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Original Article

Ang-(1−7) inhibited mitochondrial fission in high-glucose-induced podocytes by upregulation of miR-30a and downregulation of Drp1 and p53

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Abstract

Background: The aim of this study was to investigate the possible effects of angiotensin-(1−7) [Ang-(1−7)] on podocytes and the mitochondrial signaling pathway in a high-glucose (HG) environment.
Methods: We established a model of HG-induced podocytes by incubating podocytes in RPMI 1640 containing 33mM glucose. The cells were divided into the following groups: (1) normal glucose group as control incubated in Roswell Park Memorial Institute (RPMI) 1640 containing 5mM glucose; (2) Ang-(1−7), 10nM, incubated in RPMI 1640 containing 5mM glucose; (3) the HG group incubated in RPMI 1640 containing 33mM glucose; and (4) Ang-(1−7), 10nM, incubated in HG group incubated in RPMI 1640 containing 33mM glucose. After a period of 24 hours, mitochondrial fission and podocyte fusion were observed by electron microscope. Additionally, p53 and Drp1 were tested by Western blot, the position of Drp1 was detected by immunofluorescence, and miR-30a was analyzed by quantitative real-time polymerase chain reaction.
Results: Ang-(1−7) inhibited mitochondrial fission in HG-treated podocytes. However, Ang-(1−7) also significantly reduced the expression of Drp1 and p53, and improved the expression of miR-30a in HG-induced podocytes.
Conclusion: Ang-(1−7) inhibited mitochondrial fission in HG-induced podocytes by upregulation of miR-30a and downregulation of Drp1 and p53.

Keywords: Ang-(1−7); Drp1; high glucose; miR-30a; mitochondria; podocytes; p53

1. Introduction

Diabetic nephropathy (DN) is a major chronic complication of diabetes mellitus. Different angiogenic molecules and inflammation are important modulators in the pathogenesis and progression of DN. Podocytes play an important role in maintaining the structure and function of the glomerular filtration barrier. Podocyte injury is closely related to diabetic proteinuria. Angiotensin-(1−7) [Ang-(1−7)] is one of several important bioactive substances in the renin−angiotensin−aldosterone system, protecting cells of the heart and kidney, and regulating blood pressure through the Mas receptor. An earlier study indicated that chronic treatment with exogenous Ang-(1−7) produced cardiac protection in diabetic animals.[1] Also, Ang-(1−7) may partly counteract the profibrotic effects of high glucose (HG) levels.[2] It has also been suggested that glucose-induced reactive oxygen species production initiates podocyte apoptosis.[3]

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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Mitochondria play an important role in physiological and pathological activities, such as apoptosis and free radical generation. Mitochondrial morphology is an important determinant of mitochondrial function.[4] Mitochondrial fusion can inhibit apoptosis, while mitochondrial fission is involved in the initiation of apoptosis.[5] Furthermore, mitochondrial genes and nuclear genes are involved in the regulation of mitochondrial function. MicroRNAs (miRNAs) are a class of small regulatory RNAs that regulate gene expression at the post-transcriptional level, and miR-30a can induce changes in beclin-1 protein expression and alter expression of autophagy-related proteins.[6,7] Drp1 is a large GTPase of the dynamin superfamily that plays an essential role in mitochondrial fission in mammalian cells. Drp1 localizes mainly to the cytoplasm, and is involved in several important aspects of mitochondria, including size, shape, remodeling, distribution, and maintenance.[8,9] Some research suggests that Drp1 is required for mitochondrial fission in p53-dependent apoptosis.[10]

To date, the influence of Ang-(1−7) on HG-induced podocytes through mitochondrial fission has been rarely addressed. We hypothesized that Ang-(1−7) may protect podocytes in a HG environment through reducing the expression of apoptosis protein p53 and Drp1, and the expression of miR-30a may be relative to the expression of p53 and its downstream target Drp1. We observed the expression of Drp1 and p53 by Western blot, with the position of Drp1 detected by immunofluorescence, and detected the expression of miR30a by quantitative real-time polymerase chain reaction (qRT-PCR). We also analyzed the function of podocyte mitochondria to provide a possible theoretical basis for revealing the molecular biology mechanism of Ang-(1−7).

2. Methods

2.1. Cell model

Podocytes were provided by the central laboratory of Shandong University, Jinan, China. Podocytes were maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 15% heat inactivated fetal calf serum (FCS; Gibco, Bethesda, MD, USA) in 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were grown at 33°C with 10 U/mL mouse recombinant interferon-c (IFN-c; Sigma-Aldrich Corporation, Missouri, St. Louis USA). Differentiated podocytes were cultured for 24 hours in RPMI 1640 medium. The cells were divided into the following groups: (1) normal glucose control group incubated in RPMI 1640 containing 5mM glucose; (2) Ang-(1−7), 10nM, incubated in normal glucose group; (3) HG group incubated in RPMI 1640 containing 33mM glucose; and (4) Ang-(1−7), 10nM, incubated in HG group.

2.2. Electron microscopy

To determine the structural changes in podocyte morphology, we utilized customary procedures to conduct electron microscopic morphometric evaluation. The podocytes were fixed in 2.5% glutaraldehyde, and then were coated with agar, after which ultrathin sections were examined by electron microscopy. All electron microscope photomicrographs were evaluated in a blind fashion. Arising from this electron microscopic observation, we noted mitophagy and mitochondrial swelling, with fracture and fusion of cristae in HG-induced podocytes.

2.3. Western blot analysis

Protein concentration was quantified using a bicinchoninic acid protein assay kit (Beyotime Biotechnology Research Institute) and then boiled with the sample buffer in a water bath for 5 minutes. Then, a 40-μg protein sample was separated with 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels for 2 hours and transferred to a polyvinylidene difluoride membrane, which was subsequently blocked in 5% nonfat milk for 2 hours at room temperature. Primary antibodies included anti-p53(EP155Y; 1:2500) and anti-Drp1(4F6; 1:2000), and were added at 4°C overnight. The following day, the membrane was washed with tris-buffered saline and tween three times, and incubated with appropriate secondary antibodies of goat antimouse (1:5000, ZD2305, HRP marker) and goat antirabbit (1:5000, ZD2301, Horseradish peroxidase (HRP) marker) for 1 hour at room temperature, and then washed again. Antigen−antibody complexes were visualized by enhanced chemiluminescence. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:800) was applied as a protein control and the mean value was measured by comparing the other groups to obtain the relative amounts.

2.4. Immunofluorescence

Podocytes were grown on 12-mm coverslips for 24 hours at 37°C and washed with phosphate buffered saline (PBS) twice. The cells were fixed with 4% formaldehyde for 15 minutes, permeabilized with 0.25% Triton X-100 for 10 minutes, and washed with PBS three times, and then blocked with 1% bovine serum albumin in PBS for 30 minutes. Primary antibodies included anti-p53(EP155Y; 1:150) and anti-Drp1(4F6; 1:150), which were added at 4°C overnight. Podocytes were subsequently rinsed with PBS and incubated with appropriate secondary antibodies of goat antimouse (ZD2305, HRP marker) and goat antirabbit (ZD2301, HRP marker), for 1 hour at room temperature. DAPI (4',6-diamidino-2-phenylindole) was used for nuclear staining for 5 minutes, and then washed with PBS. Subsequently, podocytes were mounted in glycerin and detected under a laser scanning confocal microscope (Leica Company, Munich, Germany).

2.5. RNA extraction and qRT-PCR

Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA quantity was measured by the NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Complementary DNA

synthesis was performed using the M-MLV reverse transcriptase kit (Invitrogen) following the manufacturer's instructions. qRT-PCR was performed using a SYBR green-based PCR master mix kit (Takara, Shiga, Japan) on a Rotor Gene 3000 RT-PCR system (Corbett Research, Sydney, Australia). The PCR condition was as follows: initial denaturation at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, and thereafter 60°C for 20 seconds. During each extension the fluorescence signal was monitored at 585 nm. GAPDH and U6 served as an internal control.

2.6. Statistical analysis

Data were expressed as the mean ± standard error of the mean from the indicated number of independent experiments. Statistical analysis was performed with SPSS (Statistical Product and Service Solutions) 13.0 software using one-way analysis of variance and Student t test, and the correlation analysis was explored by use of Spearman's correlation coefficient. A p value < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Ang-(1−7) inhibited mitochondrial fission in HG-treated podocytes

To investigate whether Ang-(1−7) plays a role in mitochondrial fission of HG-induced podocytes, we conducted a HG-induced podocyte model. By way of electron microscopy, we observed normal mitochondria morphology in the control group, as indicated by the arrow in Fig. 1. Our findings suggested that compared with the control group, mitochondrial fission of the HG group increased, and the network structure noted mitochondrial collapse, as indicated by the arrow. The actual number of mitochondria increased, but the size of the structure is small (Fig. 1). However, compared with the HG group, Ang-(1−7) inhibited mitochondrial fission in HG-treated podocytes. Mitochondrial fission decreased in podocytes of the Ang-(1−7) + HG group, and the size of the structure of the mitochondria was bigger than that of the HG group, as indicated by the arrow. We also observed severe mitophagy and mitochondrial swelling with fracture and fusion of cristae in the HG group. In contrast, Ang-(1−7) + HG group had largely normal mitochondria morphology (Fig. 1).

3.2. Ang-(1−7) inhibited Drp1 and p53 expression in HG-treated podocytes

Mitochondrial distribution is closely associated with mitochondrial fission, and when the fission-promoting protein Drp1 activity is blocked, this often results in mitochondrial elongation and clustering. To investigate whether Ang-(1−7) plays a role in HG-treated podocytes by Drp1, we analyzed the expression of p53 and Drp1 by Western blot, where the levels of Drp1 and p53 were normalized to GAPDH. The expression of p53 is increased in the HG group ($p < 0.01$), but Ang-(1−7) inhibited p53 expression in HG-treated podocytes ($p < 0.05$; Fig. 2B). Similarly, compared with the control, the expression of Drp1 was increased in HG group ($p < 0.001$). Ang-(1−7) inhibited Drp1 expression in HG-treated podocytes compared with the HG group ($p < 0.001$; Fig. 2C), where after we could conclude that Ang-(1−7) inhibited Drp1 and p53 expression in HG-treated podocytes. These results provide biochemical evidence that Ang-(1−7) regulates mitochondrial fission by inhibiting Drp1 and p53 expression in HG-treated podocytes.

3.3. Ang-(1−7) decreased Drp1 expression in podocytes in a HG environment by immunofluorescence

Drp1 expression in podocytes was also determined by immunofluorescence. First, Drp1 expression was determined using a laser scanning confocal microscope. Drp1 of podocytes was stained with Drp1 antibody (green), and the nuclei were stained with DAPI (blue). The immunofluorescence data showed that the expression of Drp1 increased in the podocytes in a HG environment compared with the control group. Compared with the HG group, Ang-(1−7) significantly decreased the level of Drp1 in the Ang-(1−7) + HG group (Fig. 3A). The mean fluorescence intensity of Drp1 in each whole image was automatically quantified using Image-Pro Plus software, and expressed as mean ± SEM (in fluorescence units, FU) (Fig. 3B); the bar graph represents the average value from three independent experiments (Fig. 3B). The expression of Drp1 increased in podocytes of the HG group compared with the control group ($p < 0.05$), and Ang-(1−7) decreased the level of Drp1 in the Ang-(1−7) + HG group compared with the HG group ($p < 0.001$). This result particularly provided compelling evidence that Ang-(1−7) regulates mitochondrial fission by inhibiting Drp1 expression in HG-treated podocytes.

3.4. Ang-(1−7) improved the expression of miR-30a in HG-treated podocytes

We first observed the expression of miR-30a in podocytes by qRT-PCR. Ang-(1−7) increased the levels of miR-30a in podocytes of the control + Ang-(1−7) group, compared with the control group ($p < 0.001$); additionally, Ang-(1−7) increased the levels of miR-30a in podocytes in a HG environment, compared with the HG group ($p < 0.001$; Fig. 4).

4. Discussion

In conclusion, some studies demonstrated that DN can be considered in a similar manner to other inflammatory diseases.[11,12] Glucose has been reported to enhance the activation of the renin−angiotensin system in podocytes.[13,14] The biological effects of Ang-(1−7) are primarily mediated by interaction with the G-protein-coupled receptor Mas,[15] and include renal disease, hypertension, heart failure, pregnancy-induced hypertension (preeclampsia), and cardiac arrhythmia.[16–18] The best effects of Ang-(1−7) are its anti-proliferative actions, and it also acts as a vasodilator, with the ability to lower blood pressure. These effects apparently act

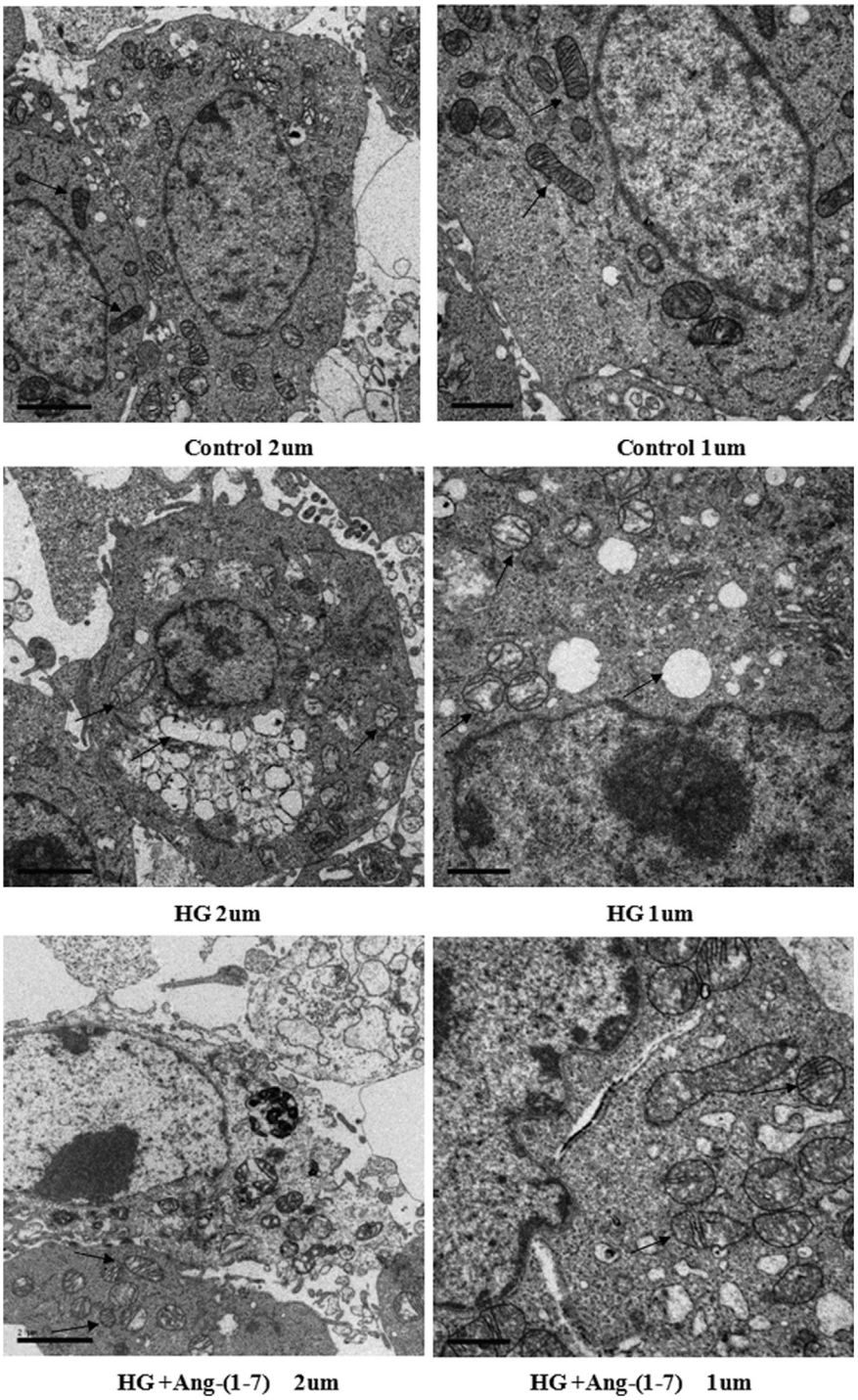

Fig. 1. Electron microscopy. (A) Normal mitochondria morphology was observed in the control group, shown at 2 μm; (B) control group, 1 μm; (C) severe mitophagy and mitochondrial swelling with fracture and fusion of cristae was observed in the HG group, shown at 2 μm; (D) HG group, 1 μm; (E) the Ang-(1−7) with HG group showed largely normal mitochondria morphology, shown at 2 μm; (F) Ang-(1−7) with HG, at 1 μm.

through a specific G protein-coupled receptor Mas for Ang-(1−7).[19−21] Some data identified that Ang-(1−7) and Mas have a significant impact on renal inflammation and may include decisive properties in the pathogenesis and progression of renal failure. Chronic Ang-(1−7) treatment preserved endothelial function in rat models of myocardial ischemia and in-stent restenosis.[22,23] Treatment with angiotensin converting enzyme-2 or Ang-(1−7) corrected diabetic defects in therapeutic angiogenesis.[24,25] In this study, we established a model of HG-induced podocytes *in vitro*, with an aim to explore the effects of Ang-(1−7) on HG-induced podocytes and find the possible mechanisms.

Mitochondria are dynamic organelles that continually undergo fusion and fission in apoptotic regulation.[26] Mutations of fission and fusion genes in mitochondria can cause human disease or death, indicating their roles in pathophysiology.

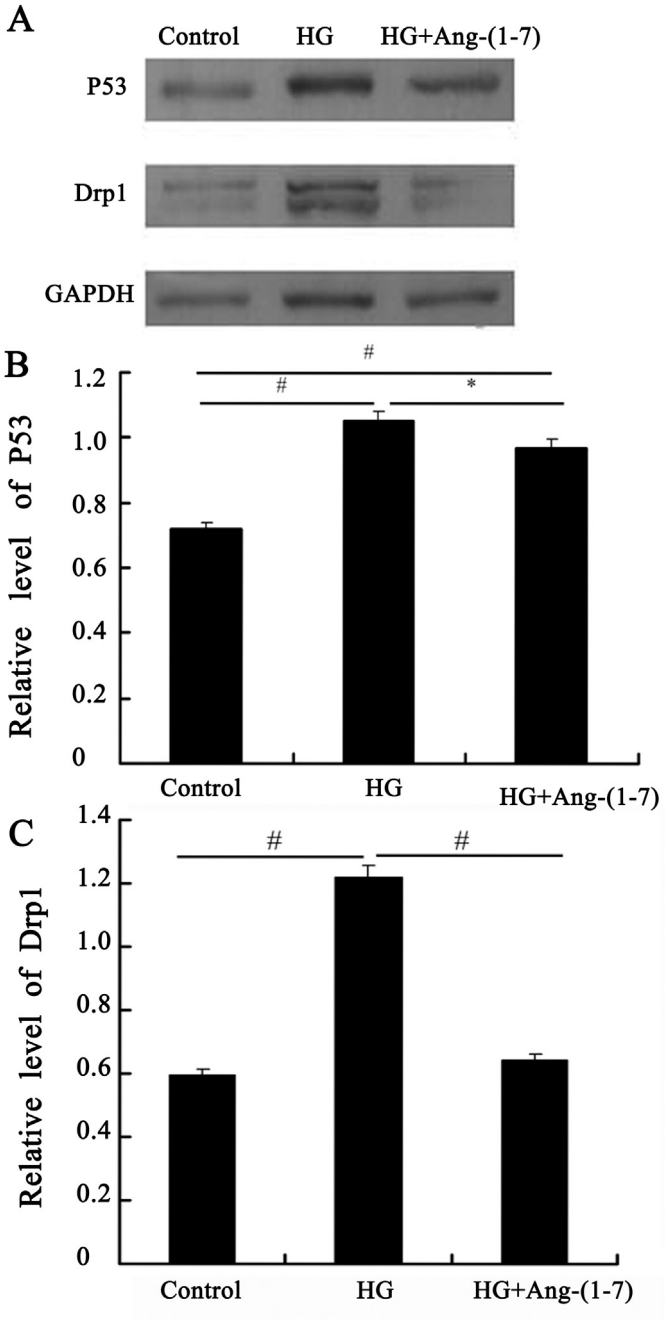

Fig. 2. The expression of Drp1 and p53 was analyzed by Western blot. The levels of Drp1 and p53 were normalized to GAPDH. (A) Western blot analysis and expression of DRP1, p53, and GADPH in the cytoplasm of podocytes; (B) the relative expression of p53; (C) the relative expression of DRP1. Each bar represents the mean ± standard error of the mean, $n = 4$. * $p < 0.001$, ** $p < 0.05$. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

Mitochondrial morphology is an important determinant of mitochondrial function.[27] The morphology alteration of the podocytes is associated with proteinuria in glomerular diseases including DN.[28] In this study, we hypothesized that elevated levels of glucose lead to an increase in the mitochondrial fission of podocytes, and we observed abnormal mitochondrial fission in HG-induced podocytes, where the number of mitochondria is increased. Alternatively, the mitochondrial fission and the number of mitochondria decreased in the podocytes of Ang-(1−7) + HG group, where the network structure collapse of mitochondria decreased in the podocytes of Ang-(1−7) + HG group compared with the HG group, and the size of mitochondria in Ang-(1−7) + HG group was bigger than that of the HG group (Fig. 1). Therefore, we have demonstrated that Ang-(1−7) acts as a protective factor against mitochondrial fission in HG-induced podocytes.

p53 has been identified as a pivotal factor in the regulation of apoptosis of podocytes.[29] The p53 protein is inactive in normal cells unless cells are exposed to various stress signals, which promotes the activation of the p53 protein by post-translational modifications.[30] Drp1 localizes mainly in the cytoplasm and is a large GTPase that controls the process of mitochondrial division.[31,32] In this study, the immunofluorescence data showed that Drp1 levels significantly increased in podocytes after stimulation with HG for 24 hours, and Ang-(1−7) decreased the expression of Drp1 in podocytes. The data suggested that Ang-(1−7) upregulated the expression of Drp1 in podocytes compared with the HG group, Ang-(1−7) downregulated the expression of Drp1 in the Ang-(1−7) + HG group compared with the HG group. At the same time, we tested p53 and Drp1 by Western blot, and our study demonstrated that p53 and Drp1 both increased in HG-induced podocytes, but the expression of Drp1 and p53 both decreased in the Ang-(1−7) + HG group compared with the HG group (Figs. 2 and 3). These results indicated that the expression of Drp1 was positively correlated with the expression of p53, and the effect of Drp1 on the regulation of podocytic apoptosis may be mediated by the p53 pathway. Thus, it is possible that Drp1 positively associated with P53 which mainly translocates to the mitochondria. In summary, our study revealed the beneficial effects of Ang-(1−7) on the treatment of HG-induced podocytes. We have showed the specific role of Ang-(1−7) in regulating p53 and p53-dependent factor Drp1. This study could provide a novel strategy for the treatment of podocyte disorders in glomerular diseases.

In mammalian cells, mitochondrial fission is mediated by the large GTPase Drp1, acting in concert with outer mitochondrial membrane proteins such as Fis1, Mff, and Mief1. Drp1 translocation to the mitochondria initiates mitochondrial fission and induces mitochondrial dysfunction and cell death.[32,33] A directly detrimental effect of p53 at the mitochondria was also documented in various cell types exposed to genotoxic, hypoxic, and oxidative stress.[34–36] Some data reveal a novel model in which a miRNA can regulate apoptosis through targeting the mitochondrial fission machinery. miR-30a inhibited mitochondrial fission through the suppression of the expression of p53 and its own team target Drp1.[37]

miRNAs are a class of small noncoding RNAs that mediate post-transcriptional gene silencing. Our findings suggested that Ang-(1−7) improves the expression of miR-30a in HG-treated podocytes. The data suggested a critical role for Ang-(1−7) as a protective factor against podocyte injury through the upregulation of the expression and activation of miR30a. The present data may provide a possible target for

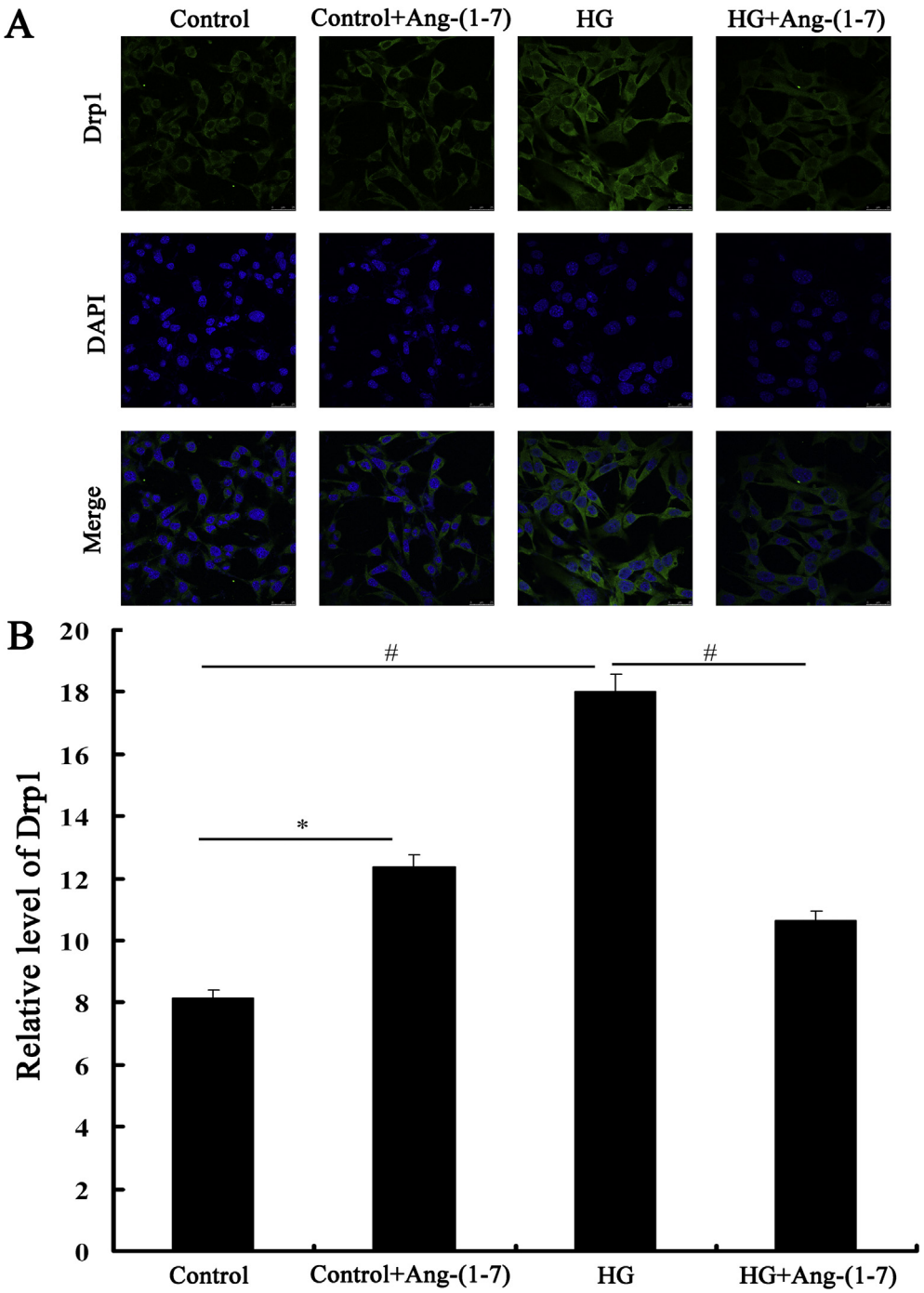

Fig. 3. Immunofluorescence showed that Ang-(1−7) decreased Drp1 expression in HG-treated podocytes. (A) Drp1 expression was determined using a laser scanning confocal microscope. Drp1 of podocytes stained with Drp1 antibody (green), with the nuclei stained by DAPI (blue); (B) the mean fluorescence intensity of Drp1 in each whole image was quantified and expressed in fluorescence units. The bar graph represents the average value from three independent experiments. Each bar represents the mean ± standard error of the mean, $n = 4$. * $p < 0.001$, ** $p < 0.05$. DAPI = .4',6-diamidino-2-phenylindole, DAPI = 4',6-diamidino-2-phenylindole.

DN treatment, although the detailed mechanism needed to be explored.

The current study complements these observations and provides direct evidence for the involvement of Ang-(1−7) in the protection of podocytes. Our data sheds new light on the understanding of the protective effect of Ang-(1−7) on HG-induced podocytes. This effect was associated with upregulation of miR-30a and downregulation of Drp1 and p53, as well as protection against mitochondrial fission in HG-induced podocytes. Our results suggest that Ang-(1−7) represents a viable therapeutic option for diabetic patients. Although we have not evaluated the exact signal pathway of Ang-(1−7) in

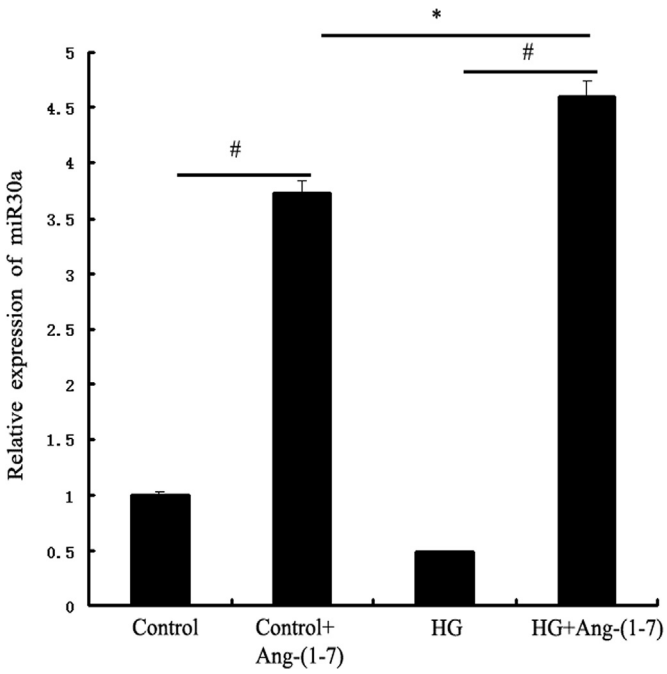

Fig. 4. Relative expression of miR-30a. The qRT-PCR analysis demonstrated that miR-30a increased in podocytes of the Ang-(1−7) group. Ang-(1−7) increased the expression of miR-30a in podocytes both of HG + Ang-(1−7) and control + Ang-(1−7) groups. Each bar represents the mean ± standard error of the mean, $n = 4$. * $p < 0.001$, ** $p < 0.05$. qRT-PCR = quantitative real-time polymerase chain reaction.

the regulation of mitochondrial fission of HG-induced podocytes in the present study, it will be important to explore this aspect in the future.

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