Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Investigating the alleviation of endothelial injury by apelin: insights from network pharmacology and in vitro experiments

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Article Info

Article history:

Received: April 25, 2024
Accepted: January 23, 2025
Published: February 28, 2025

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Abstract

Vascular lesion is the most important complication of diabetes, and vascular endothelial injury is the basis of vascular lesions. Although apelin was considered to have a positive effect on cardiovascular, the potential mechanisms remain unclear. In this work, we aimed to determine whether apelin alleviates endothelial injury through Src/Stat3 pathway. In virtue of network pharmacology, Src/Stat3 was sifted of 44 overlapping targets of diabetes and apelin. Human umbilical vein endothelial cells (HUVECs) were treated with high glucose (HG) and oleic acid (OA) to simulate the physiological environment of endothelial injury in vitro. Cell viability and migration were promoted while apoptosis rate and lactic dehydrogenase (LDH) release were reduced in the presence of apelin. Not only the protein expression of phosphorylated Src and Stat but also eNOS were raised by apelin. In conclusion, apelin dramatically improved cell status by activating Src/Stat3 pathway and increasing expression of eNOS. Apelin may provide an opportunity for the development of cardiovascular drugs.

Keywords: Network pharmacology, Gene pathway, Apelin, HUVEC, Endothelial injury.

1. Introduction

Diabetes mellitus is one of the most common endocrine and metabolic disorders, mainly divided into insulin-dependent diabetes mellitus and non-insulin-dependent diabetes mellitus. Diabetes can lead to various complications related to diabetes, which is a serious threat to human health [1] Vascular lesion is the most important complication of diabetes, and vascular endothelial injury is the basis of vascular lesions [2]. Vascular endothelial cells also have diverse physiological functions, participating in vital life activities such as coagulation, immunity, substance transport, and release of bioactive substances in the body. Their damage can cause changes in vascular tension and hemodynamics, activate the coagulation system and platelets, and increase vascular permeability, thereby causing a series of pathological and physiological changes [3] .

Apelin is a regulatory peptide, an adipokine produced and secreted by adipocytes, and a ligand for G protein-coupled receptors (APJ) [4] . It is found widely in the body, including surrounding tissues and the central nervous system. Studies have shown that the Apelin-APJ system plays an important role in various physiological processes, including homeostasis, fluid management, cell proliferation, and energy metabolism [5-8]. In addition, changes in plasma Apelin levels may be associated with related metabolic diseases [4, 6]. The benefits of apelin in the cardiovascular system and its impact on lipid and glucose metabolism pathways have been demonstrated. Apelin was deemed to induce vasodilation alongside improving cardiac contractility [6]. Apelin-13 is one of the most potent apelin isoforms present in the human heart and thus has a range of positive cardiovascular effects inducing notable improvements following myocardial infarction or heart failure. Apelin is also confirmed to improve insulin action and inhibit adaptogenic differentiation and promote lipolysis [7, 9. 10].

Network pharmacology was extensively used in finding potential therapeutic targets for drug design and discovery

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Doi: http://dx.doi.org/10.14715/cmb/2025.71.2.2

[11, 12]. To put it briefly, on the basis of existing drug and disease databases, constructing network diagrams can obtain the relationship between them. To further investigate the impact of apelin on vascular endothelium, the network pharmacology approach was used in this work.

In this work, 44 overlapping targets between diabetes and apelin were located by network pharmacology approach. Also, the protein interaction network and enrichment analysis were performed to narrow that down. Furthermore, in vitro experiments suggested that apelin improved injury and regulated Src/Stat3 expression.

2. Materials and methods

2.1. Network pharmacology analysis

2.1.1. Potential targets of apelin regulatory network

The SwissTargetPrediction database (http://www.swisstargetprediction.ch/) was used to obtain targets of apelin-13. The database is widely used to predict the probable protein targets [13] of small molecules based on the combination of 2D and 3D similarity measures of known ligands [14, 15]. SDF files of apelin were uploaded to SwissTargetPrediction database for gene symbols of these identified candidate targets. The diabetes-related genes were acquired from GeneCards (https://www.genecards.org/), and the targets with hit scores greater than 5.0 were selected as the apelin-related genes in the GeneCard database. The UniProt database (http://www.uniprot.org/) was used for comparison of target information and gene symbol standardization. The apelin-13 related targets and the targets of diabetes into venny2.1 online software (https://bioinfogp.cnb.csic.es/tools/venny/index.html) for mapping. The coincident targets of apelin-13 and diabetes mellitus were obtained [16].

2.1.2. GO and KEGG enrichment

Gene ontology (GO) analysis mainly includes three aspects of analysis: biological processes (BP), cellular composition (CC), and molecular function (MF). GO is commonly used to determine the unique biological characteristics of genes [17] . Kyoto Encyclopedia of Genes and Genomes (KEGG) is a collection of databases referring to genomes, diseases, biological pathways, drugs, and chemical materials [13]. A representation of GO and KEGG pathway enrichment analyses was shown by the "clusterProfiler","enrichplot", and "ggplot2" of R packages. The enriched GO terms and pathways with corrected P value less than 0.05 were selected and further analyzed.

2.1.3. PPI Network and topological analysis

Protein-protein interaction (PPI) network mappings of apelin and diabetes targets were performed by uploading overlapping targets to STRING (Search Tool for the Retrieval of Interacting Genes, https://string-db.org/, ver.11.0) database with a confidence score ≥0.4 [18].

2.2. In vitro experimental

2.2.1. Cell culture

SV40T-transformed HUVECs (PUMC-HUVEC-T1 cells) were cultured in endothelial cell medium (ECM, ScienCell, USA) containing 10% FBS and supplemented with 1% endothelial cell growth supplement (ECGS 100×, ScienCell, USA). The medium was changed every 2 days. Cells at passages 3 to 8 were used in the experiments.

2.2.2. Cell viability and apoptosis assay

HUVECs were seeded in 96-well plates at a density of 1×10^4 cells/well in conditioned medium (glucose 30 mM and oleic acid 400 μM simulating T2DM [19, 20]. After 24 hours, the cells were washed with PBS, and 100 μL medium comprising 10% (v/v) cell counting kit-8 (Beyotime, China) solution was added to each well, and then the plate was incubated at 37 °C for 1 h. The absorbance at 450 nm of each well was measured by microplate reader (Varioskan LUX, Thermo).

Cells were cultured with conditioned medium for 24 hours and centrifuged at 1500 rpm, and then the cell pellet was collected and washed with PBS. Then, apoptosis analysis kit (Beyotime) was added to the cells. After 30 minutes, cells were analyzed on a flow cytometer (Aria III, BD).

2.2.3. Cell migration assay

HUVECs were seeded in 6-well plates at a density of 25×10^4 cells/well then changed the conditioned medium. After 24 hours, a straight line was drawn in the bottom well with a sterile pipette tip (200 μL yellow tip). PBS was used to wash away the floating cells twice, and medium without FBS was added. After incubation for 12 hours, the cell migration ability was detected through photographing the healing of the scratches using fluorescence inverted microscopy. The pixel area was utilized to calculate relative migration rate by ImageJ.

2.2.4. Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA). cDNA was obtained using reverse transcription kit (Takala, Japan). Quantitative real-time PCR (qRT-PCR) was performed according to the manufacturer's instructions. (PowerUp™ SYBR™ Green Master Mix, ABI, USA). GAPDH was used as an endogenous control. All primers (Table 1) were obtained from Sangon Biotech Co., Ltd. The data were analyzed according to the ΔΔCT method.

2.2.5. Western blotting

HUVECs were lysed in RIPA lysis buffer containing 10 mM protease inhibitors (phenylmethyl-sulfonyl fluoride, PMSF, Beyotime). A BCA protein assay kit (Beyotime) was used to determine the protein concentrations. Before heating the samples, loading buffer (Beyotime) was added. The cellular proteins were separated by 10% SDS-poly-

Table 1 Primers for qRT-PCR.

Genes	Forward (5′-3′)	Reverse (5′-3′)
eNOS	CGGCATCACCAGGAAGAAGA	CATGAGCGAGGCGGAGAT
GAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG

Table 1 Primers for qRT-PCR.

Antibody	Dilution	Source	Code number
p-Src	1:1000	CST	6943
t-Src	1:1000	CST	2109
p-Stat3	1:1000	CST	9145
t-Stat3	1:1000	CST	12640
eNOS	1:1000	CST	9572
GAPDH	1:1000	CST	2118

acrylamide gel electrophoresis, then, the separated proteins were electrically transferred onto PVDF membranes (Millipore, USA). After that, the membranes were blocked with 3% BSA in TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.05% Tween-20) for 1 hour at room temperature. The membranes were washed with TBST and probed with primary antibodies (Table 2) overnight at 4 °C. The bound primary antibodies were detected with secondary antibodies conjugated with horseradish peroxidase and visualized by enhanced chemiluminescence (Beyotime). GAPDH was used as an internal reference.

2.3. Statistical analysis

All experiments were repeated at least three times. IBM SPSS Statistic 23 software was used and all data are presented as the means±standard deviations. One-way ANOVA was used for comparisons among six groups. Differences were considered statistically significant if a value of $p < 0.05$ was regarded as statistically significant.

4. Results

4.1. The putative targets of diabetes and predicted target proteins of apelin-13

1194 potential targets of diabetes were obtained by using GeneCards database by score > 5, and 230 potential targets of Apelin-13 were screened using SwissTargetPrediction and PharmMapper website by uploading SMILE structural formula. A total of 44 overlapping targets were selected (Figure 1).

4.2. GO function and KEGG pathway enrichment analysis

The first ten enrichment results of GO analysis were visualized as bar graphs. For apelin, BP was mainly involved in response to peptide hormone, vascular process in circulatory system, and regulation of blood pressure; CC was mainly related to membrane raft, membrane microdomain, and membrane region; MF was involved mostly in peptide binding, amide binding, and peptide receptor activity. (Figure 2).

According to the results of KEGG enrichment, the apelin pathways mainly focused on neuroactive ligand−receptor interaction, coronavirus disease-COVID-19, phospholipase D signal, etc (Figure 3). However, the pathways of diabetic cardiomyopathy, type I diabetes mellitus, vascular smooth muscle contraction, and endocrine resistance still indicated the interaction between apelin and diabetes, even though they were not at the forefront.

4.3. Integration of PPI Networks.

For apelin, a total of 44 nodes and 133 edges were mo-

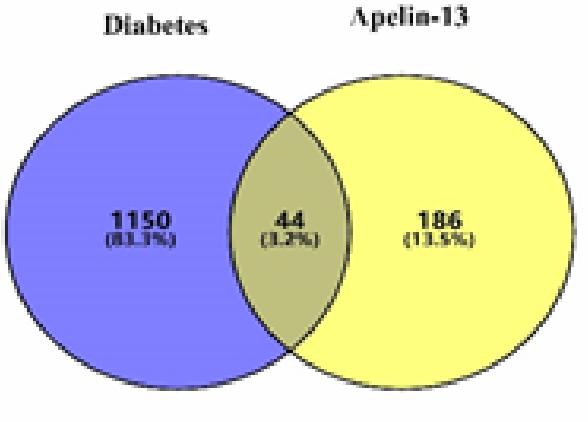

Fig. 1. Venn diagram of apelin and diabetes intersection targets.

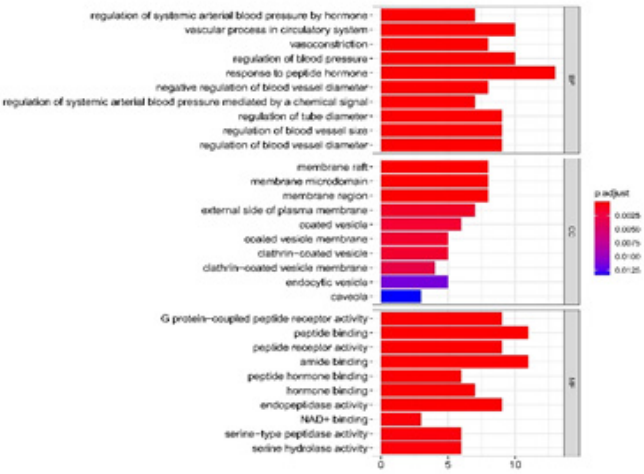

Fig. 2. GO enrichment analysis results.

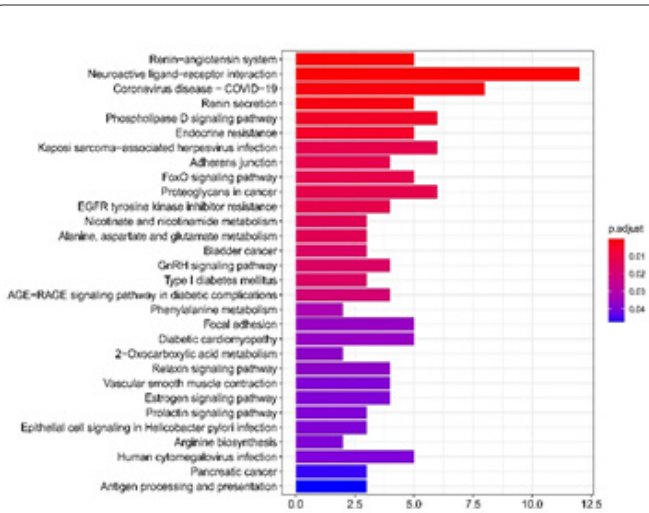

Fig. 3. KEGG pathway enrichment analysis results.

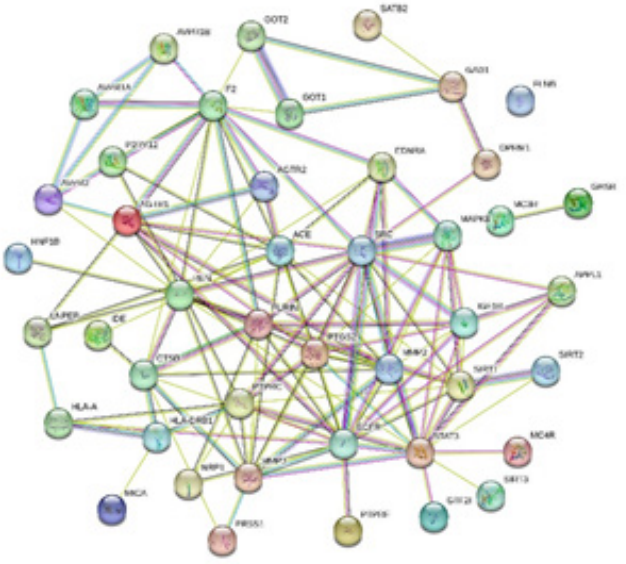

Fig. 4. Common targets protein interaction network.

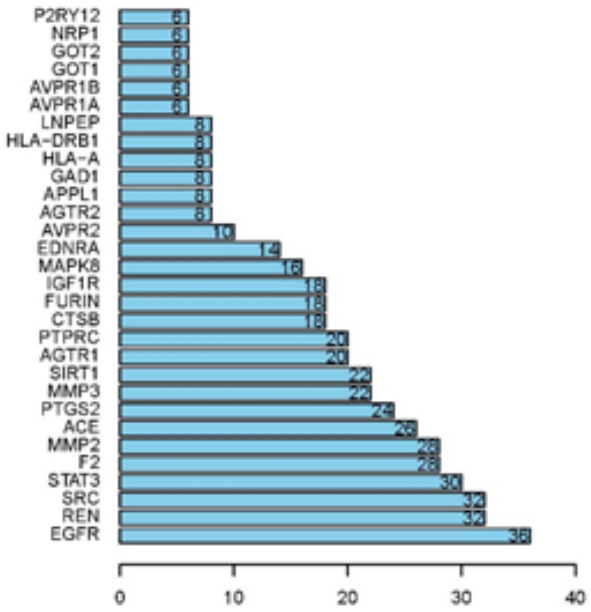

Fig. 5. The sort of top 30 proteins.

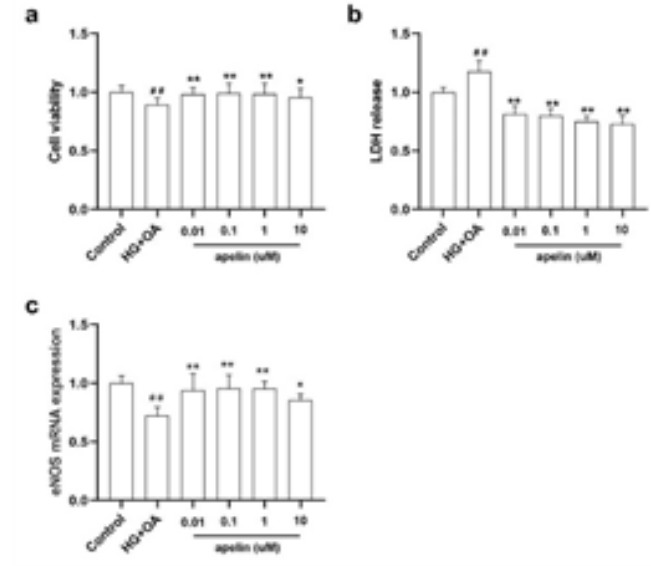

Fig. 6. Characteristics of HUVECs treated with high glucose and oleic acid. **a** Cell viability. **b** Relative release of LDH. **c** The relative mRNA level of eNOS. *p < 0.05, **p < 0.01, compared with HG + OA group. ##p < 0.01, compared with Control group. n = 3.

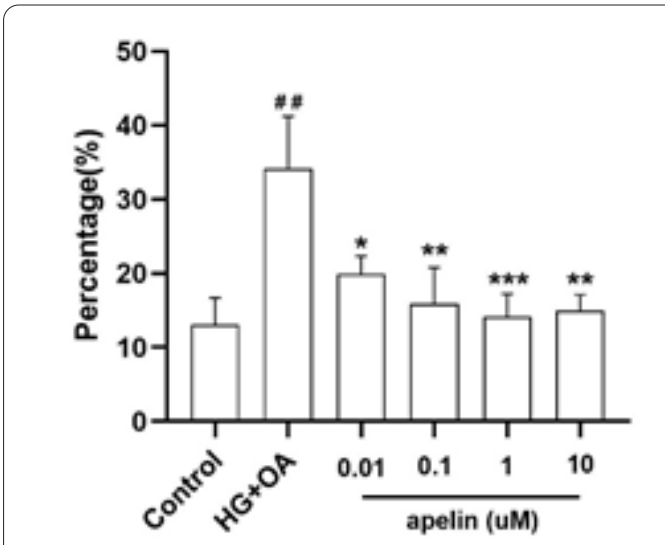

Fig. 7. HUVECs apoptosis in different experimental groups. *p < 0.05, **p < 0.01, ***p < 0.001, compared with HG + OA group. ##p < 0.01, compared with Control group. n = 3.

dulated by the PPI network (Figure 4), and the 5 central nodes were identified as EGFR, REN, SRC, STAT3, and F2 (Figure 5). The *Src/ Stat3* pathway was selected for further cellular experimental verification.

4.4. Characteristics of HUVEC treated with high glucose and oleic acid

Compared to control group, high glucose (HG) and oleic acid (OA) group significantly inhibited cellular vitality and promoted the release of LDH (Figure 6a, b). Similarly, the expressions of *eNOS*, which were the endothelial proangiogenic factors, were significantly down-regulated in high glucose and oleic acid group (Figure 6c). For the more, the crucial barometer of cell apoptosis has been increased (Figure 7). These phenomena all indicated an occurrence of endothelial injury [19-22] .

4.5. Apelin improved endothelial injury of HUVEC under high glucose and oleic acid condition

Compared to the HG + OA group, apelin group reinforces HUVECs viability and migration (Figure 6a, 8). Western blotting was performed to determine the expression of eNOS protein (Figure 9a). The results indicated that high glucose and oleic acid significantly downregulated the protein expression of eNOS, while eNOS was increased in the presence of apelin. HUVECs processed by high glucose and oleic acid showed a much higher apoptosis rate (Figure 7). On the contrary, both control group and apelin group appeared that the apoptosis rate and the release of LDH were significantly decreased (Figure 7, 6b). This significant difference indicated a better cellular status under apelin conditions in contrast with high glucose and oleic acid.

4.6. Apelin made a positive effect on HUVEC by upregulating the expression of Src/Stat3

As a transcription factor, the phosphorylated Stat3 can bind to the promoter of target genes, thereby activating

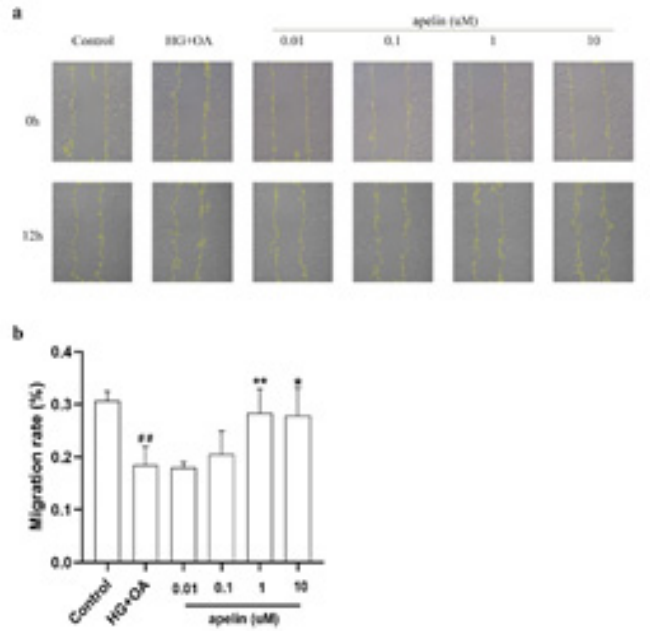

Fig. 8. The cell migration under different conditions. **a** Microscopic field from the migration assay. **b** Quantitative representation of cell migration after 12 h. *p < 0.05, **p < 0.01, compared with HG + OA group. ##p < 0.01, compared with Control group. n = 3.

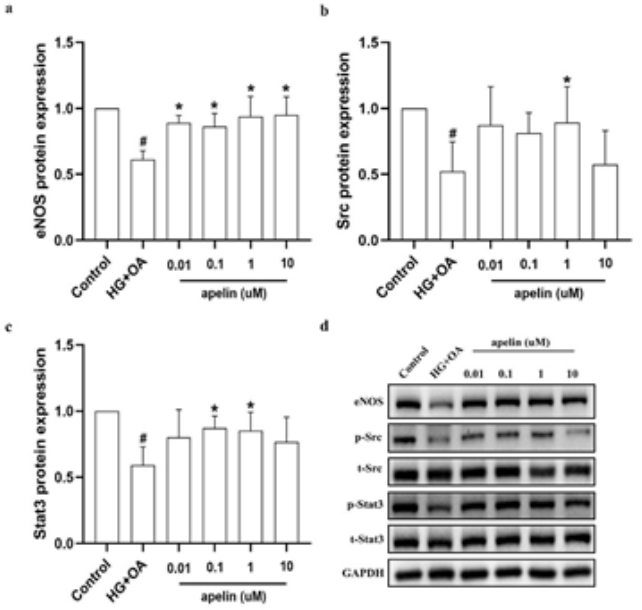

Fig. 9. The relative protein expression. *p < 0.05, compared with HG + OA group. #p < 0.05, compared with Control group. n = 3.

transcription within the nucleus. In order to confirm the expression results of Stat3, western blotting was carried out to measure the expression of Stat3 and upstream protein Src. The results indicated that high glucose and oleic acid significantly suppressed the protein expression of phosphorylated Src and Stat, while phosphorylated Src and Stat3 were increased in the presence of apelin (Figure 9).

5. Discussion

Endothelial Nitric Oxide Synthases (eNOS) play a role in protecting the health of blood vessels through its product NO. Low doses of NO can maintain vascular tension and maintain vascular homeostasis. For the short half-life, NO will quickly diffuse to the cell membrane of endothelial cells. By binding with the blood red of vascular smooth muscle cells, the concentration of Cyclic Guanosine Monophosphate (cGMP) increases. When cGMP is activated by protein kinase G (PKG), it can serve as a second messenger for the relaxation of vascular smooth muscle cells, causing them to relax and maintain the state of vasodilation [23, 24]. In addition, inhibiting the synthesis of NO can result in an increase in local arterial stiffness. In animal experiments, knocking out Nos3 (a homologous gene of human eNOS) resulted in the disappearance of endothelial-dependent vasodilation function in large blood vessels of mice. Furthermore, blood pressure increases significantly and vascular remodelling is damaged, which will lead to ischemic injury, myocardial infarction and atherosclerosis with a high probability [23-25] . For the obvious importance of eNOS in systemic cardiovascular homeostasis, eNOS was additionally selected in the present study.

Despite Src and Src family protein kinases commonly appear in tumor research as proto-oncogenes, Src family kinases have been implicated in the regulatory effects of protein kinases on processes such as cell apoptosis, proliferation, differentiation, angiogenesis, and transcription [26, 27].Src-family kinases are controlled by receptor protein tyrosine kinases, integrin receptors, G-protein coupled receptors, antigen- and Fc-coupled receptors, cytokine receptors, and steroid hormone receptors [26-28]. Src is considered to signal to a variety of downstream effectors including Stat transcription factors besides Pi3k/Akt and Fak pathways [28-31].Stat3 has been confirmed by previous studies to be involved in regulating diverse physiological pathways such as cell growth, differentiation, and apoptosis [32] . It can be specifically phosphorylated and activated, playing a crucial role in intercellular signal transduction [33]. The phosphorylated Stat3 immediately enters the nucleus and forms homologous or heterodimeric monomers as transcription factors, binding to the promoter of the target gene and activating transcription [34]. Stat3 is also a convergence point for many cytokines (such as interleukin-6, IL-10, and IL-11), growth factors (such as fibroblast growth factor), and signaling pathways activated by some oncogenes (K-Ras, Src, and cAbl). In addition to the Jak/Stat pathway [35], when growth factors bind to cell surface receptors, phosphorylated Src can further activate downstream Stat3 [36]. Stat3 can also induce the growth of endothelial cells and promote the generation of blood vessels and epithelial-mesenchymal transition in tumor tissue [37]. Although there are many studies on apelin in diabetes, there are few articles about the Src/Stat3 pathway of diabetes vascular disease. In this study, combining network pharmacology with cell experiments, we found and verified that apelin indeed made an impact on Src/Stat3. Apelin polished up the cell state by stimulating Src/Stat3 phosphorylation, and upregulated the expression of eNOS in the bargain in HUVEC.

Apelin has a significant effect on improving insulin sensitivity, and promoting glucose and lipolysis in diabetes [38-40]. Apelin also has a role in angiogenesis, migration, proliferation, and the capillary tube-like structure formation of endothelial cells [41]. There are some researches on the impact of apelin and endothelial function. The proliferation and differentiation of endothelial cells can

be promoted by apelin [42]. During muscle repair, apelin made an effect on endothelial remodelling [43]. Furthermore, apelin was considered to improve endothelial injury induced by LPS through mediating AMPK [44, 45]. Our findings are generally consistent with existing literature and indicate the direction of farther researching. Long-term apelin treatment regulated myocardial metabolism [46] and impeded development of kidney dysfunction and inflammation [47]. The long-term effects of apelin treatment on endothelial function and overall vascular health in diabetes are worth further studies. In addition, EGFR (Epidermal Growth Factor Receptor) also deserves attention (Figure 5). The EGFR protein is a transmembrane glycoprotein and a member of the protein kinase superfamily. EGFR is associated with tumor cell proliferation, angiogenesis, tumor invasion, metastasis, and the inhibition of apoptosis [48]. EGFR is the receptor for EGF, while the specific receptor for apelin is APJ. Apelin probably interact with EGF pathway through the regulation of its downstream genes, rather than directly regulating EGFR. EGFR promotes angiogenesis through high expression is likely to playing a positive role in vascular disease. The evidence of regulation between EGFR and apelin deserves further study. Endothelial dysfunction is a key event in vascular endothelial injury and inflammation. Although there are potential clinical implications, short half-life of apelin limits clinical application [4]. GLP-1 (glucagon like peptide) analogue and DPP (dipeptidyl peptidase)-4 inhibitors have become new treatment options for T2DM patients [49]. Previous studies have indicated that GLP-1 has a protective effect on the cardiovascular system by inducing vasodilation and improving endothelial function, while DPP-4 degrades GLP-1[50, 51]. The half-life of GLP-1 in serum is short (1-2 minutes) because of the hydrolysis of DPP-4[52]. Several incretin mimetics have been developed: GLP-1RA (GLP-1 receptor agonist) and DPP-4 inhibitor, on the basis of short half-life period [53]. It is similar to GLP-1 that apelin is widely expressed in various organs and tissues, and its presence has also been found on endothelial cells. The development of apelin receptor agonist, and structural modification prolongs its half-life period in vivo are perhaps promising. In addition, by nanotechnology, apelin can be wrapped in polymeric micelles to increase the stability. The nano-drugs of apelin can slow release and precisely target the vascular endothelial cell.

There are still limitations in our study. The database chosen in network pharmacology analysis may directly affect our subsequent conclusions. Besides, only a Src/Stat3 pathway was selected in this study. Thus, a more comprehensive and further study is needed.

In the present study, we found that apelin alleviated endothelial injury by promoting Src/Stat3 phosphorylation. Additionally, apelin represented positive effect on blood vessels by upregulating eNOS expression.

Conflicts of interest

The author has no conflicts with any step of the article preparation.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

Ethics approval

No human or animals were used in the present research.

Funding statement

This work was supported by the Maoming Science and Technology Plan Approval Project (Project NO.: 2022223, 2021557, 2019018023), Medical Scientific Research Foundation of Guangdong Province of China (Project NO.: A2017490, B2018061, B2022046), Natural Science Foundation of Guangdong Province of China (Project NO.: 2021A1515010884, 2023A1515010710), Science and Technology Planning Project of Guangdong Province of China (Project NO.: 2020S00049), High-level Hospital Construction Research Project of Maoming People's Hospital (Project NO.: ZX2020001, ZX2020002), Doctoral research project of Maoming People's Hospital (Project NO.: BS2020001), Outstanding Young Talents Project of Maoming People's Hospital's Double excellent Talents Plan (Project NO.: SY2020006), Science and Technology Planning Project of Guangdong Province of China (Project No.:mmkj2020020).

This research was supported by the Guangdong Engineering Technology Research Center for Precision Mass Spectrometry Analysis and Translational Medicine under grant 2022K014 awarded to Wendong Huang.

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