CXCR7 agonist TC14012 improves angiogenic function of endothelial progenitor cells via activating Akt/eNOS pathway and promotes ischemic angiogenesis in diabetic limb ischemia

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Abstract

Purpose Endothelial progenitor cells (EPCs) play a critical role in repairing damaged vessels and triggering ischemic angiogenesis, but their number is reduced and function is impaired under diabetic conditions. Improving EPC function has been considered as a promising strategy to ameliorate diabetic vascular complications. In present study, we want to investigate whether and how CXCR7 agonist TC14012 promotes the angiogenic function of diabetic EPCs.

Methods High glucose (HG) treatment was used to mimic the hyperglycemia in diabetes. Tube formation, cell scratch recovery, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were used to evaluate the angiogenic capability, cell migration and apoptosis of EPCs, respectively. Hind limb ischemia (HLI) model was used to appraise the ability of TC14012 in promoting diabetic ischemic angiogenesis *in vivo*.

Results HG treatment impaired EPC tube formation and migration, and induced EPC apoptosis and oxidative damage, while TC14012 rescued tube formation and migration, and prevented HG-induced apoptosis and oxidative damage of EPCs. Furthermore, these beneficial effects of TC14012 on EPCs were attenuated by specific siRNAs against CXCR7, validating that CXCR7 is a functional target of TC14012 in EPCs. Mechanistic studies demonstrated that HG treatment reduced CXCR7 expression in EPCs, impaired Akt and endothelial nitric oxide synthase (eNOS) phosphorylation and nitric oxide (NO) production; similarly, these signal impairments in HG-exposed EPCs could be rescued by TC14012. However, the protective effects of TC14012 on Akt and eNOS phosphorylation and NO production were almost completely abolished by siRNAs against CXCR7 or Akt specific inhibitor Wortmannin. More importantly, *in vivo* study showed that TC14012 administration enhanced blood perfusion recovery and angiogenesis in the ischemic hind limb and increased the EPC number in circulating of *db/db* mice, demonstrating the capability of TC14012 in promoting EPC mobilization and ischemia angiogenic function.

Conclusion TC14012 can prevent EPCs from HG-induced dysfunction and apoptosis, improve eNOS activity and NO production via CXCR7/Akt signal pathway, and promote EPC mobilization and diabetic ischemia angiogenesis.

Introduction

Diabetes mellitus has become a rising epidemic worldwide. Type 2 diabetes, characterized by progressive insulin resistance and subsequent hyperglycemia (1, 2), accounts for more than 90% of the diabetic patients. Diabetic vascular complications, including diabetic limb ischemia, nephropathy, retinopathy, are the major cause of morbidity and mortality in diabetic patients and put huge burden on healthcare systems (3). Diabetic vascular complications were always initiated with endothelial dysfunction (4, 5), and accompanied with dysregulation of vascular remodeling and impaired/abnormal angiogenesis.

Endothelial progenitor cells (EPCs) are the precursors of vascular endothelial cells (6). Accumulating evidence demonstrates that EPCs play a critical role in maintaining endothelial homeostasis and triggering angiogenesis (7–14). However, under diabetic conditions, the circulating EPC number was decreased and EPC function was impaired (15), which lead to attenuated repair of endothelium and ischemic angiogenesis, indicating that elevating circulating EPC number and/or improving EPC function is a potential strategy in the therapy of diabetic vascular complications.

Chemokine stromal cell derived factor 1 (SDF-1) is a key regulator of EPC mobilization, migration, retention and angiogenesis (16, 17). SDF-1 exerts its biological function via its receptors C-X-C chemokine receptor type 4 (CXCR4) and CXCR7 (15). SDF-1/CXCR4 axis plays a critical role in maintaining the quiescent state of EPCs in their bone marrow niche and specific antagonism of CXCR4 can mobilize EPCs from bone marrow into peripheral circulation to participate in vessel repair and angiogenesis (18). In addition, SDF-1/CXCR7 axis plays a primary role in maintaining EPC survival and function (17, 19). Moreover, our previous studies have demonstrated that CXCR7 expression in EPCs is downregulated under diabetic conditions, which is accompanied with EPC dysfunction and oxidative injury (15). More importantly, elevating CXCR7 expression improves survival and angiogenic function of EPCs and promotes blood perfusion in diabetic limb ischemia mouse model (15). These findings indicate the potential of targeting SDF-1/CXCR4/CXCR7 axis in ameliorating EPC dysfunction and promoting ischemic angiogenesis under diabetic conditions.

Cyclic peptide TC14012, a potent selective CXCR7 agonist (20–22), is also a strong antagonist against CXCR4 (20), has been reported to induce tube formation of human umbilical vein endothelial cells (23), reduce apoptosis in cardiovascular disease (24), and prevent lung epithelial damage and ameliorate fibrosis after bleomycin or hydrochloric acid induction (25). However, the effects of TC14012 on EPC angiogenic function and its therapeutic potential in diabetic ischemic injury remain elusive. Thus, the current study is aimed to investigate whether TC14012 can improve EPC function under diabetic conditions and clarify the underlying mechanism.

Materials And Methods

EPC isolation, identification and treatment

Human cord blood EPCs were isolated and cultured as described in our previous study (26). In brief, 50 mL human cord blood was obtained from umbilical vein of healthy post-partum women. Blood samples were diluted 1:1 with phosphate-buffered saline (PBS) and overlaid onto an equivalent volume of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). Mononuclear cells were isolated after density gradient centrifugation, then washed with Endothelial Cell Medium (ECM, ScienCell Research, Carlsbad, CA), and seeded into a culture dish ($\varphi = 6\text{cm}$) that pre-coated with fibronectin (2 mg/cm^2) and cultured with ECM supplemented with 10% fetal bovine serum (FBS, Life Technologies, Austin, TX). After 3 days of culture, the unattached cells and debris were washed with PBS. The medium was changed daily during the first week and every 3 days thereafter. All protocols were approved by the Institutional Review Board of the

First Affiliated Hospital of Wenzhou Medical University, and informed consents were all obtained from these subjects.

To mimic the hyperglycemia in diabetes *in vitro*, EPCs were treated with high glucose (HG, 33 mmol/L), and equivalent mannitol was used as osmotic control. After treatment for 24 h, EPCs were harvested for further assays. For signal test, EPCs were harvested at 10 min after mannitol or HG with or without TC14012 (5 μ mol/L, Cayman Chemical Company, Ann Arbor, MI) treatment.

siRNA transfection

To knockdown CXCR7 expression in EPCs, siRNAs against human CXCR7 or a Silencer Select Negative Control (GenePharma, Shanghai, China) were transfected into EPCs using GP-transfect-Mate (GenePharma, Shanghai, China). Three siRNAs against CXCR7 sequences are: CXCR7 siRNA-441 sense: GCUAUGACACGCACUGCUATT, antisense: UAGCAGUGCGUGUCAUAGCTT; CXCR7 siRNA-960 sense: GCAGCCGGAAGAUAUCUUTT, antisense: AAGAUGAUCUUCGGCUGCTT; and CXCR7 siRNA-1161 sense: GCUUCAUCAAUCGCAACUATT, antisense: UAGUUGCGAUUGAUGAAGCTT. The knockdown efficiency was detected by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot at 48 h post transfection, and the siRNA with the best knockdown efficiency was used in the following studies.

Tube formation and cell scratch recovery assays

Tube formation assay was adopted to investigate angiogenic capability of EPCs *in vitro* according to previous studies (14, 15) with minor modification. Briefly, a μ -Slide angiogenesis plate (Ibidi, Shanghai, China) was coated with growth factor reduced Matrigel (10 μ L per well, Corning). EPCs (1.75×10^5 cells/ml) were re-suspended in 50 μ L basal medium or medium containing HG in the presence or absence of TC14012, plated on the Matrigel, and incubated in cell incubator at 37°C with 5% CO₂ and saturation humidity. After 8 h culture, images of tube network in each well were taken using an inverted microscopy (Eclipse TI-S, Nikon, Japan) and total lengths of tube-like structures were quantified using ImageJ (NIH, Bethesda, Maryland).

Migration ability of EPCs was investigated by cell scratch recovery assay. Briefly, EPCs (1.75×10^5 /well) were seeded onto 6-well plate and maintained to confluence. Thereafter, a scratch was made using pipette tips. EPCs were cultured in the basal medium with Man or HG containing with or without TC14012 for 24 h. Mitomycin (10 μ g/mL, Sigma-Aldrich, St. Louis, MO) was used to exclude the influence of cell proliferation. Scratches were recorded under a light microscopy (Leica DMI3000B, Wetzlar, Germany) equipped with a digital camera (Olympus DP25, Japan). The recovery of scratch was measured using ImageJ.

Cell apoptosis

One-step terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay kit (Beyotime, Shanghai, China) was used to detect EPC apoptosis. According to the manufacturer's protocol, plated EPCs (2×10^5 cells/well) were rinsed with PBS and fixed with 4% paraformaldehyde. Then EPCs were incubated with immunostaining permeabilization buffer for 5 min and followed with reaction buffer at 37°C in dark for 1 h. Fluorescent images were taken with inverted microscopy (Eclipse TI-S, Nikon, Japan). TUNEL positive nuclei were quantitatively counted manually, and cell apoptosis was presented as the ratio of TUNEL positive nuclei to the total nuclei recognized by 4',6-diamidino-2phenylindole (DAPI).

Oxidative stress assay

Reactive oxygen species (ROS) assay kit (Beyotime, Shanghai, China) was used to detect oxidative stress of EPCs. According to manufacturer's instruction, EPCs were incubated with dichlorofluorescein diacetate (DCF-DA) for 20 min. Ten different fields per well were taken using a microscopy (Eclipse TI-S, Nikon, Japan) and the fluorescence intensity was analyzed using ImageJ.

Western blot

Western blot was performed as described in our previously studies (14, 15). Briefly, EPCs were lysed with cold radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) at the indicated time points and clarified by centrifugation at 12,000g at 4°C for 30 min. Supernatants were collected and the protein concentration was determined by the Bradford assay (Thermo Fisher Scientific, Waltham, MA). Total 30 µg proteins were prepared and mixed with loading buffer and dithiothreitol, and denatured by incubating at 95°C for 5 min. The proteins were separated by a 10% SDS-PAGE and then transferred to nitrocellulose membranes (Merck Millipore, Danvers, MA). After blocking by 5% milk, membranes were incubated with primary antibodies (Cell Signaling Technology) against Akt (1:5,000) and phosphor-Akt (1:1,000), eNOS (1:5,000), phosphor-eNOS (1:1,000), 3-nitrotyrosine (3-NT, 1:1000) and 4-hydroxynonenal (4-HNE, 1:1000), at 4°C overnight. Then, the membranes were washed by Tris-buffered saline with Tween-20 (TBST) for three times and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Blots were visualized by ECL Plus (Solarbio, Beijing, China) and quantified using Quantity 5.2 (Bio-Rad, Hercules, CA).

Nitric oxide (NO) assay

The NO levels in EPC culture medium were tested by NO assay (Beyotime, Shanghai, China) following the manufacturer's instructions.

Diabetic hind limb ischemia (HLI) mice models and TC14012 treatment

Male *db/db* (BKS.Cg-*Dock7*^m +/+ *Lep*^{db}/J) mice at the age of 8-12 weeks were obtained from The Jackson Laboratory (Bar Harbor, ME). HLI was performed as described previously (8, 14, 15). Briefly, right hind limbs of the mice were shaved and operated under anesthetized condition with isoflurane (1–3% isoflurane in 100% oxygen at a flowrate of 1 L/min). The entire right superficial femoral artery and vein were separated and ligated (6-0 silk sutures), cut, and excised with an electric coagulator, epidermis was sutured with 4-0 silk sutures. At 1 h after HLI surgery, TC14012 (10 mg/kg body weight) was injected intraperitoneally once and the same volume of PBS was used as vehicle control. Blood perfusion of the ischemic hind limb before surgery and at days 0, 3, 7, 14, 21, 28 after surgery was detected using a Pericam Perfusion Speckle Imager (PSI, Perimed Inc., Sweden), and presented as the ratio of the ischemic (right) to the contralateral non-ischemic (left) limb. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisville, which conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Histological assessment

Transverse sections of gastrocnemius and soleus muscles of ischemic hind limbs were cut and embedded in Optimal Cutting Temperature Compound for frozen sections. Antibody against CD31 (BD Pharmingen, San Jose, CA) was stained to recognize capillary of ischemic muscles, and 4',6-diamidino-2phenylindole (Thermo Fisher Scientific, Waltham, MA) was used to recognize nuclei. Fluorescent fields were captured by fluorescence microscopy (DP74, Olympus). The capillary number was counted using ImageJ in randomly selected fields for a total of 20 different fields per section and 3 sections per animal. The capillary density was expressed as CD31 positive capillaries per field.

Flow cytometry assay of circulating EPCs

Whole blood of *db/db* HLI mice treated with TC14012 (10 mg/kg body weight) was collected for flow cytometry analysis. According to our previous study (14, 15), 200 μ L peripheral blood was mixed with Lysing Solution (BD Biosciences, San Jose, CA) to lyse red blood cells. While blood cells were incubated with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) for 15 min for blockade of nonspecific binding and then stained with PE-conjugated anti-mouse CD34 and APC-conjugated anti-mouse VEGFR2 antibodies (BD Pharmingen, San Jose, CA) at room temperature for 1 h. The same fluorescein-labeled isotype IgG was served as control to exclude the non-specific staining. After centrifugation and resuspension with 400 μ L PBS, cells were analyzed with a flow cytometer (NovoCyte, Agilent, USA), and data were analyzed using FlowJo.

qRT-PCR

EPCs were lysed by Trizol (Tiangen Biotech, Beijing, China). Total RNA was extracted using a RNAsimple Total RNA Kit (Tiangen Biotech, Beijing, China) and reverse-transcribed using PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan) following the manufacturer's protocol. Primers of CXCR7 and β -actin were purchased from Genscript Biotechnology (Nanjing, China) and qRT-PCR was performed in duplication with a 20 μ L reaction system using a RT-PCR system (Takara, Shiga, Japan). The comparative cycle time (C_t) was used for determining fold differences, and β -actin was chosen as an endogenous reference ($2^{-\Delta\Delta C_t}$).

Statistical analysis

All data are presented as Mean $\pm$ SD. Sample size for each study was detailed in figure legends. Statistical analysis was performed using GraphPad Prism 5.0 with One-way or two-way ANOVA, followed by post-hoc Turkey's multiple comparisons. Statistical significance was considered as $P < 0.05$.

Results

TC14012 protects EPCs from HG-induced angiogenic dysfunction and oxidative stress

Matrigel tube formation assay was performed to test the protective effect of TC14012 on angiogenic function of EPCs *in vitro*. The results showed that HG treatment impaired tube formation of EPCs, while TC14012 obviously reversed this effect (Fig. 1A, B). Migration is an essential step in EPCs homing to the ischemic site and participating into vascular repair and angiogenesis. Similarly, scratch recovery assay showed that HG impaired EPC migration, while TC14012 preserved the migration ability of HG-treated EPCs (Fig. 1C, D). Furthermore, TC14012 also ameliorated HG-induced EPC apoptosis as determined by TUNEL staining (Fig. 1E, F).

As excessive generation of ROS is considered to be one of the major cause of diabetes-induced EPC dysfunction (15, 27), we investigated whether TC14012 prevents EPCs from HG-induced oxidative stress. DCF-DA staining showed that HG increased superoxide generation in EPCs, and this effect was completely abolished by TC14012 treatment (Fig. 2A, B). Moreover, the expression of oxidative stress biomarkers 3-NT (Fig. 2C) and 4-HNE (Fig. 2D) in EPCs were both elevated under HG condition, and reduced by TC14012 treatment, confirming TC14012 attenuation of HG-induced oxidative stress in EPCs. These findings demonstrate that TC14012 can prevent EPCs from HG-induced angiogenic dysfunction, apoptosis, and oxidative stress.

CXCR7 knockdown abolishes the protective effects of TC14012 against HG-induced EPC dysfunction

To test whether the protective effects of TC14012 against HG-induced EPC dysfunction relied on interaction with its receptor CXCR7, CXCR7 expression in EPCs was interfered by siRNA transfection (Supplementary Fig. S1A, B). Nonsense control-siRNA transfection did not affect TC14012 protecting HG-

treated EPCs, while CXCR7-siRNA transfection nearly abolished the protective effects of TC14012 against HG-induced impairment of tube formation (Fig. 3A, B), migration (Fig. 3C, E), and apoptosis (Fig. 3D, F). Similarly, CXCR7-siRNA transfection also significantly impaired TC14012 protecting EPCs against HG-induced superoxide generation (Fig. 4A, B) and oxidative biomarkers 3-NT (Fig. 4C, D) and 4-HNE expression (Fig. 4C, E). Taken together, these findings indicate that CXCR7 plays a vital role in TC14012 protecting EPCs from HG-induced angiogenic dysfunction and oxidative stress.

TC14012 prevents HG-induced impairment in Akt and eNOS activation and NO production in EPCs

eNOS, an endothelial-cell-specific isoform of the NO producing enzyme, plays a vital role in maintaining the pro-angiogenic function of EPCs (28–30). In addition, Akt pathway was reported to mediate eNOS activation in endothelial cells (31). In the present study, we found that HG treatment inhibited CXCR7 expression (Fig. 5A, B), Akt (Fig. 5A, C) and eNOS (Fig. 5A, D) phosphorylation, and NO production (Fig. 5E) in EPCs, while these effects were significantly prevented by TC14012 treatment (Fig. 5A-E). These findings indicate that TC14012 preserves Akt and eNOS activation and NO production in HG-treated EPCs.

CXCR7 mediates TC14012 preserving eNOS activation in HG-treated EPCs via activation of Akt pathway

To confirm whether TC14012 activates Akt and eNOS via receptor CXCR7, CXCR7 expression was interfered using CXCR7 siRNA, and nonsense-siRNA was used as control (Fig. 6A, B). The results showed that control-siRNA did not affect the effect of TC14012 on Akt (Fig. 6A, C) and eNOS (Fig. 6A, D) phosphorylation and NO production (Fig. 6E). However, CXCR7-siRNA totally abolished the protective effects of TC14012 against HG-induced impairment in Akt and eNOS phosphorylation and NO production (Fig. 6A-E).

To further determine the critical role of Akt in TC14012/CXCR7 axis preserving eNOS activation, Akt signal was blocked by Wortmannin. The results showed that Wortmannin inhibited Akt phosphorylation (Fig. 7A, B) and abolished the protective effects of TC14012 against HG-induced impairment in eNOS phosphorylation (Fig. 7A, C). These results confirm that TC14012 preserving eNOS phosphorylation is mediated by Akt in HG-treated EPCs.

TC14012 improves blood perfusion, ischemic angiogenesis, and mobilization of EPCs in diabetic HLI

To determine the therapeutic potential of TC14012 in ameliorating diabetic EPC dysfunction *in vivo*, its capability in promoting ischemic angiogenesis and blood perfusion recovery was evaluated in HLI mouse model. The PSI scanning results showed that TC14012 administration significantly improved blood perfusion recovery since day 14 to day 28 post surgery compared with vehicle treat mice (Fig. 8A, B). Moreover, TC14012-treated mice had increased capillary density in both gastrocnemius and soleus muscles measured day 28 after HLI compared with vehicle treated mice (Fig. 8C, D), indicating that TC14012 can promote angiogenesis in ischemic tissue in diabetic mice.

EPC mobilization is a critical step in ischemic angiogenesis (32). The flow cytometry results showed that TC14012-treated mice had higher circulating EPC number than that in vehicle group mice, indicating that TC14012 can also enhance EPC mobilization under diabetic ischemia conditions (Fig. 8E, F). Taken together, these *in vivo* results demonstrate that TC14012 can improve ischemic angiogenesis and blood perfusion recovery via promoting EPC mobilization and angiogenic function.

Discussion

In the present study, we demonstrate that TC14012 can improve tube formation, migration, and NO production capability of HG-treated EPCs *in vitro* and promote blood perfusion recovery in ischemic hind limb of diabetic mice via enhancing EPC mobilization and angiogenesis. Mechanistic study reveals that TC14012 alleviates oxidative stress and preserves angiogenic function of EPCs under diabetic conditions predominantly through activating CXCR7/Akt/eNOS pathways.

Emerging evidence indicates that CXCR7 is essential for regulating multiple cellular functional processes concerning endothelial hemostasis and angiogenesis (15, 26, 33). Our previous studies also demonstrate that CXCR7 plays an important role in regulating EPC function (17), and elevating CXCR7 expression can reverse EPC dysfunction under diabetic conditions (15). However, this approach requires infusion of autologous or allogenic EPCs modified by CXCR7 transgene, which has practical limitations in clinical application. If therapeutic efficacy can be achieved using a CXCR7 specific agonist, it will increase the translational value of CXCR7 as a therapeutic target in the treatment of diabetic ischemia vascular diseases. In the present study, we found that TC14012, a specific agonist of CXCR7, protected EPCs against HG-induced impairment in tube formation, migration and cell survival (Fig. 1), and improved angiogenesis and blood perfusion recovery in diabetic HLI (Fig. 8). These findings are in line with previous studies showing that TC14012 alleviates myocardial infarction (34), promotes lung alveolar repair (25) and enhances angiogenesis after myocardial infarction (24) via activating CXCR7. These facts demonstrate a promising therapeutic potential of TC14012 for treatment of diabetic ischemia vascular diseases.

In addition, TC14012 accelerated angiogenesis and blood perfusion recovery is also associated with elevation of circulating EPC number in *db/db* mice with HLI (Fig. 8E, F), indicating that TC14012 enhances EPC mobilization. Indeed, besides as an agonist of CXCR7, TC14012 can also function as an antagonist of CXCR4. SDF-1/CXCR4 axis plays a critical role in retaining the quiescent state of stem and progenitor cells in the bone marrow niche (35), and interfering SDF-1/CXCR4 interaction was considered as a feasible approach to mobilize EPCs. For example, AMD3100, a typical CXCR4 antagonist that used in clinic to mobilize stem cells for cell transplanting therapy, has been shown to accelerate blood flow restoration after HLI in diabetic mice via mobilizing EPCs (36, 37). Combining the benefits of TC14012 in improving EPC function as a CXCR7 agonist and promoting EPC mobilization as a CXCR4 antagonist to treat diabetic ischemia vascular diseases and other diabetic vascular complications in human patients needs further clinical validation.

Elevated ROS was considered as one of the major causes of diabetic EPC dysfunction and impaired peripheral vascular regeneration (15, 38), and our previous studies found that overexpression of metallothionein (14) or activating nuclear factor (erythroid derived 2)-like 2 (15) diminished oxidative stress and protected EPCs against diabetes- or HG-induced injury. In line with these findings, our present findings also showed that TC14012 alleviated ROS in HG-treated EPCs (Fig. 2), and this beneficial effect on EPCs was abolished by CXCR7-siRNA (Fig. 4). These findings indicate that TC14012 ameliorates oxidative stress in HG-treated EPCs via its receptor CXCR7, which contributes to its preservation of EPC function under diabetic conditions.

NO regulates EPC mobilization from bone marrow niches into the peripheral circulation and participating in neovascularization. Decreased NO bioavailability has been proposed as one of the determinants of EPC dysfunction under diabetic conditions (39). The activity of eNOS, the major enzyme that regulates NO production in endothelial cells, was also repressed in diabetic EPCs. Conversely, NO replenishment is beneficial for diabetic ischemia tissue reperfusion (40). In addition, few reports have specifically linked CXCR7 and eNOS to EPC angiogenic function in diabetic ischemia. In the present study, our findings demonstrated that TC14012 treatment upregulated eNOS activity and NO production (Fig. 5A, D, E) and these effects were specifically mediated by CXCR7 in EPCs (Fig. 6A, D, E).

Although the reduction in NO bioavailability could be attributable to oxidative stress in diabetic EPCs, it also may be due to impairment of the protein kinase signaling cascades that are responsible for NO production, like Akt (39). We explored the role of Akt signal in TC14012 activating eNOS pathway in EPCs under diabetic conditions, and found that the phosphorylation of Akt and eNOS were all impaired by HG and rescued by TC14012 treatment (Fig. 5A, C, D) and blockade of Akt signal repressed the phosphorylation of eNOS (Fig. 7), indicating that TC14012 protecting diabetic EPCs is likely mediated by CXCR7/Akt/eNOS signal pathways.

In summary, TC14012 activation of CXCR7 improves EPC survival and function under diabetic conditions. This is because of improved anti-oxidative potency and NO production in EPCs, which is likely mediated by Akt activating eNOS phosphorylation. Pharmacological administration of TC14012 *in vivo* enhances diabetic ischemia angiogenesis, blood perfusion recovery and EPC mobilization, highlighting a clinical relevance for diabetic vascular ischemia treatment.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Availability of data and material

All data relevant to the study are included in the article or uploaded as supplementary information.

Code availability

Not applicable

Author Contributions

Kai Wang, Xiaoqing Yan and Yi Tan conceived and designed the study. Kai Wang, Shiyue Sun, Guigui Zhang, Zixian Lu, Hui Chen, Xia Fan Chunjie Gu, Xiaohong Pan, Oscar Chen and Qian Lin performed *in vitro* cell culture, cell biology, molecular biology experiments, and histology analysis. Kai Wang and Xiaozhen Dai performed *in vivo* therapeutic studies in mice. Kai Wang, Xiaoqing Yan and Yi Tan wrote the manuscript. Kai Wang and Lu Cai edited the manuscript with important intellectual content. Xiaoqing Yan and Yi Tan supervised this study.

Ethics Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Declaration of Helsinki. All protocols were approved by the Institutional Review Board of the First Affiliated Hospital of Wenzhou Medical University.

Consent to Participate

All samples were collected with written informed consent of the participants.

Consent for Publication

Not applicable

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Figures

Figure 1

TC14012 protects EPCs from HG-induced angiogenic dysfunction and apoptosis. EPCs were exposed to high glucose (HG, 33 mmol/L) with or without TC14012 (TC, 5 µmol/L) treatment for 24 h, the equivalent concentration of mannitol (Man) was used as osmotic control (Ctrl). The angiogenic function of EPCs was determined by tube formation assay (A) and the tube length was quantified using ImageJ (B). The migration capability of EPCs was evaluated by cell scratch recovery assay (C) and the cell scratch

recovery area was quantified using ImageJ (D). The apoptosis of EPCs was detected by TUNEL staining (E) and ratio of TUNEL positive nuclei to total nuclei recognized by DAPI was calculated (F). Data were normalized to Man group and expressed as Mean $\pm$ SD. * P <0.05 vs. Man; # P < 0.05 vs. HG.

Figure 2

TC14012 attenuates HG-induced oxidative damage in EPCs. EPCs were exposed to high glucose (HG, 33 mmol/L) with or without TC14012 (TC, 5 μ mol/L) treatment for 24 h, the equivalent concentration of mannitol (Man) was used as osmotic control (Ctrl). The reactive oxygen species (ROS) generation was

detected by dichlorofluorescein diacetate (DCF-DA) staining (A) and the fluorescent intensity of ROS was quantified using ImageJ (B). The expression levels of the oxidative damage markers 3-nitrotyrosine (3-NT, C) and 4-hydroxynonenal (4-HNE, D) in EPCs were detected by Western blot and GAPDH was used as loading control. Three independent experiments were performed. Data were normalized to Man group and expressed as Mean $\pm$ SD. * P <0.05 vs. Man; # P <0.05 vs. HG.

Figure 3

Knockdown of CXCR7 abolishes TC14012 protecting EPCs from HG-induced angiogenic dysfunction and apoptosis. EPCs were transfected with the specific siRNA against CXCR7 for 48 h, the Silencer Select Negative Control was used as control siRNA (Ctrl-siRNA). Then the transfected EPCs were exposed to high glucose (HG, 33 mmol/L) for additional 24 h with or without TC14012 (TC, 5 μ mol/L) treatment, the equivalent concentration of mannitol (Man) was used as osmotic control (Ctrl). The angiogenic function of EPCs was evaluated by tube formation assay (A) and the tube length was quantified using ImageJ (B). The migration capability was evaluated by cell scratch recovery assay (C) and the scratch recovery was quantified using ImageJ (E). The apoptosis of EPCs was detected by a TUNEL staining (D) and the ratio of TUNEL positive nuclei to the total nuclei recognized by DAPI was calculated (F). Three independent experiments were performed. Data were normalized to Ctrl-siRNA-Man group and expressed as Mean $\pm$ SD. * P <0.05 vs. Ctrl-siRNA-Man; # P <0.05 vs. Ctrl-siRNA-HG; & P <0.05 vs. Ctrl-siRNA-HG+TC.

Figure 4

Knockdown of CXCR7 abolishes TC14012 protecting EPCs from HG-induced oxidative damage. EPCs were transfected with the specific siRNA against CXCR7 for 48 h, the Silencer Select Negative Control was used as control siRNA (Ctrl-siRNA). Then the transfected EPCs were exposed to high glucose (HG, 33 mmol/L) for additional 24 h with or without TC14012 (TC, 5 μ mol/L) treatment, the equivalent concentration of mannitol (Man) was used as osmotic control (Ctrl). The reactive oxygen species (ROS)

generation was detected by dichlorofluorescein diacetate (DCF-DA) staining (A) and the fluorescent intensity of ROS was quantified using ImageJ (B). The expression levels of the oxidative damage markers 3-nitrotyrosine (3-NT, C, D) and 4-hydroxynonenal (4-HNE, C, E) in EPCs were detected by Western blot and GAPDH was used as loading control. Three independent experiments were performed. Data were normalized to Ctrl-siRNA-Man group and expressed as Mean $\pm$ SD. * P <0.05 vs. Ctrl-siRNA-Man; # P <0.05 vs. Ctrl-siRNA-HG; & P <0.05 vs. Ctrl-siRNA-HG+TC.

Figure 5

TC14012 prevents HG-induced Akt and eNOS inactivation and NO reduction in EPCs. EPCs were exposed to high glucose (HG, 33 mmol/L) with or without TC14012 (TC, 5 μ mol/L) treatment for 10 min, the equivalent concentration of mannitol (Man) was used as osmotic control (Ctrl). The expression of CXCR7, and phosphorylation of Akt and eNOS were evaluated by Western blot (A-D) and GAPDH was used as loading control. The level of NO was evaluated by a NO assay kit (E). Three independent experiments

were performed. Data were normalized to Ctrl-siRNA-Man group and expressed as Mean $\pm$ SD. * P <0.05 vs. Man, # P <0.05 vs. HG.

Figure 6

Knockdown of CXCR7 abolishes TC14012 preventing HG-induced Akt and eNOS inactivation and NO reduction in EPCs. EPCs were transfected with the specific siRNA against CXCR7 for 48 h, the Silencer Select Negative Control was used as control siRNA (Ctrl-siRNA). Then the transfected EPCs were exposed to high glucose (HG, 33 mmol/L) for additional 10 min with or without TC14012 (TC, 5 μ mol/L) treatment, the equivalent concentration of mannitol (Man) was used as osmotic control (Ctrl). The expression of CXCR7 (A, B), and phosphorylation of Akt (A, C) and eNOS (A, D) were evaluated by Western blot and GAPDH was used as loading control. The level of NO was evaluated by NO assay (E). Three independent experiments were performed. Data were normalized to Man group and expressed as Mean $\pm$ SD. * P <0.05 vs. Ctrl-siRNA-Man; # P <0.05 vs. Ctrl-siRNA-HG; & P <0.05 vs. Ctrl-siRNA-HG+TC.

Figure 7

Akt inhibitor Wortmannin abolishes TC14012 preventing HG-induced eNOS inactivation in EPCs. EPCs were exposed to high glucose (HG, 33 mmol/L) with or without TC14012 (TC, 5 μ mol/L) for 10 min, the equivalent concentration of mannitol (Man) was used as osmotic control (Ctrl). The phosphorylation of Akt (A, B) and eNOS (A, C) in EPCs with or without Wortmannin (200 nmol/L) pretreatment was evaluated by Western blot, and GAPDH was used as loading control. Three independent experiments were performed. Data were normalized to Ctrl-siRNA-Man group and expressed as Mean $\pm$ SD. * P <0.05 vs. Man; # P <0.05 vs. HG; & P <0.05 vs. HG+TC.

Figure 8

TC14012 improves blood perfusion recovery and angiogenesis in ischemic limb of *db/db* diabetic mice. Real-time blood perfusion is detected and visualized by a Pericam Perfusion Speckle Imager (PSI), the images of blood perfusion were shown at before (Bef), 0, 7, 14, 21, 28 days after HLI (A) and quantified by ImageJ and expressed as the reperfusion ratio of ischemic hind limb to the contralateral non-ischemic hind limb (B). Antibody against CD31 was used to stained capillaries and 4',6-diamidino-2phenylindole

(DAPI) was used to recognize nuclei in the transverse sections of soleus muscle (SS) and gastrocnemius muscle (GS) tissue from ischemic hind limbs (C). Capillary density was shown as the number of CD31-positive capillaries per high-power field (HPF, D). At 1 h after HLI surgery, mice with or without TC14012 (10 mg/kg body weight) treatment for 1 h were euthanized and the peripheral blood was collected to evaluate the percentage of EPCs (CD34⁺/VEGFR2⁺) in circulation by a flow cytometry assay (E, F). Data were expressed as Mean $\pm$ SD. $n \geq 6$ mice per group. * $P < 0.05$ vs. Vehicle.

Supplementary Files

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