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MiRNA-324-5p inhibits inflammatory response of diabetic vessels by targeting CPT1A

G. WU[1], J. ZHANG[2], G.-G. FAN[3], Z.-Y. ZOU[4], Y.-L. YIN[5], G.-X. LI[5]

[1]Department of Neurology, Binzhou People's Hospital, Binzhou, Shandong, China
[2]Department of Fundus Diseases, Weifang Eye Hospital, Weifang, Shangdong, China
[3]Department of Cardiovascular Surgery, General Hospital of Northern Theater Command, Shenyang, Liaoning, China
[4]Department of Cardiology, The 982th Hospital of Chinese PLA, Tangshan, Hebei, China
[5]Department of Vascular Surgery, Shandong Provincial Qianfoshan Hospital, the First Hospital Affiliated with Shandong First Medical University, Jinan, Shandong, China

Abstract. – OBJECTIVE: The purpose of this study was to elucidate the regulatory role of microRNA-324-5p (miRNA-324-5p) in inhibiting inflammatory response of diabetic vessels by regulating CPT1A level, thus alleviating the development of type 2 diabetes mellitus (T2DM).

PATIENTS AND METHODS: Arterial vessels (splenic artery) and serum exosomes were extracted from 30 T2DM patients and 30 non-T2DM subjects treated in Binzhou People's Hospital from 2015 to 2019. Relative levels of miRNA-324-5p and CPT1A in each subject were detected. Then, VSMCs were induced with high-glucose, followed by detection of inflammatory factor levels. Next, the regulatory effects of miRNA-324-5p and CPT1A on viability, 5-Ethynyl-2'-deoxyuridine (EdU)-positive ratio, and release of inflammatory factors in VSMCs were determined. Finally, Dual-Luciferase reporter assay was conducted to verify the interaction between miRNA-324-5p and CPT1A.

RESULTS: The results revealed that compared with non-T2DM subjects, miRNA-324-5p was downregulated in splenic arteries and exosomes in T2DM patients. High-glucose treatment in VSMCs triggered the release of the inflammatory factors. In addition, the overexpression of miRNA-324-5p in VSMCs reduced viability and inflammatory factor levels, and the inhibited trends were partially reversed by overexpression of CPT1A. CPT1A was indicated to be the target gene binding miRNA-324-5p.

CONCLUSIONS: MiRNA-324-5p exerts an inhibitory effect on T2DM-induced inflammation in blood vessels by negatively regulating CPT1A level and reducing the release of inflammatory factors. MiRNA-324-5p might be a promising therapeutic target for T2DM.

Key Words:
MiRNA-324-5p, CPT1A, T2DM, Inflammatory response.

Introduction

Type 2 diabetes mellitus (T2DM) is a systematic metabolic disease manifested as chronic hyperglycemia[1-3]. It affects organ functions of eyes, kidneys, nerve system, heart, and blood vessels[4,5]. In recent years, the incidence of T2DM sharply increases with the improvement on economy, aging, increasing proportion of obese population, and lifestyle changes[5-7]. WHO proposed that T2DM has become the third chronic, non-infectious disease following cardiovascular diseases and cancers[5]. It is generally believed that genetics, obese, hypertension, and socio-psychological factors are risk factors for T2DM[5,8,9]. Effective targets for predicting the occurrence of T2DM are urgently required[10,11].

MicroRNAs silence or degrade target genes by recognizing and binding their 3′-untranslated region (3′-UTR), thus exerting a post-transcriptional regulation on gene expressions[12-14]. So far, over 2000 human miRNAs have been identified, and more than 60% human proteins could be regulated by miRNAs[15-17]. T2DM-related miRNAs are of significance during the development of T2DM, providing new directions in its prevention and treatment[10,11].

Oxidation and energy supply of fatty acids require the participation of a variety of enzymes. Carnitine palmitoyltransferase 1 (CPT1) has three types, namely CPT1A, CPT1B and CPT1C, with different properties and tissue distributions[18]. CPT1A is enriched in the liver and exerts a key role in the β-oxidation of fatty acids[18-20]. In brown fat cells cultured *in vitro*, CPT1A is capable of improving β-oxidation and mitochondrial function of fatty acids[20]. Here, dynamic expressions of miRNA-324-5p and CPT1A in splenic arteries

Corresponding Author: Guangxin Li, MD; e-mail: liguangxinsd@aliyun.com.cn

and exosomes extracted from T2DM patients were detected, and their potential influences on inflammatory response of diabetic vessels were further analyzed.

Patients and Methods

Patients

The study was approved by the Ethics Committee of the Hospital and all patients signed informed consent. T2DM patients with a medical history of over 10 years and those using insulin to control the blood glucose were enrolled. Arterial vessels (splenic artery) and serum exosomes were extracted from 30 T2DM patients and 30 non-T2DM subjects treated in the Binzhou People's Hospital from 2015 to 2019. Finally, the serum exosomes were extracted using a commercial kit (Invitrogen, Carlsbad, CA, USA).

Cell Culture

VSMCs were provided by American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted in 1×trypsin+EDTA (ethylenediaminetetraacetic acid; Sigma-Aldrich, St. Louis, MO, USA) at 80-90% confluence. For high-glucose induction, the cells were pretreated with serum deprivation for 24 h, followed by 12-h induction with 25 mM glucose. Meanwhile, the cells in the control group were induced with 5.5 mM glucose.

Transfection

Cells were inoculated in 6-well plates and cultured to 30-40% confluence and transfected with corresponding plasmids (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Then, the transfected cells for 48 h were harvested for functional experiments.

Cell Counting Kit-8 (CCK-8)

The cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viaility curves.

5-Ethynyl-2′-Deoxyuridine (EdU) Proliferation Assay

The cells were incubated with 50 μm EdU solution (Guangzhou ruibo biotechnology co. LTD., Guangzhou, China) for 2 h, followed by AdoLo and 4',6-diamidino-2-phenylindole (DAPI) staining (Abcam, Cambridge, MA, USA) in the dark. EdU-positive cells were captured under a fluorescence microscope. Finally, EdU-positive ratio was calculated as EdU-positive cell number (red ones) to that of DAPI-labeled cell number (blue ones).

Cytokine Measurement by Enzyme-Linked Immunosorbent Assay (ELISA)

Culture medium following different treatments was collected for determination of the release of the inflammatory factors using commercial ELISA kit (LifeSpan BioSciences, Inc., Seattle, WA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNA in cells or tissues was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Next, the obtained cDNA underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan), with β-actin as the internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. The primers used were shown below: miRNA-324-5p: forward: 5'-GAGGCCAAGCCCTGGTATG-3', reverse: 5'-CGGGCCGATTGATCTCAGC-3', U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3', CPT1A: forward: 5'-TTCCTGGGCGGACGCGCCC-3', reverse: 5'-CTGCAGAGTTCAAGTGGGCCTG-3', β-actin: forward: 5›-CCTGGCACCCAGCACAAT-3›, reverse: 5›-TGCCGTAGGTGTCCCTTTG-3›.

Western Blot

Total protein was extracted from cells or tissues. The obtained protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) and blocked in 5% skim milk for 1 hour. The specific primary antibody (CPT1A, 1:1000; Abcam, Cambridge, MA, USA) was

used to incubate with the membrane overnight at 4°C, followed by secondary antibody incubation for 2 h at room temperature. After Tris-Buffered Saline and Tween (TBST) washing for 1 min, the chemiluminescent substrate kit was used for exposure of the protein band.

Dual-Luciferase Reporter Assay

HEK293T cells were inoculated in 24-well plates. On the next day, the cells were co-transfected with WT-CPT1A/MUT-CPT1A and miRNA-324-5p mimics/NC, respectively. 48 hours later, the cells were lysed for determining the relative Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Continuous variables were analyzed by the *t*-test, and the categorical variables were analyzed by χ^2 test or Fisher's exact test. $p<0.05$ was considered as statistically significant.

Results

Relative Levels of MiRNA-324-5p and CPT1A in T2DM Patients

Compared with those from non-T2DM patients, miRNA-324-5p was downregulated, while CPT1A was upregulated in the serum exosomes extracted from T2DM patients (Figure 1A, 1B). Similarly, the expression changes of miRNA-324-5p and CPT1A in VSMCs of non-T2DM and T2DM patients were identical to their levels in exosomes (Figure 1C, 1D). Besides, protein level of CPT1A was markedly upregulated in T2DM patients (Figure 1E). In particular, a negative correlation was identified between the expression levels of miRNA-324-5p and CPT1A in VSMCs of T2DM patients (Figure 1F).

Dynamic Expressions of miRNA-324-5p and CPT1A in the In Vitro High-Glucose Model

VSMCs were induced with 5.5, 10, 15, 20, or 25 mM glucose for 12 h. It was shown that miRNA-324-5p was dose-dependently downreg-

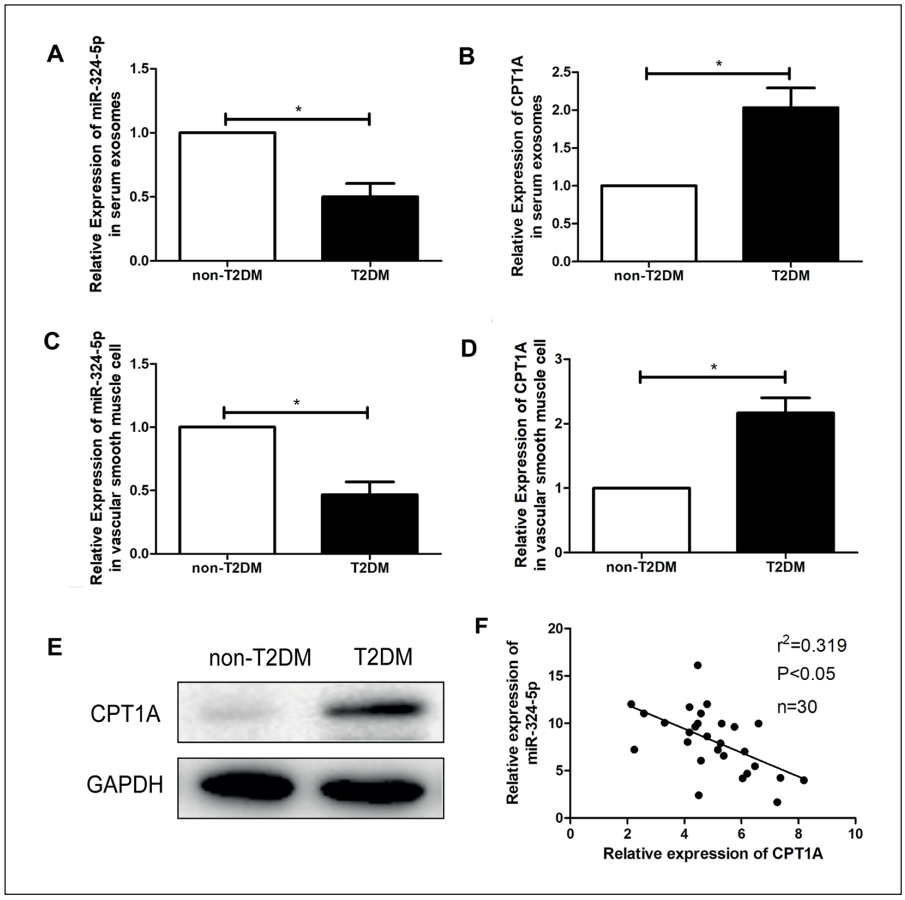

Figure 1. Relative levels of miRNA-324-5p and CPT1A in T2DM patients. **A,** MiRNA-324-5p level in serum exosomes of non-T2DM and T2DM subjects. **B,** CPT1A level in serum exosomes of non-T2DM and T2DM subjects. **C,** MiRNA-324-5p level in VSMCs of non-T2DM and T2DM subjects. **D,** CPT1A level in VSMCs of non-T2DM and T2DM subjects. **E,** Protein level of CPT1A in VSMCs of non-T2DM and T2DM subjects. **F,** A negative correlation between expression levels of miRNA-324-5p and CPT1A in T2DM patients. **p*<0.05 *vs.* non-T2DM subjects.

ulated, while CPT1A presented an opposite trend (Figure 2A). Subsequently, VSMCs were induced with 25 mM glucose for 0, 1, 6, or 12 h. MiRNA-324-5p was time-dependently downregulated, while CPT1A was upregulated (Figure 2B). After high-glucose treatment for 12 h, the contents of TNF-α (Figure 2C), IL-1β (Figure 2D), IL-6 (Figure 2E), and IL-10 (Figure 2F) were markedly elevated in VSMCs.

MiRNA-324-5p Inhibited Proliferative Ability and Inflammatory Response Following High-Glucose Induction in VSMCs

Transfection efficacy of miRNA-324-5p mimics was first verified in VSMCs (Figure 3A). It was found that transfection efficacy of miRNA-324-5p mimics markedly reduced the viability in high-glucose-induced VSMCs (Figure 3B). In addition, the overexpression of miRNA-324-5p reduced the contents of TNF-α (Figure 3C), IL-1β (Figure 3D), IL-6 (Figure 3E), and IL-10 (Figure 3F) in VSMCs.

CPT1A was the Target Gene of MiRNA-324-5p

By predicting on TargetScan, MicroRNA.org, and miRDBA, CPT1A was considered as a candidate gene binding miRNA-324-5p (Figure 4A). Luciferase activity was markedly reduced after co-transfection of WT-CPT1A and miRNA-324-5p mimics, confirming that CPT1A was the target gene binding miRNA-324-5p (Figure 4B). Both protein and mRNA levels of CPT1A were downregulated in VSMCs overexpressing miRNA-324-5p (Figure 4C, 4D).

MiRNA-324-5p Inhibited Proliferative Ability and Inflammatory Response Following High-Glucose Induction in VSMCs Through CPT1A

To uncover the involvement of CPT1A in miRNA-324-5p-regulated proliferative ability and inflammatory response of high-glucose-induced VSMCs, rescue experiments were conducted. Transfection efficacy of CPT1A overexpression plasmid was verified (Figure 5A, 5B). It was proven that the overexpression of miRNA-324-5p markedly decreased viability (Figure 5C) and EdU-positive ratio (Figure 5D) in VSMCs under high-glucose treatment. Notably, their reduced trends were partially reversed after co-overexpression of CPT1A. The inhibited release of inflammatory factors in VSMCs overexpressing miRNA-324-5p was abolished by CPT1A overexpression (Figure 5E-5H).

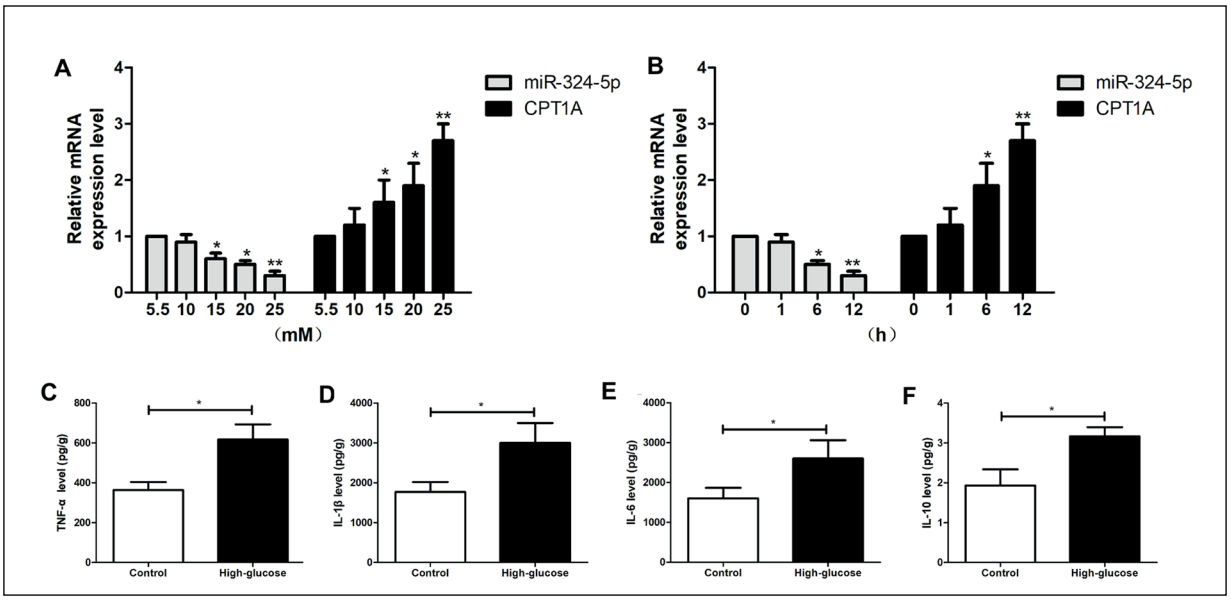

Figure 2. Dynamic expressions of miRNA-324-5p and CPT1A in the *in vitro* high-glucose model. **A,** Relative levels of miRNA-324-5p and CPT1A in VSMCs induced with 5.5, 10, 15, 20 or 25 mM glucose for 12 h. **B,** Relative levels of miRNA-324-5p and CPT1A in VSMCs induced with 25 mM glucose for 0, 1, 6 or 12 h. **C-F,** Contents of TNF-α (**C**), IL-1β (**D**), IL-6 (**E**) and IL-10 (**F**) in VSMCs treated with 5.5 or 25 mM glucose for 12 h. *$p<0.05$ *vs.* Control.

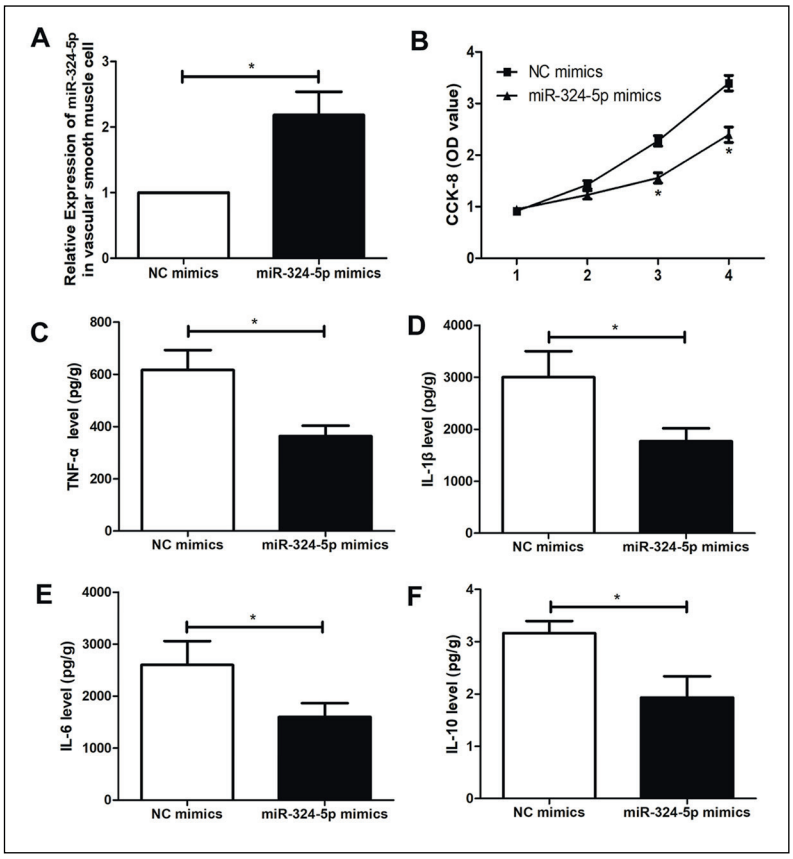

Figure 3. MiRNA-324-5p inhibits proliferative ability and inflammatory response following high-glucose induction in VSMCs. **A,** Transfection efficacy of miRNA-324-5p mimics in VSMCs. **B,** Viability in high-glucose-induced VSMCs transfected with NC or miRNA-324-5p mimics. **C-F,** Contents of TNF-α (**C**), IL-1β (**D**), IL-6 (**E**) and IL-10 (**F**) in high-glucose-induced VSMCs transfected with NC or miRNA-324-5p mimics. *p<0.05 *vs.* NC.

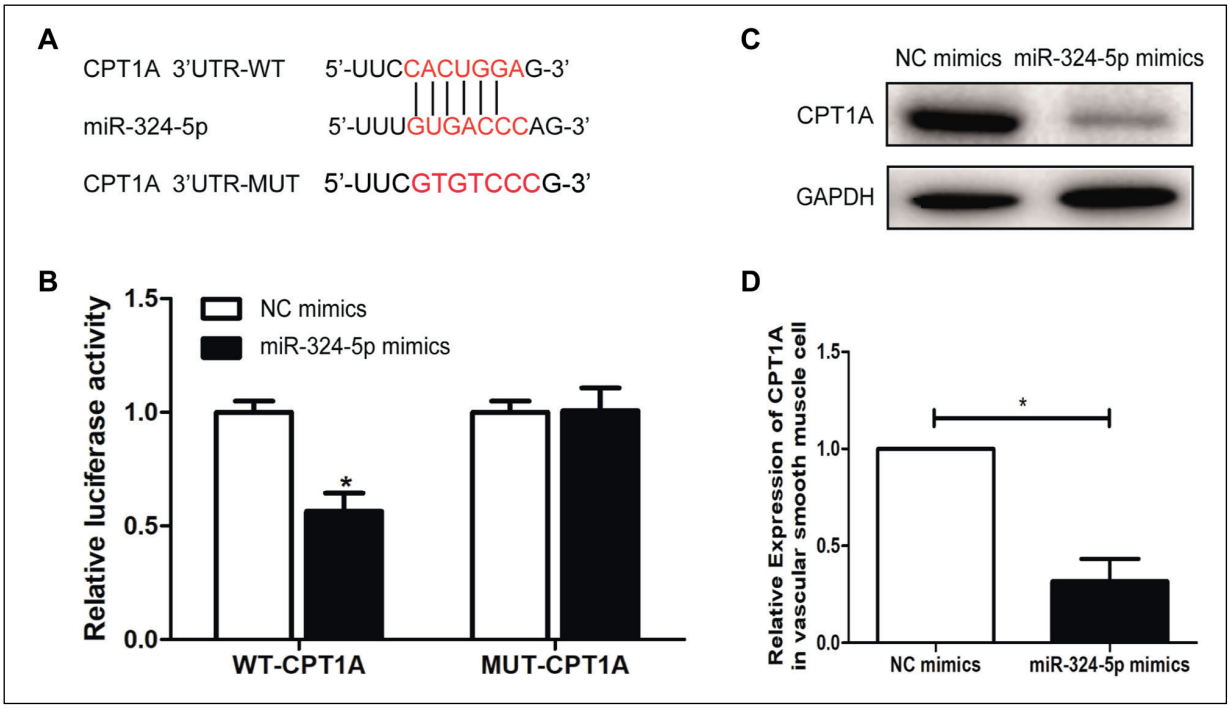

Figure 4. CPT1A is the target gene of miRNA-324-5p. **A,** Binding sequences in 3'UTR of miRNA-324-5p and CPT1A. **B,** Luciferase activity in cells co-transfected with WT-CPT1A/MUT-CPT1A and NC/miRNA-324-5p mimics. **C, D**, Protein (**C**) and mRNA (**D**) levels of CPT1A in high-glucose-induced VSMCs transfected with NC or miRNA-324-5p mimics. *p<0.05 *vs.* NC.

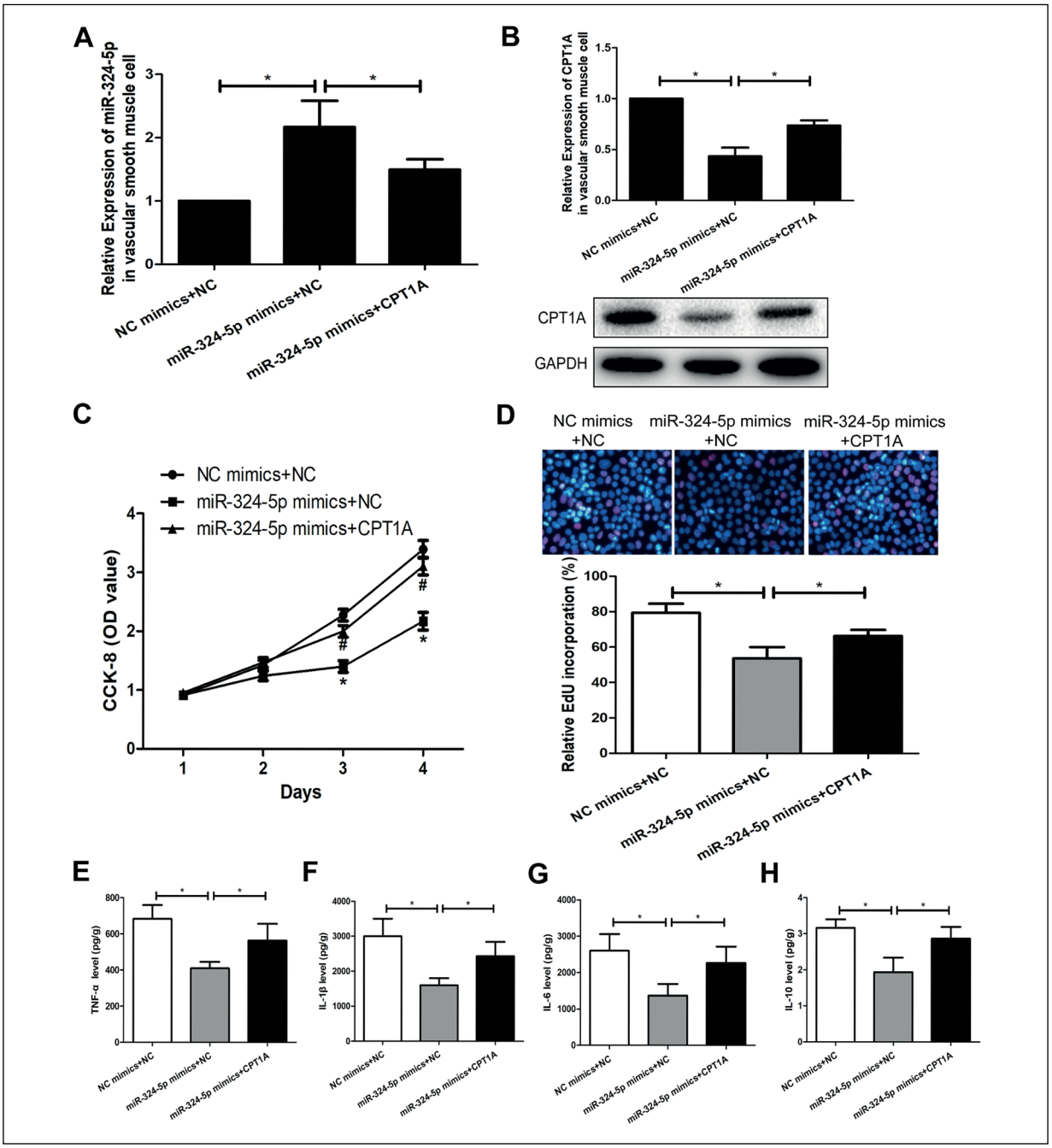

Figure 5. MiRNA-324-5p inhibits proliferative ability and inflammatory response following high-glucose induction in VSMCs through CPT1A. High-glucose-induced VSMCs are transfected with NC mimics + NC, miRNA-324-5p mimics + NC or miRNA-324-5p mimics + CPT1A. **A,** The mRNA level of miRNA-324-5p. **B,** Protein level of CPT1A. **C,** Viability. **D,** EdU-positive ratio (magnification: 200×). **E-H,** Contents of TNF-α (**E**), IL-1β (**F**), IL-6 (**G**) and IL-10 (**H**). *$p<0.05$ *vs.* NC.

Discussion

Among multiple complications of T2DM, the cardiovascular system is the most affected[4,5]. Hyperglycemia is a leading risk factor for the development of diabetic angiopathy[6-8]. Through different pathways, endothelium disorder and phenotype changes of VSMCs trigger the oxida-

tive stress and release of ROS/RNS[21,22]. Hyperglycemia-induced inflammation and oxidative stress stimulate the release of abundant pro-inflammatory factors, thus inducing vascular inflammation[23,24]. Long-term hyperglycemia is of significance in the proliferative and migratory trends of VSMCs during atherosclerosis[24].

Some studies[10,11] have demonstrated the involvement of miRNAs in inflammation, abnormal lipid metabolism, VSMCs dysfunction, and other pathological features related to atherosclerosis. MiRNAs are suitable candidates for being developed as target drugs[25,26]. It is necessary to study the synergy of miRNAs because it reduces the dosage of an individual miRNA and provides more therapeutic options[14-16]. MiRNA levels in blood fluids are stable, which could be utilized for monitoring the severity and development of T2DM[13-15]. Chen et al[27] have shown the anti-oxidation effect of miRNA-324-5p. The novelty of this work was that we elucidated the regulatory role of miRNA-324-5p in inhibiting inflammatory response of diabetic vessels *via* CPT1A, thus alleviating the development of T2DM in this paper. The results of this study showed that miRNA-324-5p was markedly downregulated in serum exosomes of T2DM patients and VSMCs induced with high-glucose.

MiRNA-gene interaction exerts vital functions in cell behaviors[28,29]. Bioinformatics analysis predicted the specific binding between miRNA-324-5p and CPT1A, which was further verified in this experiment. CPT1A is activated by extracellular stimuli, including growth factors, cytokines, ultraviolet, and cellular hyperosmotic pressure[28,29]. In this research, CPT1A level was negatively regulated by miRNA-324-5p in high-glucose-treated VSMCs. The overexpression of CPT1A partially relieved the regulatory effects of miRNA-324-5p on proliferative ability and inflammatory response in VMSCs. Hence, it was believed that CPT1A was responsible for inflammatory response in blood vessels of T2DM patients regulated by miRNA-324-5p.

Conclusions

The above findings demonstrated that miRNA-324-5p exerts an anti-inflammatory effect on blood vessels of T2DM patients by negatively regulating CPT1A level and reducing the release of inflammatory factors. MiRNA-324-5p might be a promising therapeutic target for T2DM.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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