/Angptl4 pathway.

Abbreviations: AMI = acute myocardial infarction, Angptl4 = angiopoietin-like 4, ELISA = enzyme-linked immunosorbent assay, FITC = fluorescein isothiocyanate, HCMECs = human cardiac microvascular endothelial cells, I/R = ischemia/reperfusion, OGSD/R = oxygen-glucose-serum deprivation and restoration, PPAR-α = peroxisome proliferator activated receptor-α, RT-PCR = real-time polymerase chain reaction, siRNA = small interfering RNA, TXL = Tongxinluo.

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The datasets generated during and/or analyzed during the current study are publicly available.

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1. Introduction

Myocardial ischemia/reperfusion (I/R) injury may cause myocardial cell death and limit the benefit achieved by restoration of coronary blood flow in acute myocardial infarction (AMI) patients.[1] Diabetes is characterized by endothelial dysfunction, endothelial barrier breakdown, and microvascular hyperpermeability,[2–5] which complicate and exacerbate I/R-injury.[6]

Composed of Radix ginseng, Buthus martensi, Hirudo, Eupolyphaga seu steleophaga, Scolopendra subspinipes, Periostracum cicadae, Radix paeoniae rubra, Semen ziziphi spinosae, Lignum dalbergiae odoriferae, Lignum santali albi, and Borneolum syntheticum, traditional Chinese medicine Tongxinluo (TXL) was registered in China Food and Drug Administration for the angina pectoris treatment in 1996. Recent researches demonstrated that TXL could protect the myocardium from I/R-injury, reducing the size of myocardial necrosis and no-reflow, and improving the cardiac performance in both non-diabetic[7] and diabetic[8] conditions.

The protection mechanisms of TXL involved the protection of microvascular endothelial integrity by upregulating VE-cadherin, β-catenin, γ-catenin, JAM-A and integrin-α5 and subsequent reduction of myocardial hemorrhage, inflammation, oxidization, edema, apoptosis and necrosis, while eNOS inhibitor Nω-Nitro-L-arginine, peroxisome proliferator activated receptor-α (PPAR-α) inhibitor MK886 or small interfering RNA (siRNA) against angiopoietin-like 4 (Angptl4) partly canceled these effects of TXL, indicating that endothelial barrier function and PPAR-α/Angptl4 pathway played a key role in the protection of TXL against myocardial I/R-injury.[7–11] Several signaling pathways have been implicated in this protective role of TXL, including PKA/eNOS,[7] MEK/ERK,[12] and PPAR-α/Angptl4[8] pathways.

Angptl4 is one of the dramatically upregulated cytokines by TXL,[12] and has also been shown to reduce the size of myocardial infarction and no-reflow in rats, which is attributed to the preservation of vascular integrity by protecting VE-cadherin complex.[13] Recently, we confirmed that as a key protector, Angptl4 mediated the protection of TXL by regulating endothelial barrier against myocardial I/R-injury in diabetic rats.[8] However, the relative contribution of cell-intrinsic and endothelial-specific versus circulating Angptl4 from other sources in response to I/R is not yet clear.

Rich in myocardium, PPAR-α activates Angptl4 expression under stresses.[14,15] Noticeably, as a core mechanism, PPAR-α, which could be activated by TXL to protect diabetic rats from myocardial I/R-injury,[8] is also an important regulator of renal microvascular endothelial barrier.[16] PPAR-α/Angptl4 pathway is thought to be a protective signaling pathway during I/R in diabetic hearts.[8] However, the regulation of junction substructure components by TXL in human cardiac microvascular endothelial cells (HCMECs) has not been elucidated.

Therefore, our study aims to explore whether TXL could exert protection on endothelial barrier integrity in HCMECs under high glucose condition during I/R and to identify the underlying mechanisms.

2. Methods

2.1. Cell culture and treatments

Culture of HCMECs Sciencell(San Diego, CA, USA) was performed as previously described.[17] Briefly, HCMECs were incubated in 37°C/5%CO_2. After reaching 60% confluence, cells were subcultured with Endothelial Cell Medium medium (Sciencell). All cells used in the experiments were the passage 5. The HCMECs were cultured with Endothelial Cell Medium of various final concentrations of D-glucose (normal glucose, 5.5 mM; high glucose, 18 mM; very high glucose, 30.5 mM) for a further 48 hours. To rule out the effect of osmolarity, HCMECs were exposed to normal glucose medium with various final concentrations of D-mannitol (0, 12.5 or 25 mM).

Various groups of cells were washed with phosphate buffered saline and exposed to the various treatments for 1 hour before hypoxia. HCMECs received normal glucose (5.5 mM) or high glucose (18 mM) intervention or/and treatment with insulin, rhAngptl4, and TXL. TXL at different concentrations (0, 50, 100, 200, 400, 800, 1600 μg/mL, Yiling Pharmaceutical Co., Hebei, China) were prepared in serum-free Dulbecco's Modified Eagle Medium medium low glucose, Hyclone(South Logan, UT, USA) as previously described.[18] RhAngptl4 (1 μg/ml, R&D, Minneapolis, MN, USA) and insulin 1 nM, Sigma St.Louis, MO, USA were used as control. PPAR-α inhibitor MK886 of 1 μM Tocris (Ellisville, MO, USA) or siRNA for knockdown of Angptl4 was added to further investigate the role and mechanism of TXL on endothelial barrier.

Oxygen-glucose-serum deprivation and restoration (OGSD/R) conditions were established in all groups except the control group according to published procedures,[17,19,20] with minor modifications. Briefly, in the model of oxygen-glucose-serum deprivation, cells were rinsed with phosphate buffered saline and exposed to glucose-free and serum-free medium (Hyclone) and then cells were incubated in a sealed, hypoxic GENbox jar fitted with a catalyst (BioMe rieux) to scavenge free oxygen, inducing 2 hours oxygen-glucose-serum deprivation. In the model of restoration, the plates were then taken out from the jar and the cells were exposed to medium with normal glucose (5.5 mmol/L) or high glucose (18 mmol/L) and with serum. Then cells were moved to normoxic conditions for an additional 2 hours as restoration. Oxygen tension in the medium was measured with anaerobic indicator (BioMe rieux), which enabled confirmation of successful establishment of OGSD/R model. The protocol was approved by the Committee on the Ethics of Experiments of the Fuwai Hospital (Permit Number: 2013-4/5-100/30-973).

2.2. Cell transfection

HCMECs were seeded and cultured in complete medium in 6-well plates. When HCMECs reaches 70% to 80% confluence, siRNA oligonucleotides against Angptl4 (5′-GGUCUGGA-GAAGGUGCAUAtt-3′) (Applied Biosystems, UK) or nonspecific control siRNA oligonucleotides (Applied Biosystems) were transfected in HCMECs with RNAiMAX transfection kit

Invitrogen(San Diego, CA, USA) in Opti-MEM Reduced Serum Medium Thermo Fisher Scientific (Waltham, MA, USA). The efficacy of siRNA sequences for Angptl4 was evaluated according to rational design criteria of manufacturer's protocol. Duplexes were removed after 16 hours, and cells were incubated for an additional 8 hours for further experiments.

2.3. In vitro permeability assay

Endothelial barrier function was assessed with In Vitro Permeability Assay kit 24-Well, Millipore (Billerica, MA, USA) as described previously,[21,22] with minor modifications. Briefly, 1×10^3 HCMECs were plated onto collagen-coated inserts and cultured for 72 hours to allow them to form a tight monolayer. Then the cells were treated with different interventions for 1 hour followed by OGSD/R insult. Glucose-free Dulbecco's Modified Eagle Medium was added to each receiver plate well at 500 μL. 2.5% FITC (fluorescein isothiocyanate)-dextran (40 kDa) solution was added at 150 μL per insert and allowed to permeate the monolayers for 20 minutes at 37°C, protected from light. The medium in the receiver wells was then thoroughly mixed. The extent of endothelial permeability was quantified by measuring the fluorescence intensity of bottom plate solution affected by permeated FITC-Dextran on a fluorescence spectrophotometer (TECAN GENios Plus, Switzerland) via fluorescence at 485 nm excitation/535 nm emission wavelengths.

2.4. Protein sample preparation and Western blot

Cells were lysed in 1% Triton X-100 for 15 minutes at 4°C. After centrifugation, protein concentrations were measured with the Bio-Rad (Hercules, CA, USA) protein assay (Bio-Rad). 10 μg of protein from each sample was subjected to sodium dodecyl sulfate -polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Immobilon P, Millipore Corp. Billerica, MA, USA). The membranes were then incubated with the following antibodies: rabbit monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1:1000 dilution, CST (Danvers, MA, USA) as an internal control for total protein detection; mouse monoclonal alpha 1 Sodium Potassium ATPase (Na-K-ATPase, 1:1000 dilution, Abcam, UK) as an internal control for membrane protein detection; rabbit monoclonal JAM-A (1:1000 dilution, Abcam); rabbit monoclonal occludin (1:1000 dilution, Abcam); rabbit monoclonal integrin-α5 (1:1000 dilution, Abcam); rabbit monoclonal integrin-β1 1:1000 dilution, CST (Danvers, MA, USA); rabbit monoclonal VE-cadherin 1:1000 dilution, (Santa-cruz, CA, USA), rabbit monoclonal p120-catenin (1:1000 dilution, Abcam); rabbit polyclonal Angptl4 (1:1000 dilution, Abcam). Bands were visualized with Chemiluminescent HRP Substrate (Millipore Corp. Billerica, MA, USA). For the detection of membrane protein, Cell Surface Protein Isolation Kit Thermo Pierce (Rockford, IL, USA) was utilized. Briefly, cells were surface biotinylated and lysed in 1% sodium dodecyl sulfate containing buffer. The biotinylated protein was pulled down by NeutrAvidin Agarose and loaded for western blot analysis.

2.5. Measurement of Angptl4 secretion

For measuring in vitro Angptl4 secretion by HCMECs upon stimulation, 1×10^6 HCMECs were seeded per well in a 6-well plate, then serum-starved for 24 hours and stimulated by OGSD/R. The supernatants were collected, and Angptl4 concentrations were measured via ELISA (enzyme-linked immunosorbent assay) (R&D) in 0 hours, 2 hours, and 4 hours after restoration, respectively. We measured 6 samples for each time point and normalized to the protein content of the cells.

2.6. Real-time polymerase chain reaction analysis

Total RNA was extracted from HCMECs with Trizol reagent and converted into cDNA with the M-MLV reverse transcriptional system Invitrogen(San Diego, CA, USA) in the presence of random hexamers Invitrogen(San Diego, CA, USA). The cDNA was used for quantitative real-time polymerase chain reaction (RT-PCR) with specific gene primers as follows: Angptl4 forward, 5′-AGACACAACTCAAGGCTCAG-3′; reverse, 5′-CTCATGGTCTAGGTGCTTGTG-3′; GAPDH forward, 5′-ACATCGCTCAGACACCATG-3′; reverse, 5′-TGTAGTTGAGGTCAATGAAGGG-3′. An MYIQ RT-PCR detection system and SYBR green PCR mix (Bio-Rad) were used to carry out RT-PCR. The relative abundance of transcript was quantified by the comparative C_t method with GAPDH as an internal control.

2.7. Measurement of PPAR-α activity

PPAR-α activity was measured with PPAR-α Transcription Factor Assay kit (Abcam). Nuclear protein of endothelial cells was extracted with nuclear extraction reagents (Thermo). Briefly, cells were pelleted and added with iced-cold cytoplasmic extraction reagent I (100 μL/10 μL packed cell volume) and incubated on ice for 10 minutes and ice-cold cytoplasmic extraction reagent II was added. After incubation, the Nuclear Extraction Reagent was added. The nuclear extracts were stored at −80°C until use. PPAR-α activity was measured by a non-radioactive, sensitive method for detecting specific transcription factor DNA binding activity in the nuclear extracts according to manufacturers' protocol.

2.8. Statistics

Quantitative data are presented as mean ± standard deviation. Statistical analysis was performed with 1-way analysis of variance followed by Tukey test (SPSS 20.0). Differences were considered to be significant at $P < .05$.

3. Results

3.1. TXL treatment decreases transendothelial permeability under high glucose condition undergoing OGSD/R stimulation

To evaluate whether TXL could modulate endothelial permeability in HCMECs, we treated HCMECs with various concentrations of TXL and then exposed them to OGSD/R stimulation. In vitro permeability assay was performed in the endothelial cells treated with or without TXL. As shown in Figure 1A, there was significant increase in fluorescence intensity in the wells of high glucose controls. Compared with controls without TXL treatment, TXL of different concentrations all decreased endothelial permeability remarkably in a dose-dependent way, peaked at 800 μg/mL (all $P < .01$), suggesting the optimal TXL concentration for protecting endothelial barrier was 800 μg/mL (Fig. 1A). We found similar effects in cells treated with insulin or rhAngptl4 (Fig. 1B). The TXL inhibitory effect on

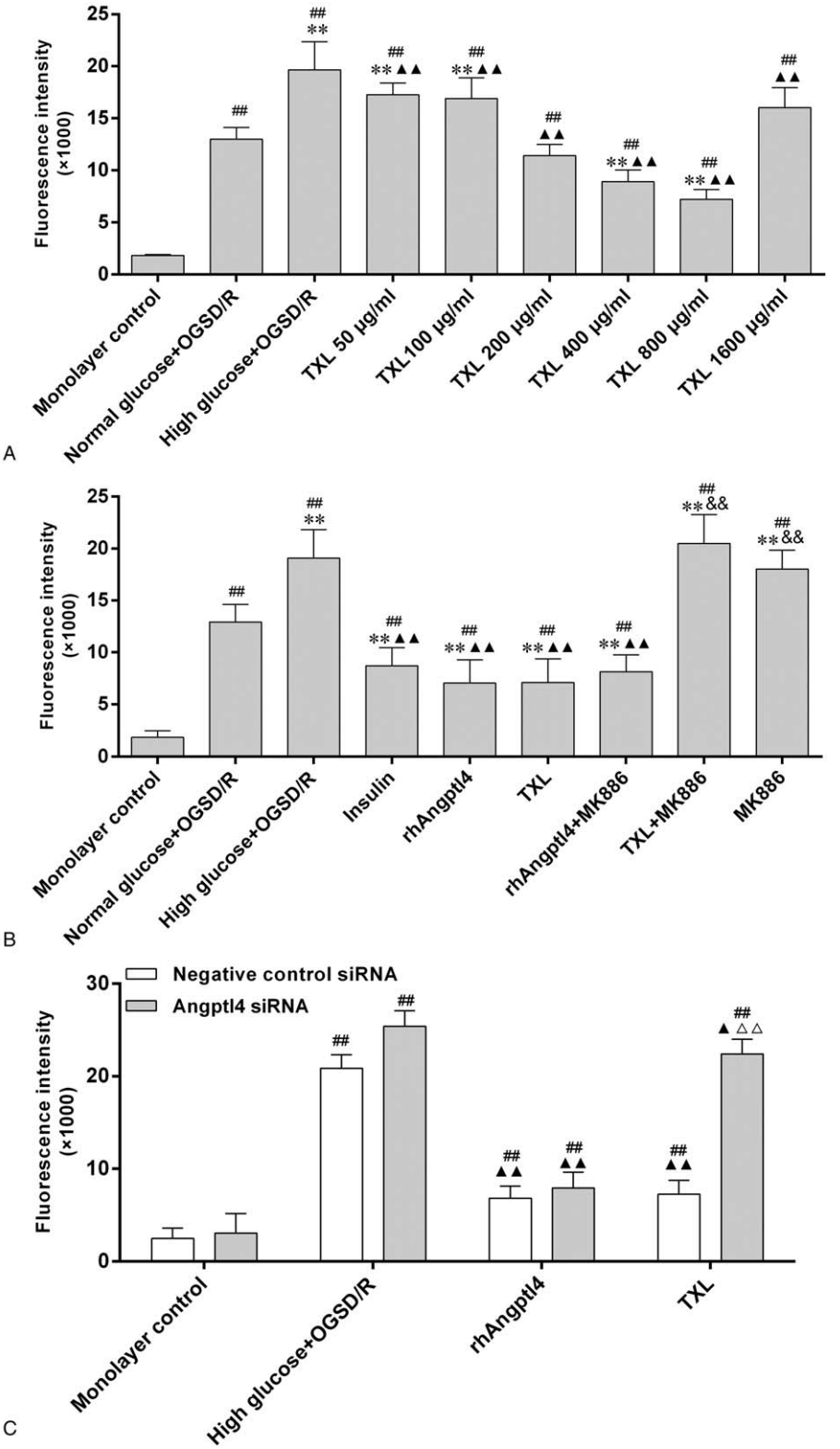

Figure 1. Endothelial permeability assessment of endothelial cells under high glucose condition treated with or without TXL in the presence or absence of signal regulators. Endothelial monolayer permeability was measured by in vitro endothelial permeability assay. n = 8; (A) TXL decreased endothelial permeability in a dose-dependent manner; (B) TXL, insulin, and rhAngptl4 decreased endothelial permeability; (C) TXL decreased endothelial permeability in Angptl4-dependent manner. $^{\#\#}P < .01$ versus Monolayer control group; $^{**}P < .01$ versus normal glucose + OGSD/R group; $^{\blacktriangle\blacktriangle}P < .01$ versus high glucose + OGSD/R group; $^{\&\&}P < .01$ versus TXL group; $^{\Delta\Delta}P < .01$ versus TXL + NC siRNA group. NC siRNA = negative control siRNA, OGSD/R = oxygen-glucose-serum deprivation and restoration, TXL = Tongxinluo.

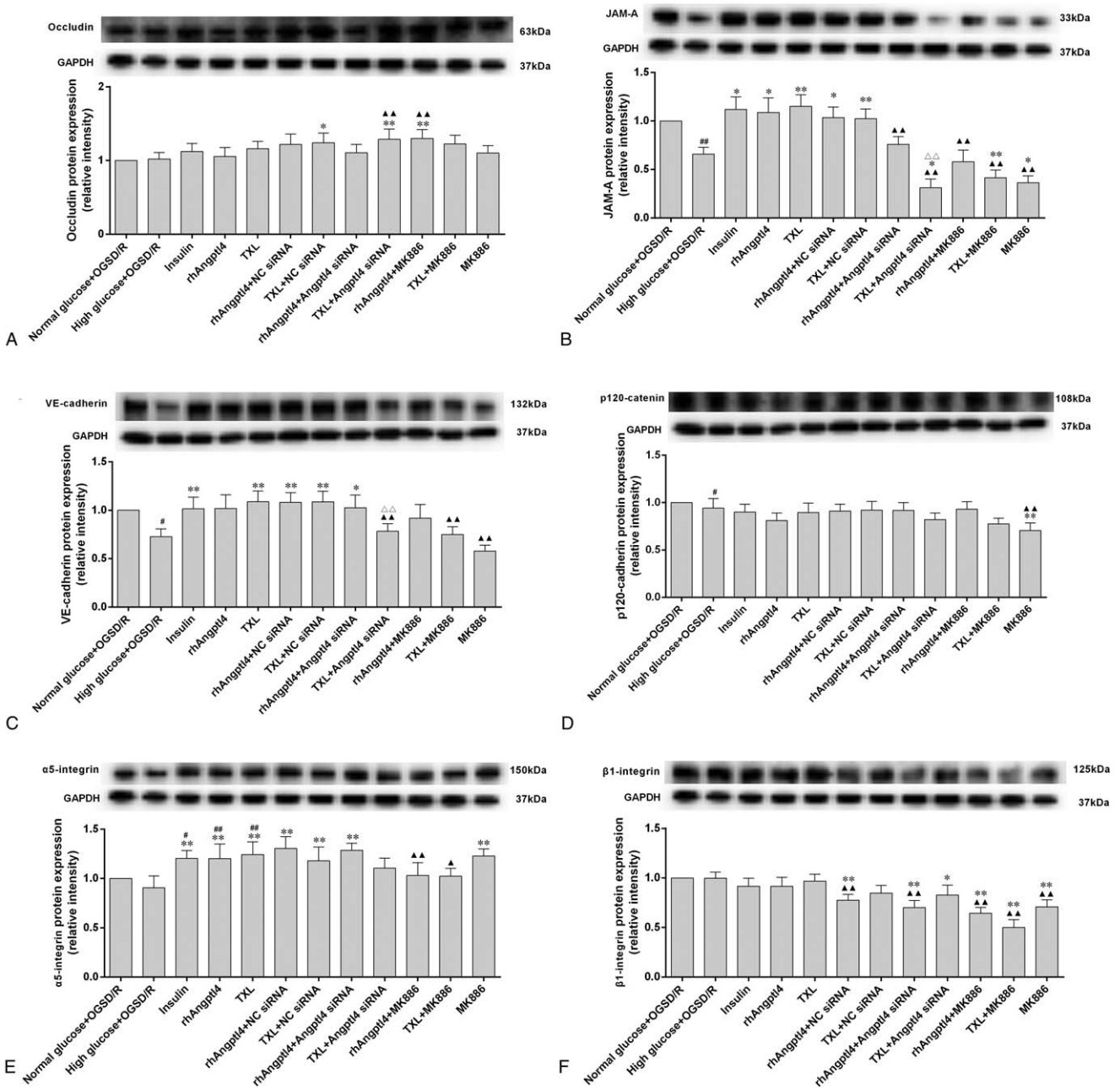

Figure 2. Immunoblotting analysis of the expression of occludin, JAM-A, VE-cadherin, p120-catenin, integrin-α5, and integrin-β1 in endothelial cells treated with or without TXL in the presence or absence of signal regulators. Total protein was extracted from the endothelial cells and analyzed by immunoblotting. (A) Occludin, (B) JAM-A. (C) VE-cadherin, (D) p120-catenin, (E) integrin-α5, (F) integrin-β1. n = 6; $^{\#\#}P < .01$ versus normal glucose + OGSD/R group; $^{**}P < .01$ versus high glucose + OGSD/R group; $\blacktriangle\blacktriangle P < .01$ versus TXL group; $^{\Delta\Delta}P < .01$ versus TXL + NC siRNA group. NC siRNA = negative control siRNA, OGSD/R = oxygen-glucose-serum deprivation and restoration, TXL = Tongxinluo.

the endothelial permeability could be partially blocked by downregulation of Angptl4 with siRNA interference ($P < .01$) (Fig. 1C), suggesting participation of HCMECs-derived intrinsic Angptl4 in TXL protective impact on endothelial hyperpermeability for FITC-dextran. The fluorescence intensity alterations in the wells observed could also be partially abolished when the PPAR-α inhibitor MK886 was delivered (Fig. 1B). Interestingly, MK886 did not affect the rhAngptl4-induced reduction in the fluorescence intensity (Fig. 1B).

3.2. TXL treatment enhances the expression of intercellular junction protein JAM-A and VE-cadherin via PPAR-α/Angptl4 pathway in OGSD/R-injured endothelial cells under high glucose condition

To evaluate the integrity of endothelial barrier in endothelial cells, we next examined by western blotting the expression levels of tight junction protein occludin and JAM-A; adhesion junction protein VE-cadherin and p120-catenin; and focal adhesion

junction protein integrin-α5 and integrin-β1 in HCMECs. In agreement with previous study,[8] there was significant reduction in the expression of JAM-A, VE-cadherin, and p120-catenin in the high glucose controls (Fig. 2B–D). Compared with the high glucose controls without TXL treatment, TXL-treated endothelial cells showed prominent increase in the expression of JAM-A, VE-cadherin, and integrin-α5 (Fig. 2B C, and E). The expression levels of JAM-A and integrin-α5 were increased at similar levels as those seen in the endothelial cells treated with rhAngptl4 (Fig. 2B and E). Interestingly, we did not find significant increase in the expression of occludin, p120-catenin, and integrin-β1 in TXL-treated endothelial cells (Fig. 2A D, and F). Similarly, there was significant increase in the expression of JAM-A and integrin-α5 in insulin-treated endothelial cells, which was at similar levels as those seen in the groups treated with rhAngptl4 or TXL (Fig. 2B and E). Furthermore, the knock-down of Angptl4 expression with siRNA interference reversed the upregulation of the expression of JAM-A and VE-cadherin, but not integrin-α5 (Fig. 2B D, and E). Addition of PPAR-α inhibitor MK886 partially blocked TXL-induced upregulation of the expression of JAM-A, VE-cadherin, and integrin-α5 (Fig. 2B, D, and E). The results indicated that the expression of JAM-A and VE-cadherin but not integrin-α5 in the HCMECs was regulated by TXL via PPAR-α/Angptl4 pathway.

3.3. TXL treatment increases the cell membrane location of intercellular junction protein integrin-α5 via PPAR-α/Angptl4 pathway in endothelial cells under high glucose condition upon OGSD/R

To further evaluate the regulation of JAM-A, VE-cadherin, and integrin-α5 by TXL, their expression in the membrane fraction was detected by western blotting analysis. As expected, there was significant reduction in the membrane location of intercellular junction protein JAM-A, VE-cadherin, and integrin-α5 in endothelial cells after OGSD/R stimulation (Fig. 3). Compared with the high glucose controls without TXL treatment, TXL-treated endothelial cells showed prominent increase in the membrane location of VE-cadherin and integrin-α5, which was at similar levels as those seen in the endothelial cells treated with rhAngptl4 (Fig. 3B and C). Interestingly, we did not find significant increase in the membrane location of JAM-A following TXL treatment under OGSD/R (Fig. 3A). Similarly, there was significant increase in the membrane location of integrin-α5 in insulin-treated endothelial cells, which was at similar levels as those seen in the groups treated with rhAngptl4 or TXL (Fig. 3C). Furthermore, the knock-down of Angptl4 expression with siRNA interference reversed the upregulation of membrane location of VE-cadherin and integrin-α5, but not

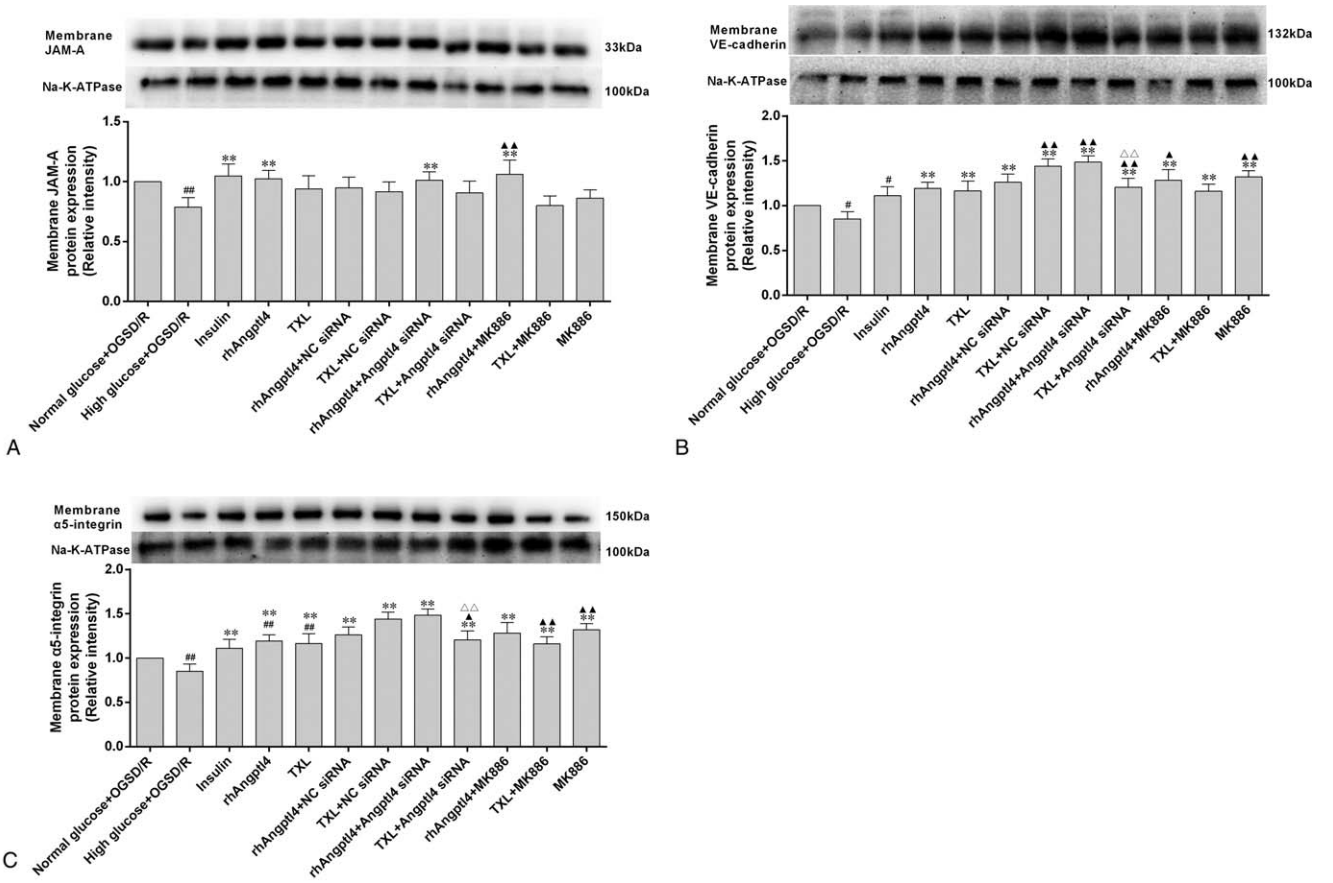

Figure 3. Immunoblotting analysis of the expression of membrane-located intercellular junction protein in endothelial cells treated with or without TXL in the presence or absence of signal regulators. (A) Membrane JAM-A, (B) membrane VE-cadherin, (C) membrane integrin-α5, n = 6; $^{\#}P < .05$, $^{\#\#}P < .01$ versus normal glucose + OGSD/R group; $^{**}P < .01$ versus high glucose + OGSD/R group; $\blacktriangle P < .05$, $\blacktriangle\blacktriangle P < .01$ versus TXL group; $^{\Delta\Delta}P < .01$ versus TXL + NC siRNA group. NC siRNA = negative control siRNA, OGSD/R = oxygen-glucose-serum deprivation and restoration, TXL = Tongxinluo.

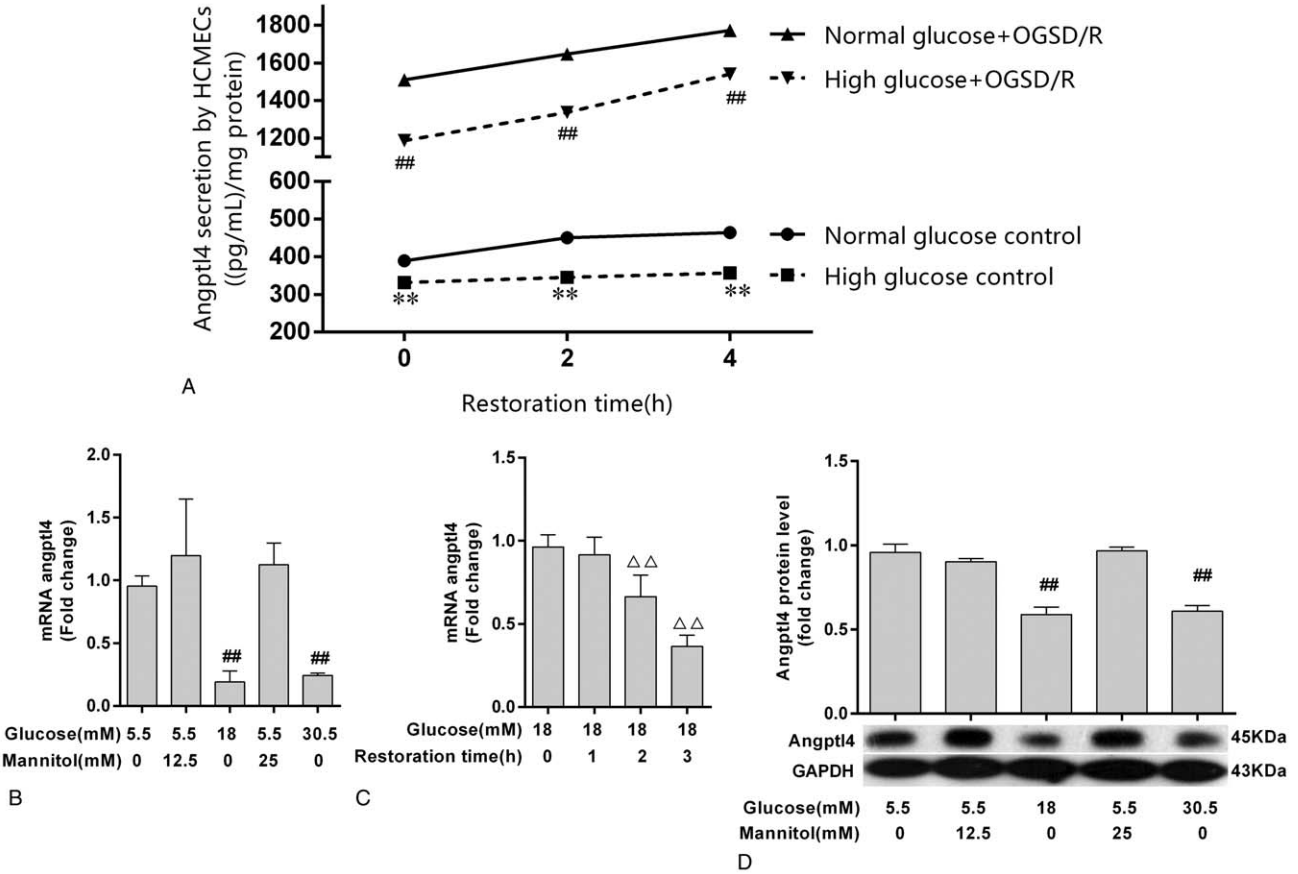

Figure 4. Measurement of the levels of Angptl4 secreted from endothelial cells and detection of the levels of the expression of angptl4 mRNA and protein in the endothelial cells treated with or without high glucose in the presence or absence of OGSD/R. (A) Angptl4 secreted by endothelial cells was measured by ELISA. n = 6; (B) angptl4 mRNA levels detected by RT-PCR, n = 3; (C) Angptl4 protein levels measured by western blotting, n = 6. $^{\#\#}P < .01$, versus normal glucose + OGSD/R group at the same time, $^{**}P < .01$ versus normal glucose control group at the same time, $^{\Delta\Delta}P < .01$ versus 0 h after restoration group. Angptl4 = angiopoietin-like 4, ELISA = enzyme-linked immunosorbent assay, OGSD/R = oxygen-glucose-serum deprivation and restoration, RT-PCR = real-time polymerase chain reaction.

JAM-A (Fig. 3). Addition of PPAR-α inhibitor MK886 partially blocked TXL-induced upregulation of the membrane location of integrin-α5, but not JAM-A or VE-cadherin (Fig. 3). These results suggested that the membrane location of integrin-α5 but not JAM-A or VE-cadherin was regulated by TXL via PPAR-α/Angptl4 pathway.

3.4. High glucose condition inhibits Angptl4 secretion in endothelial cells with or without OGSD/R stimulation

Our study confirmed that in high glucose medium-cultured and OGSD/R stimulated HCMECs, endothelial permeability was significantly increased after the knock-down of Angptl4 expression. This indicated that high glucose or OGSD/R may alter the secretion profile of Angptl4 from endothelial cells and disrupt endothelial barrier integrity. To confirm the effect of high glucose and OGSD/R on Angptl4 secretion profile, we measured Angptl4 content in the supernatant by ELISA. Figure 4A showed that without OGSD/R stimulation, Angptl4 content in the supernatant in normal or high glucose medium-cultured endothelial cells increased gradually, as identified by ELISA. However, compared with normal glucose group without OGSD/R, Angptl4 content in the supernatant significantly decreased in high glucose controls without OGSD/R (Fig. 4A). On the other hand, as shown in Figure 4A, under OGSD/R condition, Angptl4 content in the supernatant in normal or high glucose medium-cultured endothelial cells also increased gradually. Under OGSD/R condition, Angptl4 content in the supernatant also decreased in high glucose medium-cultured endothelial cells as compared with those of normal glucose controls (Fig. 4A). These data indicated that high glucose (18 mM) impaired Angptl4 secretion in HCMECs with or without OGSD/R stimulation.

3.5. OGSD/R inhibits the expression of Angptl4 mRNA and protein in endothelial cells under high glucose condition

We next measured angptl4 mRNA levels to investigate the mechanism of Angptl4 secretion impairment under OGSD/R condition at different time points (0 hours, 1 hour, and 2 hours after restoration). Figure 4C showed that under OGSD/R stimulation, the levels of the expression of angptl4 mRNA decreased gradually after restoration in high glucose medium-cultured endothelial cells. Compared with the levels of the expression of angptl4 mRNA at 0 hours after restoration, they remarkably decreased at 2 hours and 3 hours after restoration (Fig. 4C). These data suggested that the levels of the expression of angptl4 mRNA decreased upon OGSD/R in a time-dependent manner. We adopted oxygen-glucose-serum deprivation (2

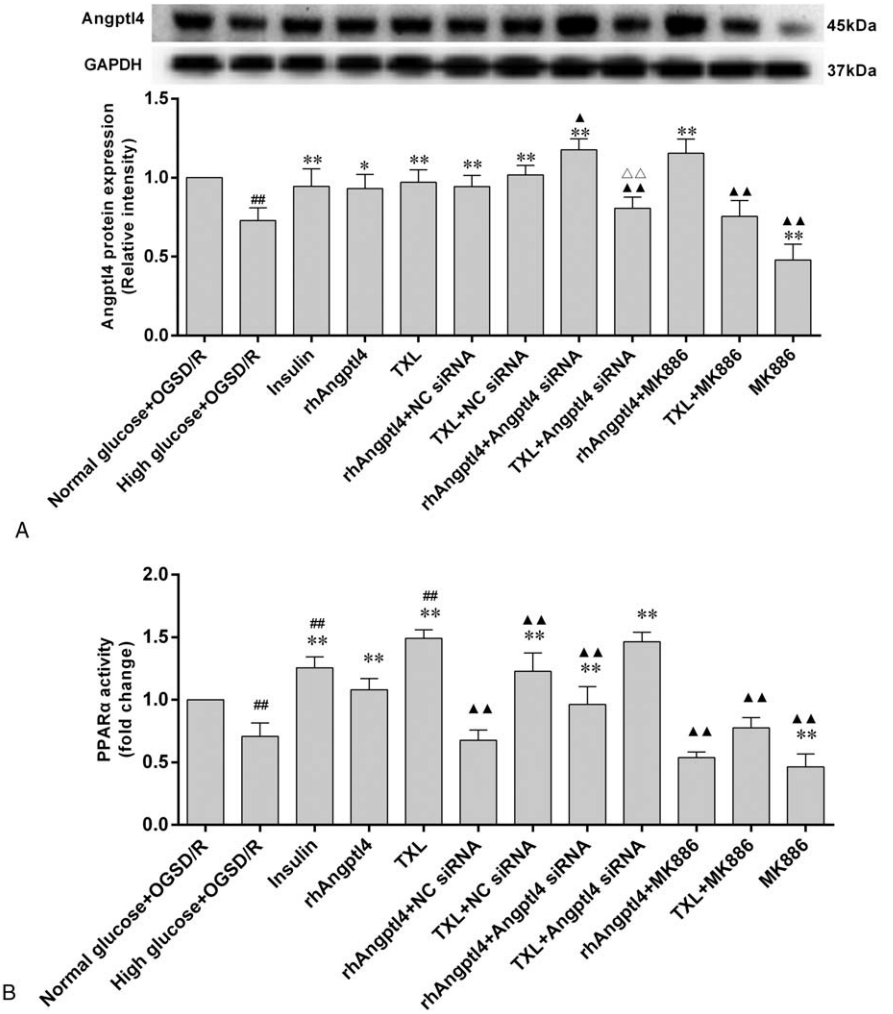

Figure 5. Immunoblotting analysis of the expression of Angptl4 and analysis of PPARα activity in endothelial cells treated with or without TXL in the presence or absence of signal regulators. (A) Total protein was extracted from the endothelial cells and analyzed by immunoblotting. n = 6; (B) PPARα activity was examined in the nuclear protein extracted from the endothelial cells treated with or without TXL in the presence or absence of signal regulators. n = 6; $^{\#\#}P < .01$ versus normal glucose + OGSD/R group; $^{**}P < .01$ versus high glucose + OGSD/R group; $▲▲P < .01$ versus TXL group; $^{\Delta\Delta}P < .01$ versus TXL + NC siRNA group. Angptl4 = angiopoietin-like 4, NC siRNA = negative control siRNA, OGSD/R = oxygen-glucose-serum deprivation and restoration, PPAR-α = peroxisome proliferator activated receptor-α, TXL = Tongxinluo.

hours) and restoration (2 hours) as the time point for further study. Meanwhile, as is shown in Figure 5A, there was significant reduction in Angptl4 expression in endothelial cells after OGSD/R.

3.6. High glucose suppresses the expression of Angptl4 mRNA and protein in endothelial cells

Compared with normal glucose controls, the levels of the expression of angptl4 mRNA and Angptl4 protein significantly decreased in high glucose (18 mM) and very high glucose (30.5 mM) groups. However, addition of mannitol had no effect on the levels of the expression of angptl4 mRNA and Angptl4 protein (Fig. 4B and D). These data indicated that high glucose decreased the levels of the expression of angptl4 mRNA and Angptl4 protein, peaked at 18 mM, as identified by RT-PCR and western blotting. We adopted high glucose (18 mM) as the glucose concentration to observe the relationship between Angptl4 expression and endothelial barrier function.

3.7. Angptl4 expression induced by TXL is dependent on PPAR-α/Angptl4 pathway in endothelial cells upon OGSD/R under high glucose condition

Compared with the controls without TXL treatment, TXL-treated endothelial cells showed prominent increase in Angptl4 expression in HCMECs upon OGSD/R under high glucose condition, which was at similar levels as those seen in the groups treated with insulin or rhAngptl4 (Fig. 5A). There is evidence showing that PPAR-α/Angptl4 pathway plays an important part in regulating endothelial barrier function.[8] To investigate whether PPAR-α/Angptl4 pathway also mediated Angptl4 upregulation stimulated by TXL, PPAR-α inhibitor MK886 was used and then the levels of the expression of Angptl4 were measured in the TXL-treated HCMECs upon OGSD/R. The results revealed that administration with MK886 reversed the effects of 800 μg/mL TXL (Fig. 5A). Interestingly, upregulation in Angptl4 expression in HCMECs treated with rhAngptl4 could not be abrogated when MK886 was delivered.

We next measured the PPAR-α activity to further explicit the mechanisms underlying TXL-induced Angptl4 upregulation. Figure 5B showed that PPAR-α activity dramatically decreased in HCMECs upon OGSD/R. Compared with the high glucose controls without TXL treatment, TXL-treated endothelial cells showed prominent increase in the levels of PPAR-α activity, which were at similar levels as those seen in the endothelial cells treated with insulin (Fig. 5B). Interestingly, we did not find significant increase in the PPAR-α activity in HCMECs treated with rhAngptl4 (Fig. 5B). Furthermore, the knock-down of Angptl4 expression failed to block the TXL-induced upregulation PPAR-α activity (Fig. 5B), suggesting TXL-induced PPAR-α activity is independent on Angptl4. But TXL impact on PPAR-α activity was compromised by PPAR-α inhibitor MK886 (Fig. 5B). These data suggested that PPAR-α/Angptl4 pathway played a role in the protection of TXL on endothelial barrier of HCMECs upon OGSD/R.

4. Discussion

Our previous animal studies have offered the data comparing the effect of TXL with other treatments on preventing from lethal I/R-injury in non-diabetic hearts, which shows ischemic preconditioning is the strongest, followed by the similar efficacy of simvastatin and TXL, and calcium channel blockers the weakest.[11] Our recent study has further confirmed the beneficial effects of TXL on I/R-injury in diabetic hearts.[8] The main findings of the present study are as follows:

(1) TXL can alleviate OGSD/R-injury by protecting endothelial barrier integrity in high glucose medium-cultured HCMECs;
(2) by enhancing the integrity of endothelial barrier structure and function, Angptl4 plays a pivotal role in the protective effect of TXL through upregulation of PPAR-α pathway.

To our knowledge, this is the first report showing the interaction of Angptl4 with endothelial barrier function in HCMECs under high glucose and OGSD/R condition and the upregulation of PPAR-α/Angptl4 pathway as a protective mechanism of TXL in HCMECs.

Our study confirmed the high glucose medium cultured-HCMECs were more sensitive to OGSD/R-injury compared with those under normal glucose condition, demonstrated by the increase of endothelial barrier permeability, the decrease of the expression of cellular junction protein in both total and membrane fraction of HCMECs. Furthermore, the results also showed that high glucose condition decreased the levels of the expression of angptl4 mRNA and Angptl4 protein with the effects peaked at 18 mM in HCMECs. In addition, the levels of the expression of angptl4 mRNA were significantly decreased by OGSD/R intervention in a time-dependent manner in HCMECs under high glucose condition.

Timely reopening of the occluded coronary artery is the most effective therapy to limit infarct size and improve the clinical outcome after AMI.[1] However, I/R-injury may cause myocardial cell death in AMI patients with or without diabetes. For diabetes patients receiving reperfusion therapy, insulin not only lowers the levels of blood glucose but also decreases myocardial I/R-injury by reducing myocardial apoptosis.[8] Endothelial cell injury caused by diabetes may be ameliorated via insulin receptor-mediated activation of PI3K/Akt/eNOS pathway.[23] Importantly, previous study showed that endothelial tight junction protein ZO-1 was upregulated by insulin and was related with enhancing endothelial barrier function[24] and decreasing apoptosis of endothelial cells via activating PI3K/Akt pathway.[25] Our recent study confirmed that as effective as insulin, TXL protected endothelial barrier integrity during I/R in diabetic hearts.[8] We chose insulin as positive control in our research.

Various mechanisms mediate the protection of TXL in reducing infarct size and alleviating I/R-injury. Previous study confirmed that TXL provided protection via anti-apoptotic and pro-autophagic mechanisms by activating AMPK pathway in infarcted hearts of non-diabetic rats.[26] TXL could also upregulate adhesion junction protein VE-cadherin and β-catenin in cardiac microvascular endothelial cells in mini-swine[27] and tight junction protein occludin and claudin.[28] HCMECs research showed that induction of autophagy by TXL through the MEK/ERK pathway protected HCMECs from hypoxia/reoxygenation injury in normal glucose condition.[17] Our present study revealed that TXL greatly reduced OGSD/R-injury under high glucose condition, which could further supplement the endothelial barrier integrity protection mechanisms of TXL under high glucose condition in vitro.

It has been demonstrated that TXL could modulate cytokine secretion by HCMECs in I/R-injury, Angptl4 was one of the protein upregulated by TXL.[17] Highly expressed in adipose tissue, liver, placenta, and ischemic tissue, Angptl4 can increase angiogenesis, regulate the survival and adhesion of endothelial cells, lipid metabolism and vascular permeability tissue-specifically.[13,29] Previous study showed that Angptl4 expression was upregulated by HIF-1α in articular cartilage cells, endothelial cells, cardiomyocytes, and tumor cells during hypoxia.[13,29–31] Moreover, transcriptional factor can activate angptl4 gene expression under ischemia, hypoxia, stress and high glucose conditions.[14,15] Our previous study confirmed that Angptl4 mediated the protection of TXL in diabetic hearts from I/R-injury.[8] The originality of Angptl4 may be circulating, HCMECs-derived or cardiomyocytes-derived. In agreement with these results, we demonstrated that, by maintaining the structure and function of endothelial barrier, HCMECs-derived Angptl4 played a pivotal role in the protection of endothelial cells upon OGSD/R under high glucose condition. Moreover, TXL decreased endothelial permeability and the effects of TXL were as effective as that of rhAngptl4. In studies of cancer, overexpression of Angptl4 was found to be able to inhibit the formation of endothelial stress fibers and increase the membrane location of intercellular junction protein vinculin.[32] Angptl4 produced by hypoxic tissue could enhance microvascular endothelial barrier function,[33] which is also in accordance with our study. However, Angptl4 derived from some kinds of tumors could damage microvascular endothelial barrier.[29,34] The discrepancy may be due to the different cell types, stimuli, and duration of treatment applied in the studies. Our study demonstrated that upon OGSD/R insult, Angptl4 expression was significantly decreased under high glucose condition compared with that under normal glucose condition. Interestingly, suppressing Angptl4 by siRNA resulted in endothelial barrier dysfunction in OGSD/R-treated HCMECs; in contrast, supplement with extrinsic rhAngptl4 could reverse the endothelial barrier disruption upon OGSD/R under high glucose condition and rhAngptl4 was as effective as that of insulin and TXL. These data suggested that Angptl4 was the protector in HCMECs under OGSD/R and the insufficiency of Angptl4 may partially explain the disruption of endothelial barrier upon

OGSD/R under high glucose condition. Modulating the expression of Angptl4 may be a new target to improve endothelial barrier function of HCMECs under OGSD/R in vitro, more importantly, to solve myocardial I/R-injury problem.

Not only a simple physical barrier, endothelial cells could also protect its own endothelial barrier integrity against OGSD/R-injury through regulating Angptl4 secretion in endocrine, paracrine, and autocrine manners. Angptl4, either extrinsic or induced by TXL showed protective effects. OGSD/R upregulated the expression of Angptl4 in HCMECs, which was related to endogenous protection against OGSD/R-injury. However, high glucose could weaken the endogenous protection induced by OGSD/R by diminishing Angptl4 production in HCMECs upon OGSD/R. Being treated with TXL, the expression of Angptl4 was markedly upregulated, together with increasing the expression of intercellular junction protein. Knock-down Angptl4 expression with siRNA blocked the upregulation of intercellular junction protein, indicating the key role of Angptl4 both in endogenous and TXL-induced protection of endothelial barrier in HCMECs upon OGSD/R under high glucose condition.

The endothelial barrier is mainly composed of tight junction, adhesion junction, and focal adhesion junction. The OGSD/R insult may regulate the expression of junction substructure components, leading to disconnection of neighboring endothelial cells, disconnection of endothelial cells from base membrane and microvascular hyperpermeability. Occludin and JAM-A are the important protein that constitute the tight junctions between adjacent endothelial cells. Previous study showed that tight junction protein occludin and claudin were upregulated by TXL in Ang II-induced inflammation in cardiac microvascular endothelial cells.[28] In the present study, the results further confirmed that administration of either rhAngptl4 or TXL could upregulate the expression of tight junction protein JAM-A and enhance the endothelial barrier function in "diabetic pathological condition." VE-cadherin and p120-catenin are the important protein that constitute the adhesion junctions between neighboring endothelial cells. It has been demonstrated that Angptl4 could decrease endothelial permeability[35] by decreasing of the dissociation of VE-cadherin-VEGFR2 complex, the mechanism may be related with activation of PI3K/Akt pathway and inhibition of VEGF-induced translocation of Src family kinases to cell membrane.[13,30] A study conducted by Li etc. showed that adhesion junction protein VE-cadherin was upregulated by TXL via upregulating KLF5.[28] In consistence with these reports, we further confirmed that VE-cadherin expression was remarkably increased by TXL in HCMECs in "diabetic pathological condition," together with strengthened endothelial barrier function. So far, the role of integrin, which constitutes the focal adhesion junctions connecting the endothelial cells with base membrane, remains elusive in the protection of TXL in HCMECs from OGSD/R-injury under high glucose condition. Intriguingly we found that TXL can not only remarkably increase integrin-α5 expression, but also significantly increase the levels of the membrane location of integrin-α5 and VE-cadherin in HCMECs treated by TXL, which may indicate that TXL-induced Angptl4 upregulation protects endothelial barrier through increasing the expression and membrane location of intercellular junction protein. This is in agreement with the report that exogenous Angptl4 protected endothelial barrier integrity by inhibiting VEGF-driven dissociation of the VEGFR2/VE-cadherin complex.[36]

To date, many signaling pathways have been suggested in Angptl4 regulation and PPAR-α is thought to be an important positive regulator of Angptl4 under I/R and other stresses.[14,15] PPAR-α is highly expressed in the heart, liver, kidney, intestine, and brown adipose tissue, all of which are characterized by an elevated rate of fatty acid catabolism. PPAR-α regulates the cardioprotection effect in AMI rats of diabetes, through anti-inflammation and anti-oxidization via the PI3K/Akt/eNOS/NO pathway.[37] Previous study confirmed that activating PPAR-α could enhance cerebrovascular endothelial barrier function upon oxygen-glucose deprivation,[10] ameliorating diabetic micro-angiopathy.[38] In accordance with our previous study in vivo,[8] the protection of TXL on endothelial barrier in this study was also related with upregulating Angptl4 via activating PPAR-α in HCMECs in vitro. PPAR-α may become the common target of diabetic microangiopathy,[38] and also the target of cardioprotective drugs for diabetic patients with AMI in the reperfusion era.

The study has several limitations. First, although targets of TXL in endothelial barrier function have been confirmed in our study. The underline mechanisms of action were not clear. Further studies are needed to confirm whether TXL targets receptors expressed on the cell membrane or enters into the cells. And studies are also needed to confirm whether it mediates effects through genomic or non-genomic mechanism. Second, the crosstalk between cardiomyocytes and HCMECs remains unclear in TXL cardioprotection. Further studies are needed to confirm whether TXL mediates effects through the crosstalk mechanism.

5. Conclusion

In a manner similar to insulin and rhAngptl4, TXL could protect endothelial barrier structure and function during OGSD/R under high glucose condition. Activating PPAR-α/Angptl4 pathway may provide an attractive strategy to defense endothelial barrier integrity against I/R-injury in diabetic patients with AMI in reperfusion era.

Author contributions

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