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Effect and mechanism of the Ang-(1-7) on human mesangial cells injury induced by low density lipoprotein

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ABSTRACT

Hyperlipidemia is an independent risk factor for renal disease, and lipid deposition is associated with glomerulosclerosis. The angiotensin converting enzyme 2-angiotensin-(1-7)-Mas axis (ACE2-Ang-(1-7)-Mas axis) has been reported to participate in lipid metabolic regulation but its mechanism remains unclear. We hypothesized Ang-(1-7) would reduce lipid uptake in human mesangial cells (HMCs) by regulating the low density lipoprotein receptor–sterol regulatory element binding proteins 2–SREBP cleavage activating protein (LDLr–SREBP2–SCAP) negative feedback system, and improve glomerulosclerosis by regulating the transforming growth factor-β1 (TGF-β1). In this study we found that ACE2 was undetected in HMCs. The administration of LDL caused normal LDLr–SREBPs–SCAP negative feedback effect. Exogenous Ang-(1-7) enhanced this negative feedback effect via down-regulating LDLr, SREBP2, and SCAP expression, and effectively inhibited LDL-induced lipid deposition and cholesterol increases. This enhanced inhibitory effect was reversed by the Mas receptor antagonist A-779. Meanwhile, Ang-(1-7) significantly decreased the high LDL-induced production of TGF-β1, an effect blocked by A-779. Interestingly, HMCs treated with Ang-(1-7) alone activated the TGF-β1 expression. Our results suggested that Ang-(1-7) inhibits LDL accumulation and decreases cholesterol levels via modulating the LDLr–SREBPs–SCAP negative feedback system through the Mas receptor. Moreover, Ang-(1-7) exhibits a dual regulatory effect on TGF-β1 in HMCs.

1. Introduction

Hyperlipidemia contributes to lipid accumulation in the kidney and renal disease progression, causing pathological effects such as glomerulosclerosis [1,2]. Recently, investigators have compared glomerulosclerosis with atherosclerosis because of similarities regarding their pathogenesis and pathological effects. The role of HMCs in glomerulosclerosis is regarded as analogous to the role of vascular smooth muscle cells in atherosclerosis. In HMCs, a specific receptor-mediated endocytosis of LDL has been clearly demonstrated [1] and mesangial lipid accumulation proved to be an important risk factor for glomerulosclerosis [2].

LDLr, the primary receptor for binding LDL, is highly expressed in HMCs [3,4]. The expression of LDLr is mediated by SREBP2 and SCAP. They constitute a tight feedback system that has a crucial role in absorbing LDL cholesterol, thereby regulating plasma and cellular LDL concentrations. When cells are short of cholesterol for their normal needs, SREBP2 is transported from the endoplasmic reticulum (ER) to the Golgi by SCAP and then hydrolyzed by the Site 1 protease and Site 2 protease. The hydrolyzed SREBP2 enters into the nucleus and binds to the sterol regulatory element (SRE-1) site within the LDLr promoter, activating LDLr gene transcription. Conversely, when cells are in a high cholesterol environment, the SREBP2–SCAP complex remains in the ER, thus, *LDLr* gene transcription is suppressed to a relatively low level [4].

There are three known isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) [5]. TGF-β1, the most abundant, is a well-known fibrogenic factor with a pathogenic role in glomerulosclerosis and TGF-β1 is activated under high lipid conditions [1,6,7]. Activation of the TGF-β1 results in cell proliferation and fibrosis [8–10]. In addition, TGF-β1 can induce the synthesis of the extracellular matrix (ECM) and inhibit ECM degradation [11,12]. Apart from that, as an inflammatory cytokine, TGF-β also increases LDLr promoter activity and promotes the expression of LDLr [2,13].

Abbreviations: ACE2, angiotensin converting enzyme 2; Ang-(1-7), angiotensin-(1-7); HMCs, human mesangial cells; LDLr, low density lipoprotein receptor; SREBP2, sterol regulatory element binding proteins 2; SCAP, SREBP cleavage activating protein; TGF-β1, transforming growth factor-β1; ECM, extracellular matrix.

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The ACE2-Ang-(1-7)-Mas axis is a recently identified branch of the renin–angiotensin system [14–18]. Ang-(1-7), the core component of the ACE2-Ang-(1-7)-Mas axis, is chiefly produced via the catalysis of Angiotensin II (Ang II) by ACE2 *in vivo*. When Ang-(1-7) binds to its specific receptor, the Mas receptor, it antagonizes the effect of Ang II by maintaining blood pressure [19,20], balancing electrolytes [21], and inhibiting cell proliferation [22,23]. Recent studies have shown that the ACE2-Ang-(1-7)-Mas axis participates in lipid metabolism regulation through reducing total cholesterol, triglyceride levels in serum [24–26]. Overall, these results indicate the Ang-(1-7) may provide an effective strategy for the treatment of lipid-mediated renal injury.

The aim of this study was to evaluate whether Ang-(1-7) alleviates LDL induced HMCs injury through regulating the LDLr–SREBP2–SCAP negative feedback system and inhibiting the expression of TGF-β1.

2. Materials and methods

2.1. Cell culture

Established stable HMC line cells, a generous gift from Dr. Xiongzhong Ruan (Royal Free Hospital, UK), were used for all experiments in this study. HMCs were grown in 35 cm [2] type culture flasks and cultured in a 37 °C and 5% CO_2 air incubator. For Oil Red O experiment, HMCs were grown on cover slips in 6 well plates. Cells between the 3th and the 6th passage were used for each experiment.

2.2. Experimental groups

Confluent HMCs were synchronized and starved by replacing their medium with fresh serum-free RPMI-1640 for 24 h. HMCs were then divided into six groups: control group, LDL group (100 μg/ml), LDL (100 μg/ml) +Ang(1-7) (10^{-7} mol/L) group, LDL + Ang-(1-7)+A-779 group (10^{-6} mol/L A-779 pre-incubated for 15 to 30 min before adding 100 μg/ml LDL and 10^{-7} mol/L Ang-(1-7)), Ang-(1-7) group (10^{-7} mol/L), and A-779 group (10^{-6} mol/L).

2.3. Oil Red O staining on HMCs

After a 24 h incubation under the conditions described above, HMCs were washed twice with phosphate-buffered saline, fixed with 10% formal saline for 30 min, incubated with 1,2-propanediol for 2 min, stained with Oil Red O for 30 min, stained with Carazzi's haematoxylin for 2 min, and then washed in tap water for 5 min. Finally, micrographs of the HMCs were taken with a microscope at a magnification of 200× and 400×.

2.4. Measurement of total cellular cholesterol

The stimulated HMCs were washed twice with PBS and total cholesterol measured using a total cholesterol assay kit according to the manufacturer's instructions. An absorbance of 490 nm was selected in the SpectraMax M2 system. The total cholesterol content in each group was normalized relative to total cell proteins determined using the modified Lowry assay.

2.5. RNA isolation and real-time quantitative PCR

Total RNA was extracted from cultured HMCs using the Total RNA Extraction Kit, and used as a template for reverse transcription using the RT Reagent Kit. Real-time quantitative PCR was performed using the ABI 7300 Real-time PCR System using SYBR Premix Ex TaqII. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ Method. The sequences of the forward (F) and reverse (R) primers are in Table 1.

2.6. Western blotting

The protein samples transferred onto 0.45 μm polyvinylidene fluoride (PVDF) transfer membranes, and then incubated with rabbit anti-human ACE2, LDLr, SREBP2, SCAP, TGF-β1, GAPDH antibodies. The proteins were measured using the enhanced chemiluminescence kit on a Bio-Rad ChemiDoc Imaging System.

2.7. Statistical analysis

Significance was assessed by analysis of variance, values of $P < 0.05$ considered significant, with Bonferroni correction in all cases involving multiple comparisons. Results are presented as means ± SD.

3. Results

3.1. Expression of ACE2 in human mesangial cells

ACE2 expression in HMCs was assessed through real-time PCR and Western blotting. ACE2 mRNA (Fig. 1A) and protein (Fig. 1B) were undetected which suggested ACE2 was not expressed in HMCs. Thus, exogenous Ang-(1-7) was used to treat HMCs in this study.

3.2. LDL absorption capacity of human mesangial cells

Oil Red O Staining was performed to assess the LDL absorption capacity of HMCs. A few lipid droplets were detected in HMCs incubated with LDL at concentration of 25 μg/ml (Fig. 1C1) and 50 μg/ml (Fig. 1C2). The lipid droplets in HMCs were obviously observed when the LDL 75 μg/ml was chosen (Fig. 1C3). However, the cytoplasm of HMCs was full of lipid droplets when the concentration of LDL added to 100 μg/ml (Fig. 1C4). This suggested that with the increasing concentration of LDL, the absorbed lipid in HMCs was also enhanced.

3.3. Effect of different concentrations of Ang-(1-7) on LDL-stimulated TGF-β1 expression in HMCs

After HMCs had been treated with different concentrations of Ang-(1-7) and LDL at concentration of 100 μg/ml for 24 h, the expression of TGF-β1 was evaluated by Western blot (Fig. 1D and E). The results suggested that LDL lead to the significantly increase of TGF-β1. Although Ang-(1-7) at the concentration of 10^{-9} mol/L and 10^{-8} mol/L decreased the LDL-induced TGF-β1 expression,

Table 1
Real-time PCR primer sequences.

Gene	Primer sequences	
ACE2	F	GATCCCATGGCTACAGAGGA
	R	GCCAGGAAGAGCTTGACATC
LDLr	F	GTGTCACAGCGGCGAATG
	R	CGCACTCTTTGATGGGTTCA
SREBP2	F	CCGCCTGTTCCGATGTACAC
	R	TGCACATTCAGCCAGGTTCA
SCAP	F	GGGAACTTCTGGCAGAATGACT
	R	CTGGTGGATGGTCCCAATG
TGF-β1	F	GCAACAATTCCTGGCGATAC
	R	AAGGCGAAAGCCCTCAAT
β-Actin	F	CCTGGCACCCAGCACAAT
	R	GCCGATCCACACACGGAGTACT

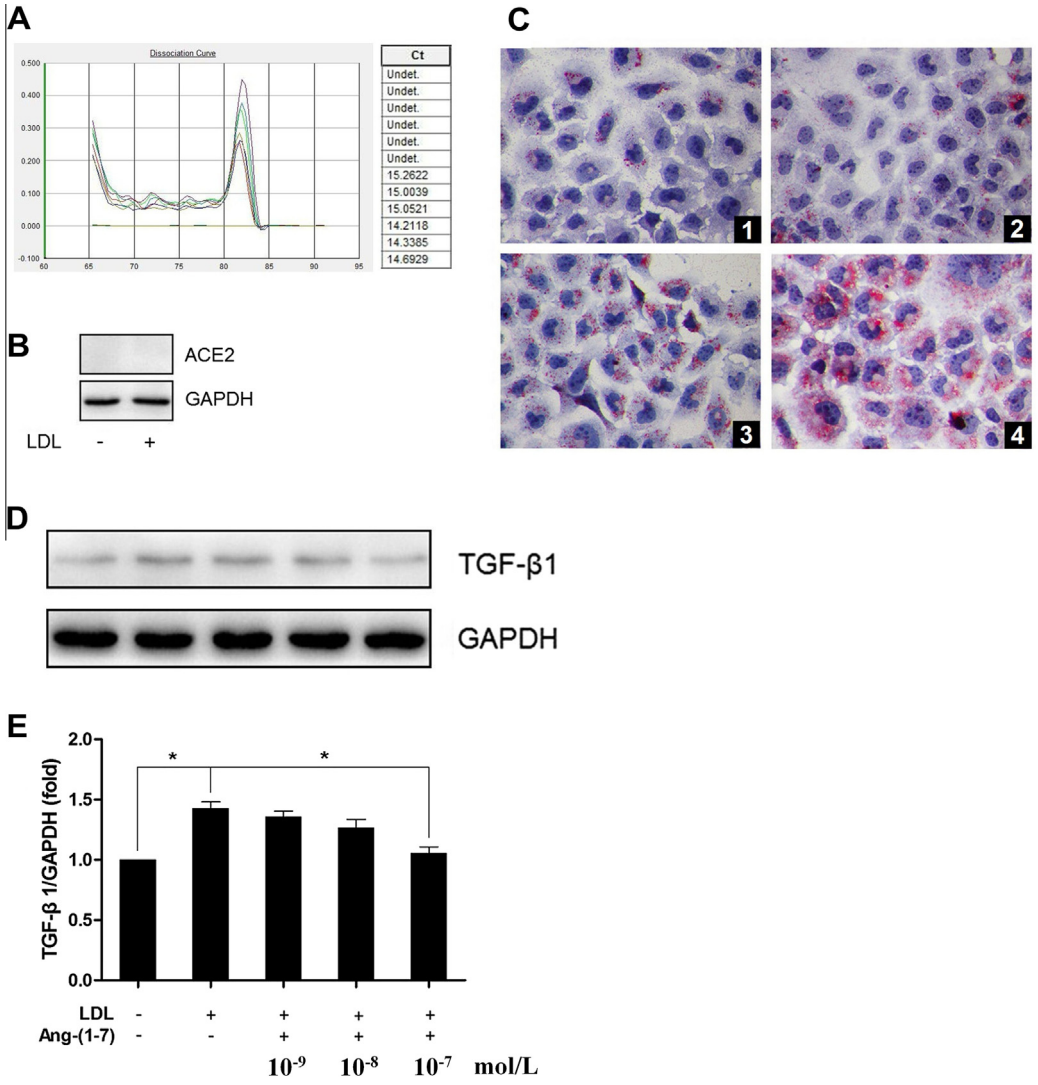

Fig. 1. Expression of ACE2 in human mesangial cells. HMCs were starved in fresh serum-free RPMI-1640 for 24 h. HMCs were divided into two groups: untreated (control), treated with LDL (100 μg/ml). (A) The mRNA expression of *ACE2* was detected by real-time PCR, the *β-actin* gene served as the internal control. (B) Representative immunoblot for ACE2. LDL induces concentration-dependent absorption in human mesangial cells. Starved HMCs were divided into four groups: (C1) treated with LDL (25 μg/ml), (C2) treated with LDL (50 μg/ml), (C3) treated with LDL (75 μg/ml), (C4) treated with LDL (100 μg/ml). After 24 h, cells were fixed and stained with Oil Red O. Magnification = 400×. Different concentrations of Ang-(1-7) on LDL-stimulated TGF-β1 expression in HMCs. Starved HMCs were divided into five groups: untreated (control), treated with LDL (100 μg/ml), treated with LDL (100 μg/ml) and Ang-(1-7) (10^{-9} mol/L), treated with LDL (100 μg/ml) and Ang-(1-7) (10^{-8} mol/L), treated with LDL (100 μg/ml) and Ang-(1-7) (10^{-7} mol/L). (D) Representative immunoblot for TGF-β1. (E) Representative quantitative analysis for TGF-β1. $^{*}P < 0.05$, $n = 3$.

they had no statistical significance compared to the LDL group. Ang-(1-7) 10^{-7} mol/L effectively inhibited the TGF-β1 expression. The results indicated that Ang-(1-7) inhibited LDL-induced TGF-β1 expression in a dose-dependent manner.

3.4. Oil Red O Staining of human mesangial cells

Oil Red O Staining was performed to determine whether Ang-(1-7) is capable of inhibiting LDL uptake in HMCs. There was a difference between HMCs incubated with (Fig. 2A2 and B2) or without (Fig. 2A1 and B1) LDL. When HMCs were co-incubated with LDL and Ang-(1-7), the intracellular lipid droplets markedly decreased (Fig. 2A3 and B3). This process was reversed by pre-incubating HMCs with A-779 (Fig. 2A4 and B4). Almost no lipid was observed in HMCs cultured with Ang-(1-7) (Fig. 2A5 and B5) or A-779 (Fig. 2A6 and B6). These results suggested that Ang-(1-7) inhibits the absorption of LDL in HMCs, and this effect was blocked by A-779.

3.5. Cholesterol content in human mesangial cells

The incubation of HMCs with LDL for 24 h resulted in an approximately threefold rise in total cellular cholesterol. The Ang-(1-7) + LDL group displayed a significant reduction in total cellular cholesterol compared with the LDL group. However, this reduction by Ang-(1-7) was reversed by A-779. Moreover, the results showed that treating HMCs with Ang-(1-7) or A-779 alone had no effect on total cellular cholesterol content compared with the control group (Table 2).

3.6. Effect of Ang-(1-7) on LDL-induced LDLr, SREBP2, and SCAP mRNA expression in HMCs

The results of real-time PCR suggested that the administration of LDL inhibited *LDLr*, *SREBP2*, and *SCAP* mRNA expression in HMCs. Compared to the LDL group, the expression of these mRNAs decreased in HMCs treated with LDL and Ang-(1-7). Furthermore, A-779 overrode the suppression of those mRNAs induced by the

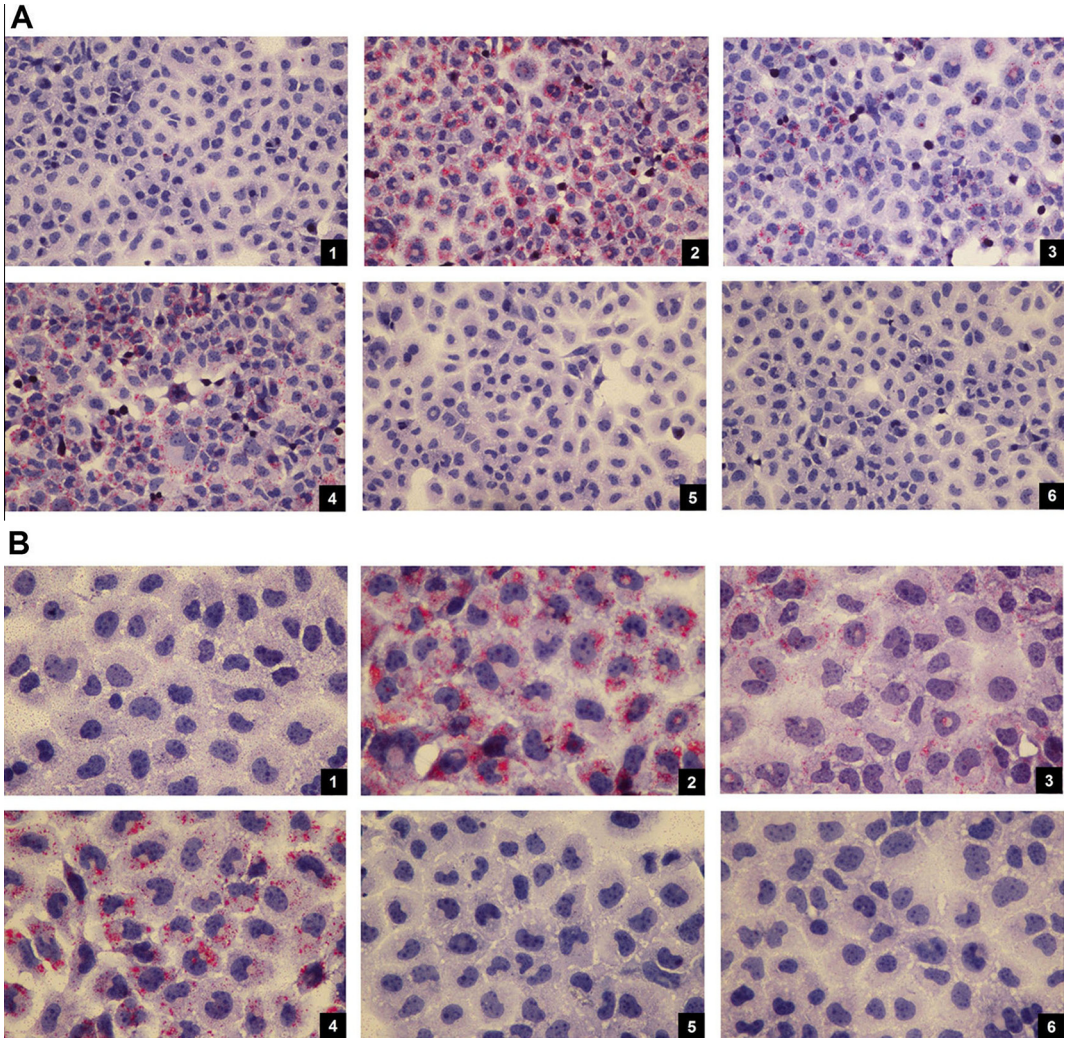

Fig. 2. Oil Red O staining of human mesangial cells. HMCs were treated as described for Table 2. After 24 h, cells were fixed and stained with Oil Red O. (A1 and B1) Control group, (A2 and B2) LDL group, (A3 and B3) LDL + Ang-(1-7) group, (A4 and B4) LDL + Ang-(1-7) + A-779 group, (A5 and B5) Ang-(1-7) group, (A6 and B6) A-779 group. Magnification = 200× (A) and 400× (B).

Table 2
Total cholesterol content in human mesangial cells. Confluent HMCs were synchronized and starved in fresh serum-free RPMI-1640 for 24 h. HMCs were then placed into one of six treatment groups: untreated (control), treated with LDL (100 μg/ml), treated with LDL (100 μg/ml) and Ang-(1-7) (10^{-7} mol/L), preincubated with A-779 (10^{-6} mol/L) for 30 min prior to administration of LDL (100 μg/ml) and Ang-(1-7) (10^{-7} mol/L), incubated with Ang-(1-7) (10^{-7} mol/L), and incubated with A-779 (10^{-6} mol/L). Values are expressed as means ± SD.

Group	Cholesterol (μg cholesterol/mg cell protein)
Control	10.92 ± 3.21
LDL	42.82 ± 1.68*
LDL + Ang-(1-7)	32.19 ± 2.20**
LDL + Ang-(1-7)+A-779	40.89 ± 3.78
Ang-(1-7)	12.85 ± 4.67
A-779	13.83 ± 2.31

* $P < 0.05$ vs. control group.
** $P < 0.05$ vs. LDL group or vs. LDL + Ang-(1-7)+A-779 group, $n = 6$ for each.

co-incubation of LDL and Ang-(1-7). Moreover, the addition of Ang-(1-7) alone to HMCs also decreased the *LDLr*, *SREBP2*, and *SCAP* mRNA expression. These results demonstrated that Ang-(1-7) regulates the LDLr–SREBP2–SCAP negative feedback system (Fig. 3A–C).

3.7. Effect of Ang-(1-7) on LDL-induced LDLr, SREBP2, and SCAP protein synthesis in HMCs

The expression of LDLr, SREBP2, and SCAP protein was also examined (Fig. 3D–F). LDL significantly decreased the LDLr, SREBP2, and SCAP protein expression in HMCs. Co-incubating HMCs with Ang-(1-7) and LDL further inhibited LDLr, SREBP2, and SCAP protein expression. However, the enhanced inhibitory effect of Ang-(1-7) on LDL-induced LDLr, SREBP2, and SCAP protein synthesis were reversed in the presence of A-779. In addition, the addition of Ang-(1-7) to HMCs alone also exerted an inhibitory effect. However, A-779 alone did not affect the expression of LDLr, SREBP2, and SCAP.

3.8. Effect of Ang-(1-7) on LDL-stimulated TGF-β1 expression in HMCs

The results of real-time PCR and Western blot revealed that LDL induced a significant increase in TGF-β1 expression. Ang-(1-7) could partially counteract the LDL-stimulated TGF-β1 expression. However, this inhibitory effect was blocked when HMCs were preincubated with A-779. Unexpectedly, HMCs incubated with Ang-(1-7) alone also increased TGF-β1 expression. These results

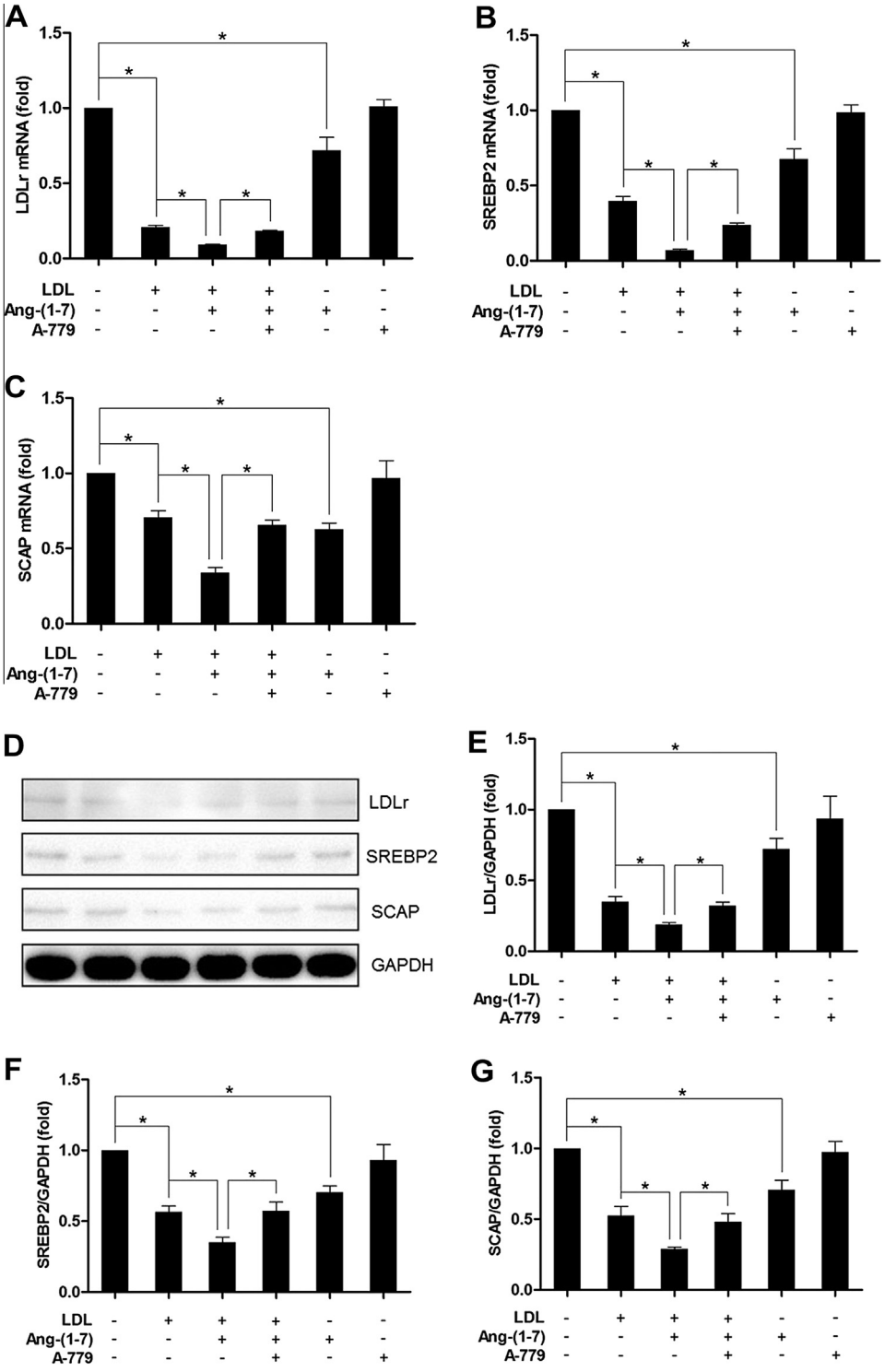

Fig. 3. A-779 reverses the inhibitory effect of Ang-(1-7) on LDL-induced LDLr, SREBP2, and SCAP expression. HMCs were treated as described for Table 2. The mRNA expression of (A) *LDLr*, (B) *SREBP2*, and (C) *SCAP* were detected by real-time PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, The *β-actin* gene served as the internal control. $^{*}P < 0.05$, $n = 6$ for each. (D) Representative immunoblot for LDLr, SREBP2 and SCAP. (E) Representative quantitative analysis for LDLr. (F) Representative quantitative analysis for SREBP2. (G) Representative quantitative analysis for SCAP. $^{*}P < 0.05$, $n = 5–6$.

indicate that LDL and Ang-(1-7) modulate TGF-β1 expression at the mRNA and protein level. (Fig. 4)

4. Discussion

ACE2 is chiefly expressed in the renal proximal tubules, but inside glomeruli ACE2 is mainly expressed in podocytes [27]. In this study ACE2 was undetected in HMCs. Because Ang-(1-7) is primarily formed from Ang II by the catalytic effect of ACE2 [27–30], we speculated that HMCs barely generate bioactive Ang-(1-7). However, HMCs contain the Mas receptor because the effect of Ang-(1-7) was partly inhibited after the addition of A-779. We hypothesize that Ang-(1-7) was produced elsewhere in the kidney, or in other tissues/organs, transported to the mesangial area, and

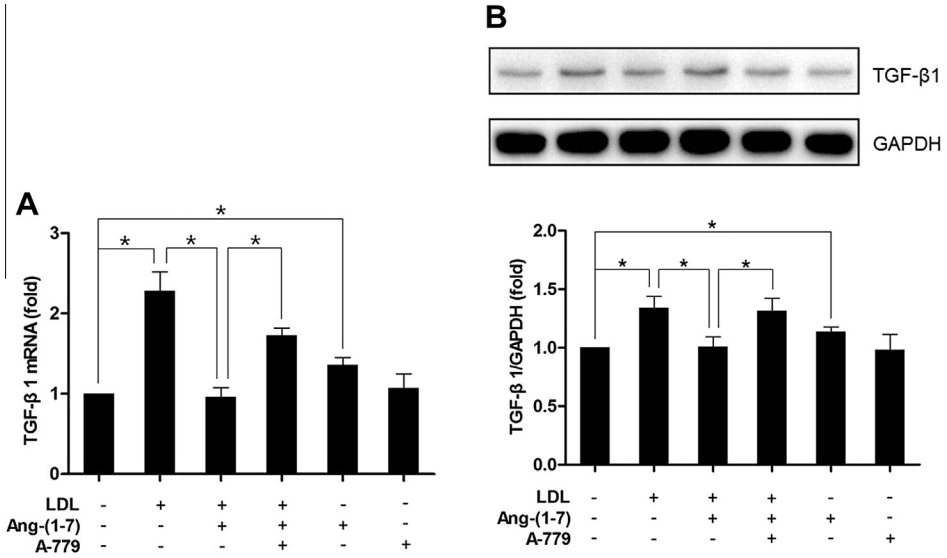

Fig. 4. A-779 reverses the inhibitory effect of Ang-(1-7) on LDL-stimulated TGF-β1 expression. HMCs were treated as described for Table 2. (A) Representative real-time PCR analysis for *TGF-β1* mRNA. Relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ Method, the *β-actin* gene served as the housekeeper gene. (B) Representative immunoblot and quantitative analysis for TGF-β1 protein. $^{*}P < 0.05$, $n = 6–7$.

then combined with the Mas receptor to perform its regulatory functions.

The beneficial effects of the ACE2-Ang-(1-7)-Mas axis in the regulation of lipid metabolism has been identified. In high-fat diet rats, Ang-(1-7) produced a reduction in body weight and cholesterol levels [31]. Mas deficiency in FVB/N mice induced a dyslipidemic state [24]. Singh et al. [32] pointed out that Ang(1-7) decreases dyslipidemia in diabetic rats. However, the molecular mechanisms are unclear. Santos et al. [25] have suggested Ang(1-7) increased adipose lipid-binding protein expression and stimulated adiponectin production. Moreover, Oh et al. [33] demonstrated that Ang-(1-7)-induced lipolysis occurs via the Ang-(1-7)/Mas receptor/PI3K pathway.

Our research has revealed a new mechanism that the ACE2-Ang-(1-7)-Mas axis participates in lipid metabolism via regulating the LDLr–SREBP2–SCAP negative feedback system in HMCs. First of all, Ang-(1-7) alone resulted in a reduced expression of LDLr, SREBP2, and SCAP in HMCs at normal circumstance. Moreover, when HMCs were treated by LDL, it caused the LDLr–SREBP2–SCAP negative feedback effect, the expression of LDLr, SREBP2, and SCAP were sharply inhibited. However, Ang-(1-7) could enhance this negative feedback effect, that means even if HMCs were under high lipid conditions, Ang-(1-7) could also effectively prevented LDL uptake, inhibited lipid accumulation, and decreased the total cholesterol concentration by down-regulating the LDLr expression. Finally, A779 strongly reversed the inhibitory effect of Ang-(1-7), suggesting the inhibitory effect of Ang-(1-7) on the LDLr–SREBP2–SCAP negative feedback system is mediated through binding to the Mas receptor.

The physiological functions of the TGF-β1 in the kidney are well-known. Isaka et al. [34] found that transfection of the *TGF-β1* gene into rat kidneys resulted in glomerulosclerosis. TGF-β1 is involved in ECM synthesis and deposition [8,35]. Reports have also proved that lipid accumulation and TGF-β1 expression are closely related. LDL induced a persistent increase in TGF-β1 release [6,36].

Our results also proved that LDL considerably up-regulated TGF-β1 expression in HMCs, suggesting TGF-β1 was activated in a high lipid circumstance. Another major finding was that when co-incubating HMCs with Ang-(1-7) and LDL, the TGF-β1 expression was decreased, manifesting that Ang-(1-7) could effectively inhibit the expression of TGF-β1 induced by LDL both in gene and protein levels, and in a dose-dependent manner. Moreover, the inhibitory effect of Ang-(1-7) on TGF-β1 was reversed by A-779, which indicated that it is the Mas receptor that mediates the effect of Ang-(1-7). Finally but interestingly, we found that incubating HMCs with Ang-(1-7) alone increased TGF-β1 expression. It is beyond our expectation and seems contrary to many other studies [37–41].

Nevertheless, in previous experiments we found the regulatory effect of Ang-(1-7) on LDLr–SREBP2–SCAP negative feedback system in human mesangial cells. Accordingly, we considered that Ang-(1-7) decreased the expression of LDLr by regulating the LDLr–SREBP2–SCAP negative feedback system at first, and then inhibited the TGF-β1 expression induced by LDL. Interestingly, Zimpelmann [42] found that Ang-(1-7) stimulated TGF-β1 in HMCs through p38 MAPK pathway. Similarly, Shao et al. [43] reported that an exogenous Ang-(1-7) injection resulted in elevated TGF-β1 expression in diabetic rats. In addition, Tom et al. [44] mentioned that Ang-(1-7) owns the low affinity of binding to AT1 receptors. Ang-(1-7) also acted as an agonist in the absence of Ang II. Taking the above into consideration, we concluded that Ang-(1-7) exerts different regulatory effects on the TGF-β1 depending on the organ, tissue, or cell type involved, or depending on the different physiological status in the same kind of organ, tissue, or cell type.

In summary, our research enables a better understanding of how Ang-(1-7) participates in lipid metabolism regulation and shows the dual regulatory effect of Ang-(1-7) on TGF-β1 in human mesangial cells.

Acknowledgments

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