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Angiotensin-(1–7) decreases glycated albumin-induced endothelial interleukin-6 expression via modulation of miR-146a

Huang-Joe Wang [a,b], Wan-Yu Lo [c,d,e,*], Li-Jen Lin [f]

[a] School of Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan
[b] Division of Cardiology, Department of Medicine, China Medical University Hospital, No. 2, Yuh-Der Road, Taichung 40447, Taiwan
[c] Graduate Integration of Chinese and Western Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan
[d] Department of Medical Research, China Medical University Hospital, No. 2, Yuh-Der Road, Taichung 40447, Taiwan
[e] Department of Life Science, National Chung Hsing University, Kuo Kuang Rd., Taichung 40227, Taiwan
[f] School of Chinese Medicine-Basic Chinese Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan

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ABSTRACT

The presence of glycated albumin (GA) is associated with increased diabetic complications. This study investigated the effect of angiotensin-(1–7) on the expression of GA-induced endothelial interleukin-6 (IL-6) in human aortic endothelial cells (HAECs). We also evaluated whether miR-146a is involved in the post-transcriptional regulation of angiotensin-(1–7). HAECs were stimulated with GA with or without angiotensin-(1–7) pretreatment. Inflammatory cytokine screening approach identified that angiotensin-(1–7) (10^{-7} M) potently inhibited GA (200 μg/mL)-stimulated endothelial IL-6 expression in conditioned medium. ELISA confirmed this finding. Real-time PCR showed that angiotensin-(1–7) decreased GA-induced intracellular IL-6 mRNA expression and western blotting showed that angiotensin-(1–7) decreased GA-induced intracellular IL-6 protein expression. Bioinformatics' miR target analysis identified homology between miR-146a and the 3′-UTR of the human IL-6 mRNA, suggesting a potential regulation of IL-6 by miR-146a. Treatment with GA decreased endothelial miR-146a expression to 37.2% of the albumin control, while angiotensin-(1–7) increased endothelial miR-146a expression to 1.9-times that of the medium control. Pretreatment with angiotensin-(1–7) inhibited the GA-mediated downregulation of miR-146a to 78.9% of the albumin control levels. Furthermore, the inhibitory effect of angiotensin-(1–7) on IL-6 expression was abolished in GA-treated, miR-146a inhibitor-transfected HAECs. In conclusion, these results suggest that angiotensin-(1–7) exerted an endothelial protective effect through IL-6 down-regulation, and miR-146a modulation is involved in this protective effect.

1. Introduction

The prevalence of diabetes mellitus is increasing in all age groups worldwide. In 2000, the prevalence was 2.8%, and in 2030 it is estimated to be 4.4% [1]. Diabetes can cause microvascular diseases and macrovascular diseases. Vascular diseases account for most morbidity and mortality in patients with diabetes mellitus and endothelial dysfunction is a common starting point of diabetic microvascular and macrovascular diseases. Chronic hyperglycemia and enhanced oxidative stress in diabetes result in Amadori rearrangement, a reaction that causes non-enzymatic linkages and the reduction of carbohydrates with reactive amine residues in proteins, lipids, and nucleic acids to form reversible early glycation products. Earlier Amadori products then undergo further complex reactions such as dehydration, condensation, and cross-linking to become irreversible heterogeneous derivates termed advanced glycation end products (AGEs) [2]. Glycated albumin (GA) is a predominantly Amadori-modified early glycated protein *in vivo* [3]. Due to its abundance in plasma and intermediate half-life (~21 days), GA is regarded as an intermediate glycation index [4]. Furthermore, GA is correlated with diabetic complications and its role as a pathogenic protein has received increased attention [5,6].

Abbreviations: ACE2, angiotensin-converting enzyme 2; AGE, advanced glycation end product; ELISA, enzyme-linked immunosorbent assay; GA, glycated albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAEC, human aortic endothelial cell; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; LPS, lipopolysaccharide; miRNA, microRNA; MTS, methoxyphenyl tetrazolium; NAPDH, reduced phosphorylated nicotinamide adenine dinucleotide; Nox-4, NADPH oxidase 4; ox-LDL, oxidized low-density lipoprotein; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor-α.

* Corresponding author at: Graduate Integration of Chinese and Western Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan. Fax: +886 4 22056139.
E-mail address: wylo@mail.cmu.edu.tw (W.-Y. Lo).

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The angiotensin-converting enzyme 2 (ACE2)/angiotensin-(1–7)/Mas axis is a newly found counter-regulatory pathway in the renin-angiotensin system [7]. There are several lines of evidence for the amelioration of endothelial dysfunction by the angiotensin-(1–7)/ACE2/Mas axis. *In vitro*, angiotensin-(1–7) stimulation was reported to cause endothelial eNOS activation and nitric oxide production via an Akt-dependent pathway [8]. *In vivo*, overexpression of ACE2 improved endothelial dysfunction and reduced blood pressure in spontaneously hypertensive stroke-prone rats [9], and chronic angiotensin-(1–7) infusion improved endothelial function and reduced atherosclerotic lesion formation in apolipoprotein E-deficient mice [10]. Taken together, angiotensin-(1–7) holds promise as an agent against endothelial dysfunction.

Interleukin-6 (IL-6) is a multifunctional pro-inflammatory cytokine that is produced by a variety of cell types, including endothelial cells. Increased IL-6 expression has been associated with a variety of diseases, including atherosclerosis, obesity, myocardial infarction, and diabetes [11]. In addition, IL-6 has been shown to contribute to both atherosclerotic development and plaque destabilization through its diverse cellular actions [12]. In type 1 diabetes, IL-6, C-reactive protein, and tumor necrosis factor-α (TNF-α) are independently associated with microvascular complications and cardiovascular disease [13], emphasizing the importance of IL-6 level modulation for the prevention of diabetic complications.

MicroRNAs (miRNAs) are an evolutionarily conserved class of endogenous ~22-nucleotide RNAs that recognize the 3′-untranslated region of specific messenger RNA (mRNA) targets. These small RNAs regulate expression of their target gene at the post-transcriptional level, mainly by inhibiting translation or causing degradation of the target mRNA [14]. Among the multiple miRNAs, miR-146a is an anti-inflammatory miR-RNA since it acts as a negative regulator of NF-κB [15]. Recently, miR-146a has been implicated in the negative feedback loop restricting excess IL-6 expression in human fibroblasts [16]. In addition, miR-146a was also reported to be a negative regulator of constitutive NF-κB activity in the breast cancer cell line MDA-MB-231, and suppress the NF-κB target genes IL-6 [17]. This evidence suggested that miR-146a might modulate IL-6 expression.

Since angiotensin-(1–7) is known to improve endothelial dysfunction, the present study investigated whether angiotensin-(1–7) influenced the GA effect on endothelial IL-6 expression. In addition, we tested whether miR-146a is involved in the angiotensin-(1–7)-mediated modulation of IL-6 expression.

2. Materials and methods

2.1. Cell culture

Human aortic endothelial cells (HAECs) were purchased from Cell Applications, Inc. (San Diego, CA, USA) and cultured in endothelial cell growth medium (Cell Applications, Inc.) according to the manufacturer's recommendations. The cells were grown to near confluence in a 75-T flask before the experiment. Angiotensin-(1–7) (Sigma–Aldrich, St. Louis, MO, USA) at a dose of 10^{-7} M was added to the HAECs 1 h before stimulating the cells with human GA (Sigma–Aldrich). Human albumin (Sigma–Aldrich) was used as a non-glycated control.

2.2. Methoxyphenyl tetrazolium inner salt cell viability assay

HAECs were seeded into a 96-well plate at a density of 1×10^4 cells/well and treated with different concentrations of GA (50 μg/mL, 100 μg/mL, or 200 μg/mL; for 12 h). Cell viability assay was analyzed with methoxyphenyl tetrazolium (MTS, 0.5 mg/mL; Promega, Madison, WI, USA), as previously described [18].

2.3. Inflammatory cytokine screening

HAECs were seeded into in a 24-well plate at a density of 1×10^5 cells/well and stimulated with GA (200 μg/mL, for 12 h), or GA (200 μg/mL, for 12 h) + angiotensin-(1–7) (10^{-7} M, for 13 h). After treatment, the supernatants were collected and separated by centrifugation at 500*g* for 5 min at 4 °C and stored at −80 °C until analysis. A panel of cytokines was simultaneously quantified using the Human Inflammatory Cytokines Kit (BD Biosciences Pharmingen, San Diego, CA, USA) by flow cytometry (BD FACSCanto™ System; Becton Dickinson Corp., San Jose, CA, USA). In the cytometric bead array, 6 bead populations with distinct fluorescence intensities were coated with specific antibodies to capture different cytokines in conditioned medium. The cytokine-captured beads were then mixed with phycoerythrin-conjugated detection antibodies to form sandwich complexes. After incubation, washing, and acquisition of sample data, the results were generated in a graphic format using BD cytometric bead array analysis software.

2.4. IL-6 enzyme-linked immunosorbent assay (ELISA)

After stimulation, cells were collected, and then the conditioned medium was centrifuged to pellet any detached cells, and tested using the Quantikine human IL-6 immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All plates were read using an ELISA reader set at 450 nm.

2.5. Real-time polymerase chain reaction (PCR)

The mRNA expression level in the HAECs was analyzed by real-time PCR, as previously described [18]. The primer sequences used were as follows:

2.5.1. GAPDH

Forward primer: 5′-CTCTGCTCCTCCTGTTCGAC-3′
Reverse primer: 5′-ACGACCAAATCCGTTGACTC-3′

2.5.2. IL-6

Forward primer: 5′-GATGAGTACAAAAGTCCTGATCCA-3′
Reverse primer: 5′-CTGCAGCCACTGGTTCTGT-3′

2.6. Western blot analysis

The protein expression level in the HAECs was analyzed with Western blot analysis, as described previously [18]. Antibodies against IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at concentration of 1:500 and 1:2000, respectively. Whole cell lysates were used in all blots, which were normalized to GAPDH expression.

2.7. miRNA extraction and analysis

Small RNAs were extracted from the cell pellet using the High Pure miRNA Isolation Kit (Roche Diagnostics). Reverse transcription of total small RNA was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) with reverse transcription primers. PCR amplification was performed using the Roche LightCycler 1.5 sequence detection system and the hydrolysis TaqMan probes Kit (Roche) according to the manufacturer's protocol. The expression of RNU6B served as a loading control.

The reverse transcription primers and the PCR primer sequences of RNU6B and miR-146a used were as follows:

2.7.1. RNU6B

Reverse transcription primer: 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAAAAATAT-3′

Forward primer: 5′-TTCCTCCGCAAGGATGACACGC-3′

Reverse primer: 5′-GTGCAGGGTCCGAGGT-3′

2.7.2. miR-146a

Reverse transcription primer: 5′-GTTGGCTCTGGTGCAGGGTCC-GAGGTATTCGCACCAGAGCCAACAACCCA-3′

Forward primer: 5′-CCGCCGTGAGAACTGAATTCCA-3′

Reverse primer: 5′-GTGCAGGGTCCGAGGT-3′

2.8. miR-146a inhibitor-transfection

The hsa-miRNA-146a inhibitor (mirVana™ miRNA inhibitor, ID: MH:10722) was obtained from Ambion (Austin, TX, USA). HAECs were transfected with hsa-miRNA-146a inhibitor (30 nM) using the Lipofectamine 2000 transfection reagent (Invitrogen) in serum-free M-199 medium for 2 h. Mock transfection groups were treated with Lipofectamine 2000, but not with hsa-miRNA-146a inhibitor. After transfection, the M-199 medium was changed to the endothelial cell growth medium. The mock and transfected HAECs were then treated with GA (200 μg/mL, for 6 h) or GA (200 μg/mL, for 6 h) + angiotensin-(1–7) (10^{-7} M, for 7 h). After treatment, the supernatants were collected and preserved for IL-6 protein analysis by ELISA. The HAECs were collected and IL-6 mRNA and miR-146a expression were examined as described above.

2.9. Statistical analysis

Statistical analyses were performed using SPSS 12.0 statistical software for Windows (SPSS Inc., Chicago, IL, USA). All data are presented as the mean ± SEM. The significance was determined using ANOVA and unpaired t tests, as appropriate. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. GA was not toxic to endothelial cells at concentrations up to 200 μg/mL for 12 h

The MTS cell viability assay revealed that treatment with different concentrations of GA (50 μg/mL, 100 μg/mL, and 200 μg/mL) for 12 h caused no significant cytotoxicity (data not shown). In all subsequent experiments, 200 μg/mL GA was used.

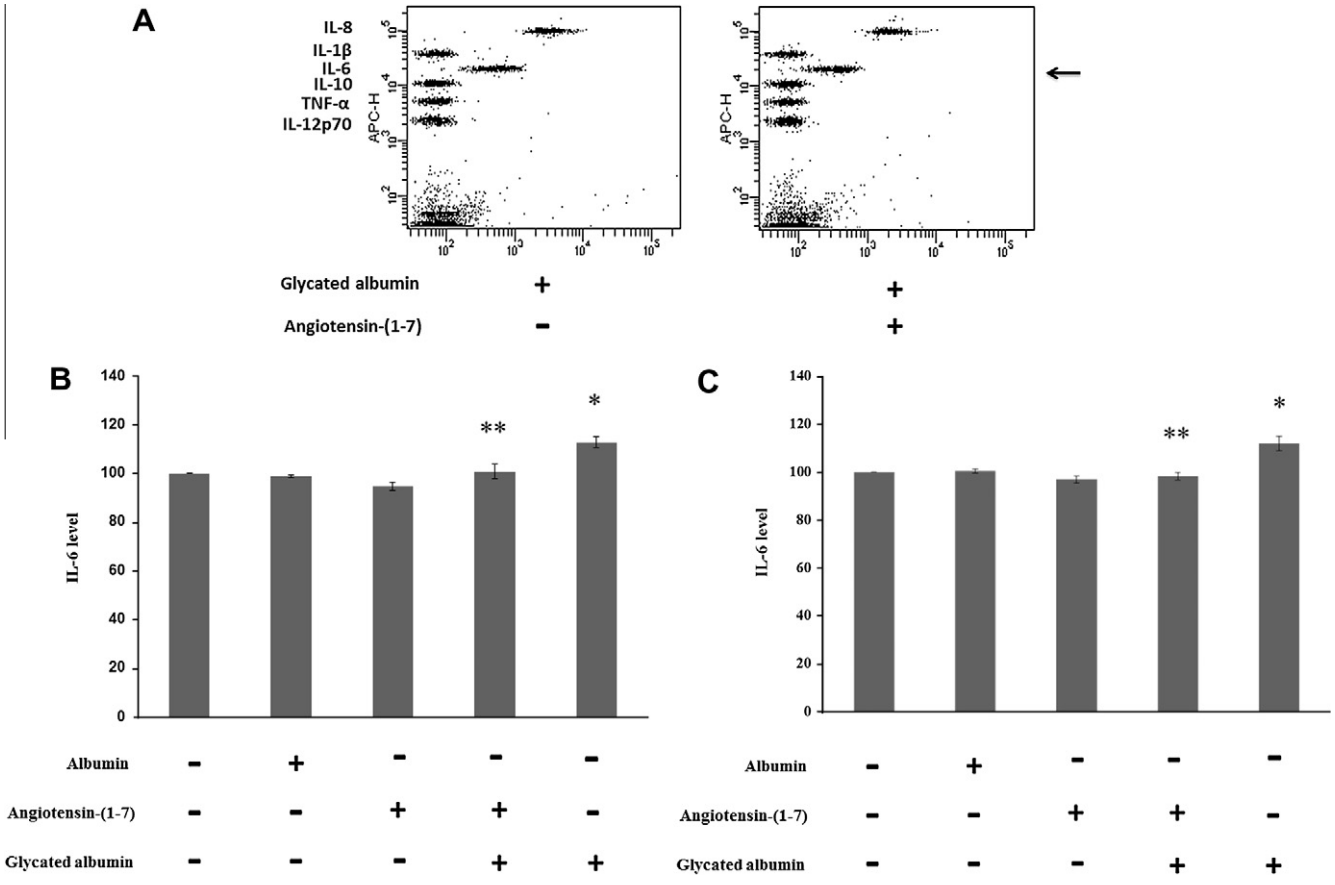

Fig. 1. Angiotensin-(1–7) pretreatment downregulated GA-induced IL-6 protein expression in conditioned medium. (A) From top to the bottom, multiple dots accumulated into 6 lines, which represented the expression levels of IL-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12p70 proteins in conditioned medium. The rightward shift of the dotted lines represented the relative protein expression levels. The inflammatory cytokines kit showed that angiotensin-(1–7) pretreatment decreased IL-6 expression in GA-stimulated HAECs (arrow). The images shown represent the results from 3 independent experiments. (B) The relative quantity of IL-6 expression in conditioned medium was determined by ELISA. ELISA showed that stimulation of HAECs with GA for 6 h induced a 1.14-fold increase in IL-6 protein levels compared to the albumin control, while angiotensin-(1–7) pretreatment reversed this increase. The bars represent the means ± SEM from 5 experiments. $^{*}P < 0.001$ for albumin vs. GA. $^{**}P < 0.005$ for GA vs. GA/angiotensin-(1–7). (C) ELISA showed that stimulation of HAECs with GA for 12 h induced a 1.11-fold increase in IL-6 protein levels compared to the albumin control. Similarly, angiotensin-(1–7) pretreatment reversed this increase. Bars represent the means ± SEM from 7 experiments. $^{*}P = 0.05$ for albumin vs. GA. $^{**}P < 0.05$ for GA vs. GA/angiotensin-(1–7).

3.2. Inflammatory cytokine screening revealed that angiotensin-(1–7) downregulated GA-induced IL-6 protein expression in conditioned medium

GA-induced inflammatory cytokine changes were probed using the Human Inflammatory Cytokines Kit. Both IL-6 and IL-8 expression level increased in conditioned medium, as shown by the rightward shift of the dotted lines. However, IL-1β, IL-10, TNF, and IL-12p70 expression levels did not change. According to the screening results, GA treatment at 12 h increased IL-6 expression, while angiotensin-(1–7) pretreatment of the GA-stimulated HAECs caused a decrease in GA-induced IL6 expression (Fig. 1A).

3.3. ELISA confirmed that angiotensin-(1–7) downregulated IL-6 protein expression in conditioned medium

ELISA showed that compared to the albumin control in conditioned medium, GA treatment alone at 6 and 12 h caused a 1.14-fold and 1.11-fold increase in IL-6 expression, respectively. Consistent with the above experiments, angiotensin-(1–7) pretreatment caused a significant downregulation of IL-6 expression at both 6 and 12 h (Fig. 1B and C).

3.4. Angiotensin-(1–7) downregulated intracellular IL-6 mRNA expression

In the real-time PCR analysis, GA alone caused a 1.20-fold and 1.20-fold increase in IL-6 mRNA expression at 6 and 12 h, respectively, compared with expression in the albumin control. Angiotensin-(1–7) pretreatment decreased the IL-6 mRNA level to near that of the albumin control (Fig. 2A and B).

3.5. Angiotensin-(1–7) downregulated intracellular IL-6 protein expression

In the western blot analysis, GA alone caused an increase in IL-6 expression compared with albumin control, while angiotensin-(1–7) pretreatment downregulated IL-6 protein expression in GA-stimulated HAECs (Fig. 2C).

3.6. Angiotensin-(1–7) inhibited the downregulation of miR-146a expression in GA-stimulated HAECs

Bioinformatics' miR target analysis from miRNA databases, MicroRNA.org (http://www.microrna.org/microrna/getGeneForm.do) identified homology between miR-146a and the 3′-UTR of the human IL-6 mRNA, which suggests this gene as a potential

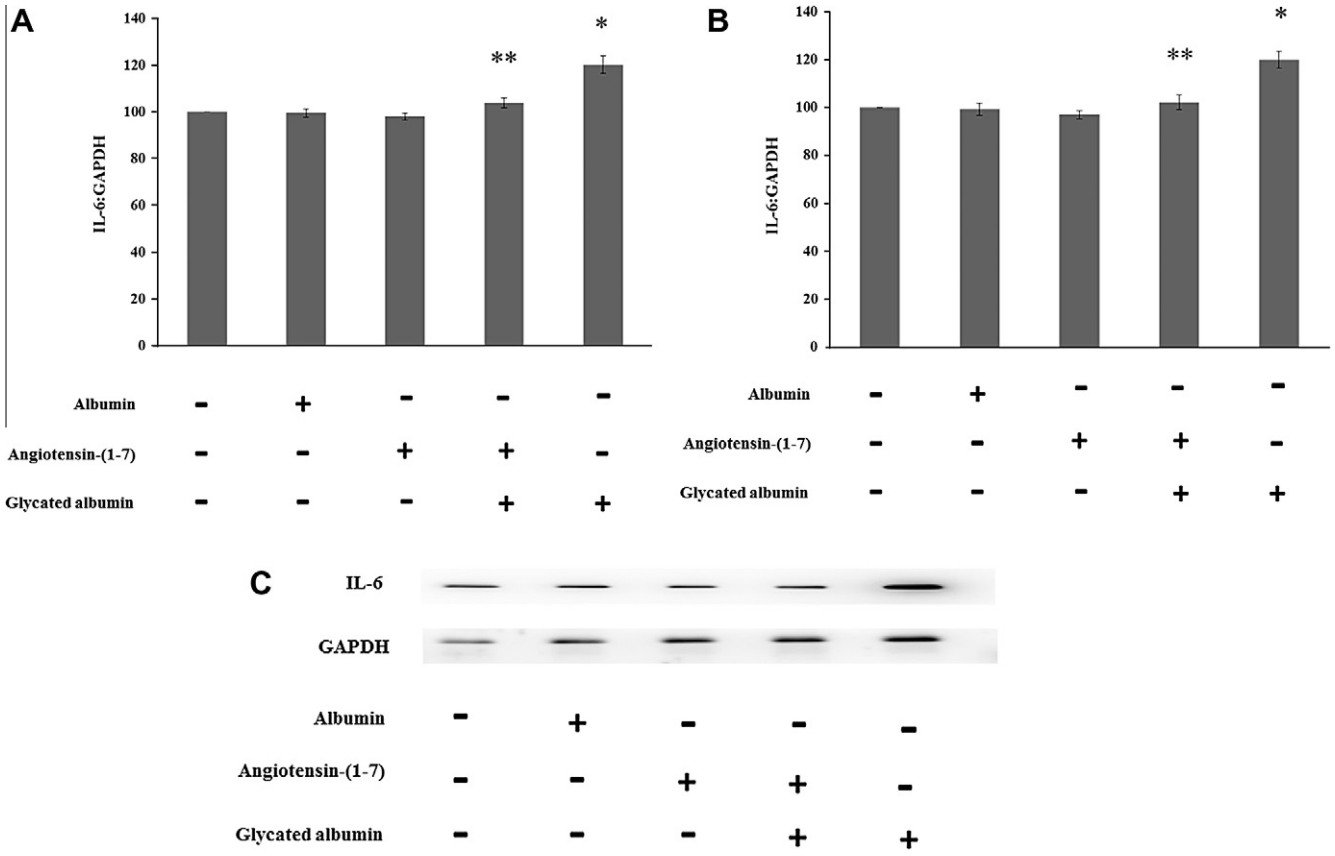

Fig. 2. Angiotensin-(1–7) downregulated intracellular IL-6 mRNA and protein expression. (A) Real time PCR showed that stimulation of HAECs with GA for 6 h induced a 1.20-fold increase in IL-6 mRNA levels compared to the albumin control, while angiotensin-(1–7) pretreatment reversed this increase. The bars represent the means ± SEM from 7 experiments. $^{*}P < 0.01$ for albumin vs. GA. $^{**}P < 0.05$ for GA vs. GA/angiotensin-(1–7). mRNA levels are expressed as a ratio of GAPDH levels. (B) Real time PCR showed that stimulation of HAECs with GA for 12 h induced a 1.20-fold increase in IL-6 mRNA levels compared to the albumin control, while angiotensin-(1–7) pretreatment reversed this increase. The bars represent the means ± SEM from 8 experiments. $^{*}P < 0.01$ for albumin vs. GA. $^{**}P < 0.05$ for GA vs. GA/angiotensin-(1–7). mRNA levels are expressed as a ratio of GAPDH levels. (C). In the western blotting analysis, stimulation of HAECs with GA for 6 h increased IL-6 expression compared to the albumin control, while angiotensin-(1–7) pretreatment downregulated intracellular IL-6 protein expression in GA-treated HAECs. The blot represents the results from 4 independent experiments.

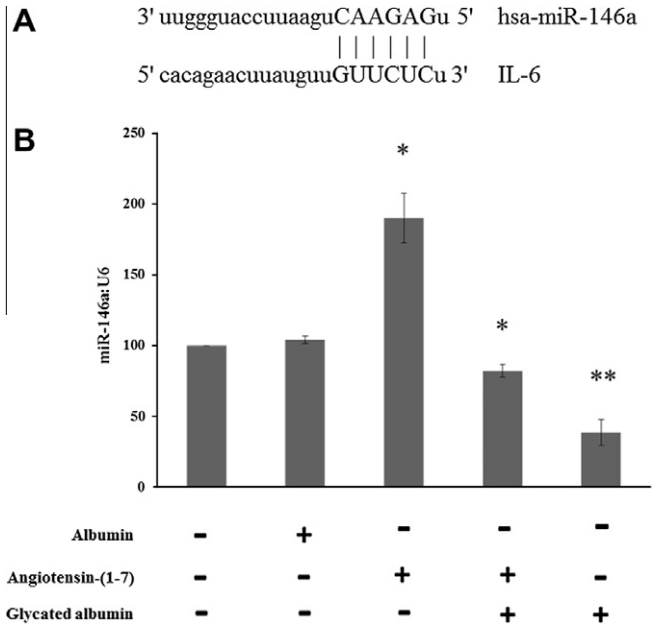

Fig. 3. Angiotensin-(1–7) recovered the downregulated miR-146a expression in GA-stimulated HAECs. (A) Bioinformatics' miR target analysis from miRNA databases identified homology between miR-146a and the 3′-UTR of the human IL-6 mRNA, suggesting a potential regulation of IL-6 by miR-146a. (B) miR-146a expression was measured by real time PCR. GA stimulation for 6 h decreased miR-146a expression to 37.2% of the albumin control. In contrast, angiotensin-(1–7) pretreatment alone caused a 1.90-fold increase in miR-146a expression compared to the medium control. Pretreatment of GA-stimulated HAECs with angiotensin-(1–7) recovered the downregulated miR-146a expression to 78.9% of the albumin control. The bars represent the means ± SEM from 4 experiments. $^{*}P = 0.05$ for medium control vs. angiotensin-(1–7) and for GA vs. GA/angiotensin-(1–7), $^{**}P < 0.05$ for albumin vs. GA. miRNA levels are expressed as a ratio of RNU6B levels.

molecular target for miR-146a (Fig. 3A). Since miR-146a is an important anti-inflammatory regulator, we determine whether miR-146a levels changed in GA-stimulated HAECs, and the effect of angiotensin-(1–7) pretreatment was also examined. GA stimulation for 6 h decreased miR-146a expression to 37.2% of the albumin control. In contrast, angiotensin-(1–7) stimulation for 7 h caused a 1.90-fold increase in miR-146a expression compared to the medium control. Pretreatment of GA-stimulated HAECs with angiotensin-(1–7) reversed the GA-mediated downregulation of miR-146a to 78.9% of the albumin control (Fig. 3B).

3.7. The inhibitory effect of angiotensin-(1–7) on IL-6 expression was abolished in miR-146 inhibitor-transfected, GA-treated HAECs

To determine whether miR-146a is involved in the angiotensin-(1–7)-mediated inhibitory effect on GA-stimulated IL-6 expression, we transfected HAECs with miR-146a inhibitor and examined the effect of GA and angiotensin-(1–7) on IL-6 expression. miR-146a expression was measured to assess transfection efficiency. The data showed a good transfection efficiency in miR-146a inhibitor-transfected, GA-stimulated HAECs and miR-146a inhibitor-transfected, GA/angiotensin-(1–7)-stimulated HAECs (Fig. 4A). Regarding IL-6 mRNA expression, the inhibitory effect of angiotensin-(1–7) was preserved in mock-transfected, GA-stimulated HAECs. In contrast, angiotensin-(1–7) was unable to inhibit IL-6 mRNA expression in miR-146a inhibitor-transfected, GA-stimulated HAECs (Fig. 4B). Regarding IL-6 protein expression, the inhibitory effect of angiotensin-(1–7) was also preserved in mock-transfected, GA-stimulated HAECs. In contrast, angiotensin-(1–7) lost the ability to inhibit IL-6 protein expression in miR-146a inhibitor-transfected, GA-stimulated HAECs (Fig. 4C).

4. Discussion

This study demonstrated that angiotensin-(1–7) can downregulate GA-induced IL-6 expression in HAECs. Angiotensin-(1–7) inhibits GA-induced IL-6 expression at the post-transcriptional level via inhibition of GA-induced miR-146a downregulation. Our findings showed that angiotensin-(1–7) exerted a protective effect in endothelial cells upon GA stimulation, and such a property may be useful for treating diabetic complications.

Early glycation Amadori-modified proteins (i.e. GA) can affect cellular biology through interaction with the RAGE receptor [3]. Similar to AGE, GA upregulates several inflammatory mediators through the NFκB and AP-1 signaling pathways in several cells types (smooth muscle cells, fibroblasts, and macrophages) [19–21]. In endothelial cells, GA induced reactive oxygen species via upregulation of reduced phosphorylated nicotinamide adenine dinucleotide oxidase 4 (NADPH oxidase 4, Nox-4) [22]. Interestingly, angiotensin-(1–7) has been reported to decrease the angiotensin II- induced NAD(P)H activity in endothelial cells [8], alleviate Nox-4-mediated oxidative stress, and reduce renal dysfunction in diabetic hypertensive rats [23]. Therefore, it is reasonable that angiotensin-(1–7) can downregulate IL-6 expression in GA-stimulated HAECs. We demonstrated that GA could induce IL-6 expression at both the protein and mRNA levels, suggesting that GA-mediated IL-6 regulation is under some form of transcriptional control.

Recently, miR-146a was reported to be downregulated in high glucose-stimulated endothelial cells and diabetic rats [24–25]. Consistent with these experimental data, Balasubramanyam et al. reported that impaired miR-146a expression was associated with subclinical inflammation and insulin resistance in type 2 diabetes [26], Interestingly, they noted that miR-146a levels in peripheral blood mononuclear cells were negatively correlated with plasma levels of glycated hemoglobin and IL-6, a finding that shares the same regulating directions as our data. All the above *in vitro* and *in vivo* evidence implied that miR-146a is a specific modulator involved in diabetic complications. Our data showed that miR-146a was downregulated by GA, which in turn upregulated endothelial IL-6 expression, suggesting that impaired miR-146a expression decreased the inhibitory effect of miR-146a on IL-6 expression. Furthermore, we noted that angiotensin-(1–7) itself increased miR-146a, and this angiotensin-(1–7)-mediated increase in miR-146a could reverse GA-mediated downregulation of miR-146a. Transfection experiments confirmed that the anti-inflammatory effect of angiotensin-(1–7) was at least partially mediated by a post-transcriptional regulatory mechanism via miR-146a.

The control of miR-146a expression is intriguing, and 2 types of miR-146a regulation have been reported. In the type 1 response, an increased level of cellular miR-146a is noted upon acute stressor stimulation. This type 1 response is typified in lipopolysaccharide (LPS)-stimulated monocytic THP-1 cells [27], interleukin-8 (IL-8)/TNF-α/interleukin-1β (IL-1β)-stimulated gastric epithelial cells HGC-27 [28], IL-1β-stimulated human airway smooth muscle cells [29], phorbol 12-myristate 13-acetate (PMA)-stimulated human microvascular endothelial cells [30], and oxidized low density lipoprotein (ox-LDL)-stimulated dendritic cells [31]. In the type 2 response, decreased cellular miR-146a levels are noted upon acute stressor stimulation. The type 2 response is typified by ox-LDL-stimulated THP-1 cells [32] and high glucose-stimulated human umbilical-vein endothelial cells [24]. Our data regarding the miR-146a response in GA-stimulated HAECs fit the type 2 response. Although the differences in miR-146a regulation can be explained by the cell-type (e.g. ox-LDL in THP-1 vs. dendritic cells), other possibilities exist. LPS, TNF-α, IL-1β, PMA, and IL-8 may be stronger stressors that directly induce miR-146a expression, which serves

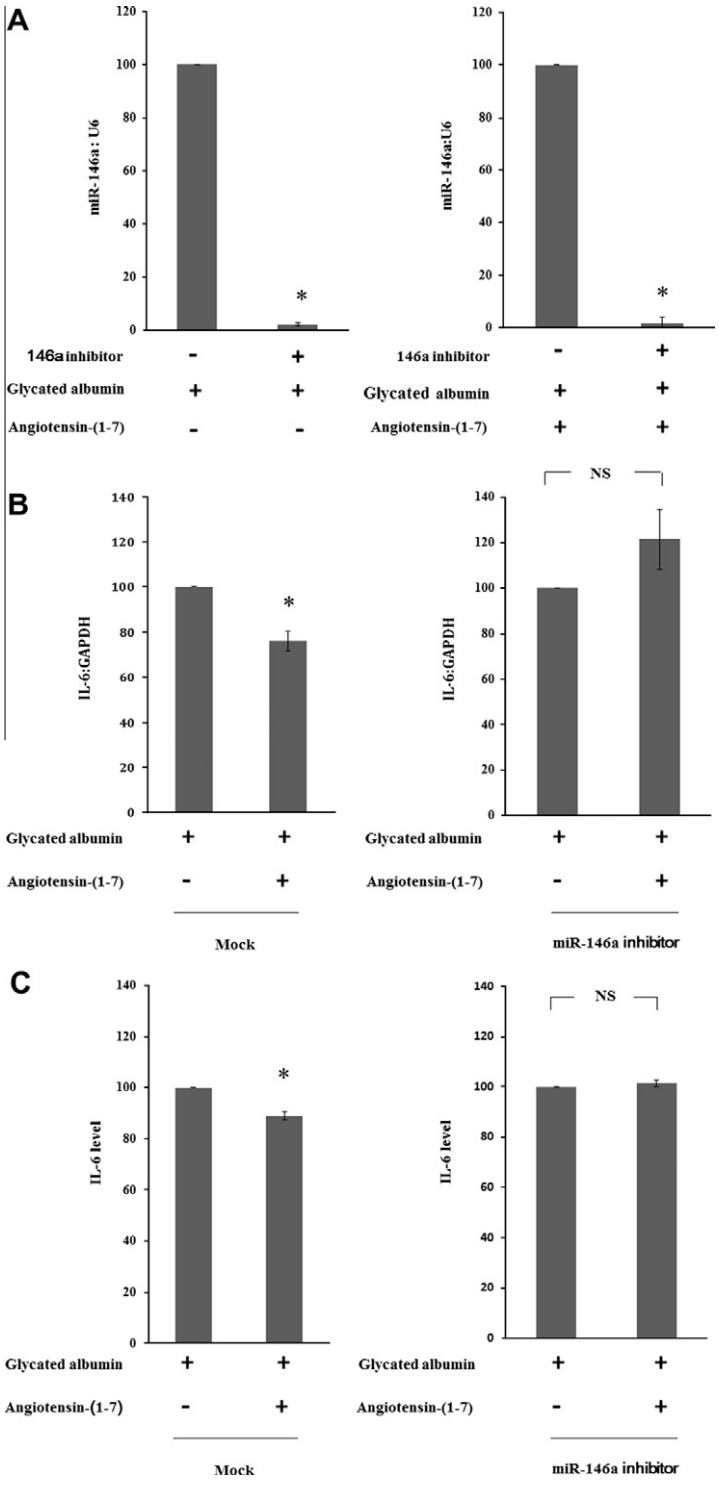

Fig. 4. The inhibitory effects of angiotensin-(1–7) on IL-6 expression were abolished in miR-146a inhibitor-transfected, GA-stimulated HAECs. (A) Transfection efficiency was assessed by measuring miR-146a expression. In the miR-146a inhibitor-transfected, GA-stimulated HAECs, miR-146a expression was 49-fold lower than in mock-transfected, GA-stimulated HAECs. In addition, in miR-146a inhibitor-transfected, GA/angiotensin-(1–7)-stimulated HAECs, miR-146a expression was 58-fold lower than in the mock transfected, GA/angiotensin-(1–7)-stimulated HAECs. $^{*}P < 0.001$ for GA (mock) vs. GA (146a inhibitor) and for GA/angiotensin-(1–7) (mock) vs. GA/angiotensin-(1–7) (146a inhibitor). (B) The inhibitory effect of angiotensin-(1–7) on GA-stimulated IL-6 mRNA expression was preserved in mock-transfected HAECs. Angiotensin-(1–7) pretreatment decreased IL-6 mRNA expression to 75.9% of that in GA-treated HAECs. In contrast, angiotensin-(1–7) did not inhibit IL-6 mRNA expression in miR-146a inhibitor-transfected, GA-stimulated HAECs. NS = not significant. $^{*}P < 0.005$ for GA (mock) vs. GA/angiotensin-(1–7) (mock). (C) The inhibitory effect of angiotensin-(1–7) on GA-stimulated IL-6 protein expression in conditioned medium was preserved in mock-transfected HAECs. Angiotensin-(1–7) pretreatment decreased IL-6 protein expression to 88.9% of that in GA-treated HAECs. In contrast, angiotensin-(1–7) did not inhibit IL-6 protein expression in the miR-146a inhibitor-transfected, GA-treated HAECs. NS = not significant. $^{*}P < 0.01$ for GA (mock) vs. GA/angiotensin-(1–7) (mock). The bars represent the means ± SEM from 5 experiments.

as a negative, restraining loop that alleviates NF-κB activation and associated inflammatory mediator expression. In contrast, high glucose and GA may be weaker stressors, since both stimulators exist in the circulating blood of diabetic patients for a long

duration, and these weaker stressors control the inflammatory response by downregulating miR-146 to attenuate the repressive effects of different inflammatory mediators release by constitutive miR-146a. Whatever the mechanisms, the concept that miR-146a as a negative NF-κB feedback loop may have additional control mechanisms beyond our current understanding.

In summary, this study demonstrated that angiotensin-(1–7) can inhibit endothelial IL-6 expression upon GA stimulation by modulating miR-146a. This finding provides insight into developing a new treatment strategy for diabetic complications.

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