# THE ROLE OF SREBP-1 IN KIDNEY FIBROSIS

# THE ROLE OF SREBP-1 IN MEDIATING ANGIOTENSIN II-INDUCED KIDNEY FIBROSIS

By

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## ABSTRACT

End stage renal disease (ESRD) has been a world-wide cause of renal disease-related fatality. Most patients with ESRD progress through chronic kidney disease (CKD). There are various causes of CKD including mechanical stress, infections, high blood pressure or immune conditions. Renal fibrosis is known to play an important pathogenic role in CKD and ESRD.

Transforming growth factor-beta (TGF-ß) is one of the major mediators of fibrosis, and inhibition of TGF-ß could reduce renal fibrosis in in vitro models. One of the well-known inducers of TGF-ß transcription is Angiotensin II (ANGII). [1] This is a major contributor to the matrix accumulation and glomerular fibrosis leading to a decline in kidney function and kidney failure in patients with chronic kidney disease (CKD). [2] A recent study demonstrated that TGF-ß could be increased by SREBP-1c overexpression. [3] My laboratory's data showed that ANGII could induce TGF-ß on a transcriptional level, and this induction could be blocked by Fatostatin, an SREBP/SCAP inhibitor. My data showed that ANGII could induce SREBP-1 in a time and dose dependent manner. Therefore my study focused on understanding the role of SREBP-1 in ANGII-induced TGF-ß upregulation in primary mesangial cells (MCs).

We found that ANGII-induced TGF-ß promoter activity requires Angiotensin type 1 receptor (AT1R) and SREBP-1 activation as well as ER stress. Interestingly, the activation of SREBP-1 by ANGII stimulation relies on ER stress and PI3K/AKT signaling. Both ANGII and ER stress inducers could induce AKT phosphorylation, indicating that ER stress is an upstream mediator in this signaling pathway. We also found that ER stress inhibition could block SREBP-1 activation by ANGII stimulation and, more importantly, could also block ANGII-induced TGF-ß promoter activity. In C57BL/6 mice infused with ANGII for one week, we found that both SREBP-1 and GRP78 were significantly upregulated in glomeruli in the ANGII infusion group compared with the control group. Taken together, this data indicates that ER stress is required for SREBP-1 activation, both of which are mediators of kidney fibrosis.

**ACKOWLEDGMENTS**

First of all I would like to thank my supervisor, Dr. Joan Krepinsky for accepting me as a Master student candidate. Over the last two years, I have learned both academic knowledge and experimental skills from you. When I had difficulty with my research work, you were always there to help me find out the problem and guide me to the right direction. You have always been encouraging to me.

I also would like to thank my committee members and my course directors. Dr. Ingram, Dr. Austin and Dr. Tang, thank you for your help and guidance with my research work and thank you for giving me knowledge of renal function and disease. I would also like to thank everyone in Dr. Krepinsky's lab for your kindness and support for my work. Thank you, Lalita, Guang, Bo, Richard, Maria, and Xing. Thank you to all lab members of the surrounding labs who were always willing to help and answer any of my questions.

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**LIST OF ABBREVIATIONS**

| | |
|---|---|
| ANGII | Angiotensin II |
| AT1R | ANGII type 1 receptor |
| ATF-4 | Activating transcription factor-4 |
| CKD | Chronic kidney disease |
| CTGF | Connective tissue growth factor |
| ECM | Extracellular matrix |
| eIF2α | Eukaryotic translation initiation factor α |
| ER | Endoplasmic reticulum |
| ESRD | End stage renal disease |
| FA | Fatty acid |
| GFR | Glomerular filtration rate |
| GRP78 | Glucose regulated protein 78 |
| IP3 | Inositol-1, 4, 5-triphosphate |
| LLC | Large latent complex |
| LTBP | Latent TGF-β-binding protein |
| MAPK | Mitogen - activated protein Kinase |
| MC | Mesangial cells |
| PI3K | Phosphoinositide 3-kinase |
| PLC | Phospholipase C |
| S1P | Site-1 protease |
| S2P | Site-2 protease |
| SCAP | SREBP-cleavage activating protein |
| SLC | Small latent complex |
| SREBP | Sterol regulatory element binding protein |
| TGF-ß | Transforming growth factor-beta |
| UPR | Unfolded protein response |
| UUO | Unilateral ureteral obstruction |
| VEGF | Vascular endothelial growth factor |

# I. INTRODUCTION

## *1.1 Chronic Kidney Disease and Renal Fibrosis*

1) Overview of Chronic Kidney Disease

Chronic kidney disease (CKD) has been considered as one of the most common causes of morbidity and mortality all over the world, with an estimated 1.9 million and 2.3 million people affected in Canada. [4] CKD can be caused by a large variety of acute or chronic injurious stimuli such as metabolic diseases, mechanical injury, infections, or immune conditions. [5] It can be identified in five stages according to the values of the glomerular filtration rate (GFR). Patients who have a GFR below 15mL/min/1.73m$^2$ are considered to have end-stage renal disease (ESRD).[5] Even though only 1% of CKD patients have ESRD, this is still a considerable fraction of the general population and leads to extremely high medical and socioeconomic costs. Since renal fibrosis is the histological hallmark of CKD and ESRD, its attenuation is an important goal in diminishing the burden of renal disease.

2) TGF-ßand Renal Fibrosis

TGF-ßis believed to be a central mediator of fibrosis, and was one of the first profibrotic factors described. TGF-ßis involved in biological and immunological functions via a complex signaling pathway.[6] First, latency-associated peptide (LAP) forms homodimers called the small latent complex (SLC) with TGF-ß. SLC binds to latent TGF-β-binding protein (LTBP), forming the large latent complex (LLC). LLC is secreted and binds to extracellular matrix (ECM). Active TGF-β is released after cleavage of LLC and SLC by, for example, plasmin, matrix metalloproteases and activated by integrin αV. Further posttranscriptional regulation is effected by molecules that regulate TGF-β activity in a negative (e.g. decorin) or a positive (e.g. CTGF) manner. TGF-β binds to cell surface type II receptors (TβRII), which then recruit the type I receptors (TβRI, ALK5) with kinase activity. This activated heteromeric receptor complex phosphorylates downstream proteins of the Smad

family, but also interact with other pathways such as p38 MAPK, JNK or Rho proteins.

The prominent profibrotic effects of TGF-β1 in renal fibrosis were shown in mice with inducible tubular-specific TGF-β1 activation. After four days of TGF-β1 activation, animals developed prominent interstitial fibrosis, proliferation of interstitial fibroblasts, tubular dedifferentiation and autophagy.[7] However, knock-out of TGF-β1 in mice results in a fatal multifocal autoimmune syndrome. [8] The prominent regulatory effects of TGF-β1 on the immune system might therefore hinder the clinical use of complete and long-term blockade of TGF-β1 in patients.

As one of the most important intracellular proteins that transduce extracellular signals from TGF-β to the nucleus where they activate downstream gene transcription, the Smad family has also been well investigated in its role in renal fibrosis. Overexpression of Smad7, which acts as an inhibitory factor in the TGF-β signaling pathway, could ameliorate renal fibrosis in the unilateral ureteral obstruction (UUO) model. [9] Key members of the Smad family are Smad2 and 3, mediating TGF-β signaling. Smad2 may also limit fibrosis in some cases via inhibition of TGF-β1/Smad3 signaling.[10] However, it should be noted that TGF-β is most likely one of many factors involved in fibrogenesis, acting with other molecules. Inhibition of TGF-β reduces renal fibrosis in animal models to a similar extent as studies inhibiting other single factors.

### *1.2 Mesangial Cells*

Mesangial cells are specialized cells around capillaries glomeruli in the kidneys, forming the mesangium. They are specialized smooth muscle cells that function to regulate blood flow through the capillaries. Mesangial cells constitute the central stalk of the glomerulus and are in continuity with the extraglomerular mesangium and the juxtaglomerular apparatus.[11] When the glomeruli are normally functioning, the mesangium tightly controls the amount of mesangial matrix synthesis. However, during kidney disease matrix synthesis could be considerably increased.[11]

### *1.3. Angiotensin II*

1) Introduction:

Angiotensin II (ANGII) is a pleiotropic hormone that influences the function of many cell types and regulates many organ systems. The diverse actions of ANGII are mediated via AT1R and AT2R receptors, which further mediate many signaling molecules, including small G proteins, phospholipases, mitogen-activated protein kinases (MAPK), phosphatases, tyrosine kinases, NADPH oxidase, and transcription factors. MC expresses both AT1R and AT2R. Increasing evidence shows that ANGII plays a key role in chronic disease, particularly in hypertensive and diabetic nephropathy. [12] The blockade of ANGII with ANGII-converting enzyme inhibitors and/or ANGII type 1 (AT1R) receptor antagonists slows the progression of both experimental and human kidney diseases[13].

2) ANGII signaling pathways and matrix synthesis:

Once ANGII binds to the AT1R, it activates a series of signaling cascades which in turn regulate the various physiological effects of ANGII. Traditionally, the pathways induced by ANGII have been divided into two classes: G protein-related and non-G protein-related signaling. [14] G protein related pathways start with activation of the AT1R and subsequent activation of downstream effectors including phospholipase C (PLC). Activation of PLC produces inositol-1, 4, 5-triphosphate (IP3) which binds to its receptor, allowing calcium efflux into the cytoplasm. Non-G protein-related signaling includes NAD (P)H oxidase and ROS signaling . It is now understood that ANGII mediates renal fibrosis by stimulating endogenous synthesis of transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF).[15] It was shown that the downstream TGFβ mediator Smad3 plays an essential role in ANGII–mediated renal fibrosis, and ANGII–induced tubular CTGF and collagen I mRNA and protein increases were regulated positively by activated Smad2/3, but negatively by Smad7. It was suggested that targeting Smad3 may have therapeutic

potential for hypertensive nephropathy. [16]

Stimulation of the ANGII type 1 receptor (AT1R) in murine mesangial cells could result in activation of the EGF receptor with subsequent signaling through PI3 kinase and MAP kinase, thereby regulating TGF-β mRNA levels. It was suggested that AT1R signaling through EGFR may serve as a therapeutic target to inhibit the development of CKD.[17]

The other pathway of ANGII-induced hypertrophy and fibronectin accumulation that has been well investigated is through PDK-1 (3-phosphoinositide-dependent protein kinase-1) kinase activation. Inhibiting NADPH oxidase 4, Src, or PDK-1 prevents the stimulatory effect of ANGII on fibronectin accumulation.[18]

### *1.4 SREBP*

1) Background:

The sterol regulatory element binding protein (SREBPs) family is a group of transcription factors that regulate lipid homeostasis by controlling the activation of a range of enzymes required for endogenous cholesterol, fatty acid (FA), triacylglycerol and phospholipid synthesis. As different isoforms of this family, SREBP-1a, SREBP-1c and SREBP-2 all have different roles in lipid synthesis. [19]

SREBP-1 associates with and forms a complex with another ER membrane protein, the SREBP-cleavage activating protein (SCAP). SCAP binds to another ER membrane protein, Insig. When cells become loaded with cholesterol, this will increase the concentration of cholesterol in the ER membrane. This leads to a conformational change in SCAP through direct cholesterol binding to its sterol-sensing domain and induces SCAP detachment from Insig.[20] The SREBP/SCAP complex is then transported to the Golgi apparatus. In the Golgi apparatus, SREBP will first be cleaved by site-1 protease (S1P), a membrane-bound serine protease. After S1P cleavage, a second membrane-bound protease, site-2 protease (S2P), clips the N-terminal domain and releases the active transcription factor from the membrane[20]**Fig. 1**. My data show that ANGII activates SREBP-1 in a time and dose-dependent manner. The purpose of this study was to investigate how ANGII

activates SREBP-1 and its role in the MC fibrotic response.

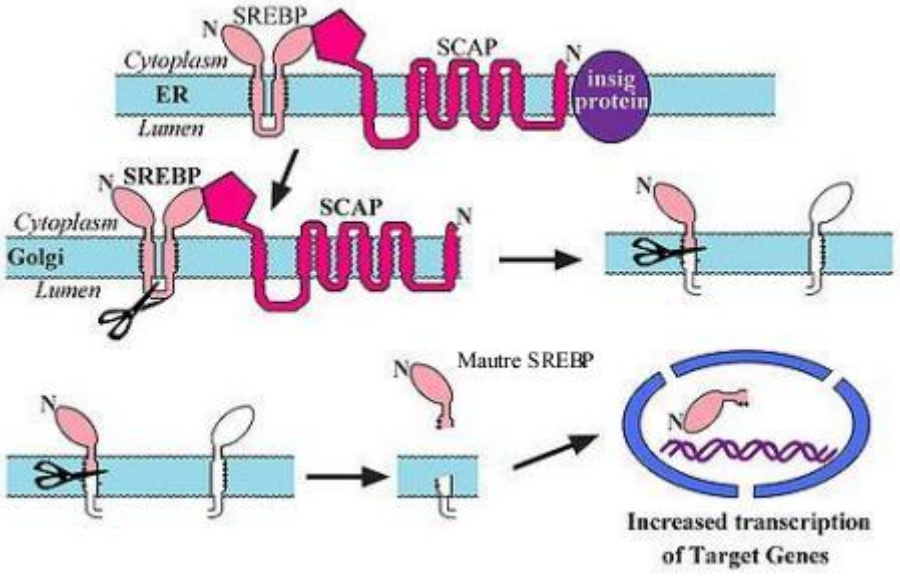


**Fig.1 Activation of SREBP**

The activation of SREBP started with SREBP/SCAP complex combing with Insig on ER. When blood cholesterol increase, SCAP detach from Insig and translocate to Golgi and S1P and S2P proteinase will cleave mature SREBP (mSREBP) from SCAP and mSREBP will tanslocate to nuclease to mediate transcription its target gene

2) SREBP and glomerular fibrosis:

The involvement of SREBP-1 in kidney disease has been investigated in transgenic mice with PEPCK-driven activation of SREBP-1, as well as mice with obesity-related diabetes. The activation of SREBP-1c in kidney has been associated with increased activation of profibrotic growth factors, enhanced mesangial expansion with accumulation of extracellular matrix proteins, and proteinuria.[21,22] It was suggested that SREBP-1c could contribute to kidney disease through lipotoxicity. Thus, without hyperglycemia or systemic dyslipidemia, the increased activation of SREBP-1c in the kidney could also result in lipid accumulation and increased activation of TGF-β, plasminogen activator inhibitor (PAI)-1, and vascular endothelial growth factor (VEGF). It was suggested that such signaling could mediate renal hypertrophy, accumulation of extracellular matrix proteins, and mesangial expansion, resulting in glomerulosclerosis and proteinuria.

3) SREBP regulation by PI3K/Akt Signaling:

It has been well established that ANGII could activate phosphoinositide 3-kinase (PI3K)/Akt signaling to mediate different biological functions such as promotion of cardiac hyperthrophy.[23,24] The AT1R regulates PI3K transduction of extracellular signals.[25] PI3K was shown to mediate SREBP-1c transcriptional activity in rat hepatocytes.[26] As one of the most important upstream regulator of Akt activation, PI3K's role in mediating ANGII-induced SREBP activation is unclear. [27] The relationship between ANGII and PI3K/Akt signaling pathway and its function in mediating SREBP1-c activity are investigated in this work.

### *1.5 ER Stress:*

1) Overview of ER stress

The endoplasmic reticulum (ER) functions to fold and modify lipids and proteins. In conditions such as depletion of ER $Ca^{2+}$ stores, disturbance of disulfide bond formation or through infection, protein misfolding and subsequent protein aggregation may result. The accumulation of misfolded and unfolded proteins could induce the ER stress response through the Unfolded Protein Response (UPR) pathway. [28] There are three arms of the UPR **Fig.2**. The first is protein kinase RNA (PKR)-like ER kinase double-stranded RNA-dependent protein kinase-like ER kinase (PERK), which is a transmembrane protein that has a structure that includes an ER luminal stress-sensing domain that binds GRP78, and a cytosolic kinase domain.[29] PERK induces phosphorylation of eukaryotic translation initiation factor α (eIF2α) and activates activating transcription factor-4 (ATF4). Phosphorylated-eIF2α (eIF2α) could further activate NFκB, although the exact mechanism remains unclear [30,31]

The stress inositol-requiring 1α (IRE1α)/XBP-1 pathway could activate MAPK (JNK), NF-κB and apoptosis signal-regulating kinase (ASK1), which is further involved in autophagy and the apoptotic pathway. [32,33,34,35,36] ATF6 is the third ER stress sensor which is bound as an inactive precursor in the ER membrane. The cleavage of activating transcription factor-6 ATF6 occurs after its transportation from

ER to Golgi during the UPR. Site 1 protease (S1P) and site 2 protease (S2P) are both involved in this process. The product of ATF6 cleavage, the cytoplasmic bZIP domain, could translocate to the nucleus and activate the transcription of target genes [37,38.]

It is now understood that in unstressed cells the three proteins that mediate the downstream function of ER stress, IRE1α, PERK and ATF6 are all maintained in an inactive state via their association with the ER protein chaperone glucose regulated protein 78/ immunoglobulin heavy chain-binding protein (GRP78). During UPR, GRP78 will be released and bind to unfolded proteins, allowing activation of PERK, IRE1α and ATF6. GRP78 therefore is the major mediator of ER stress initiation.

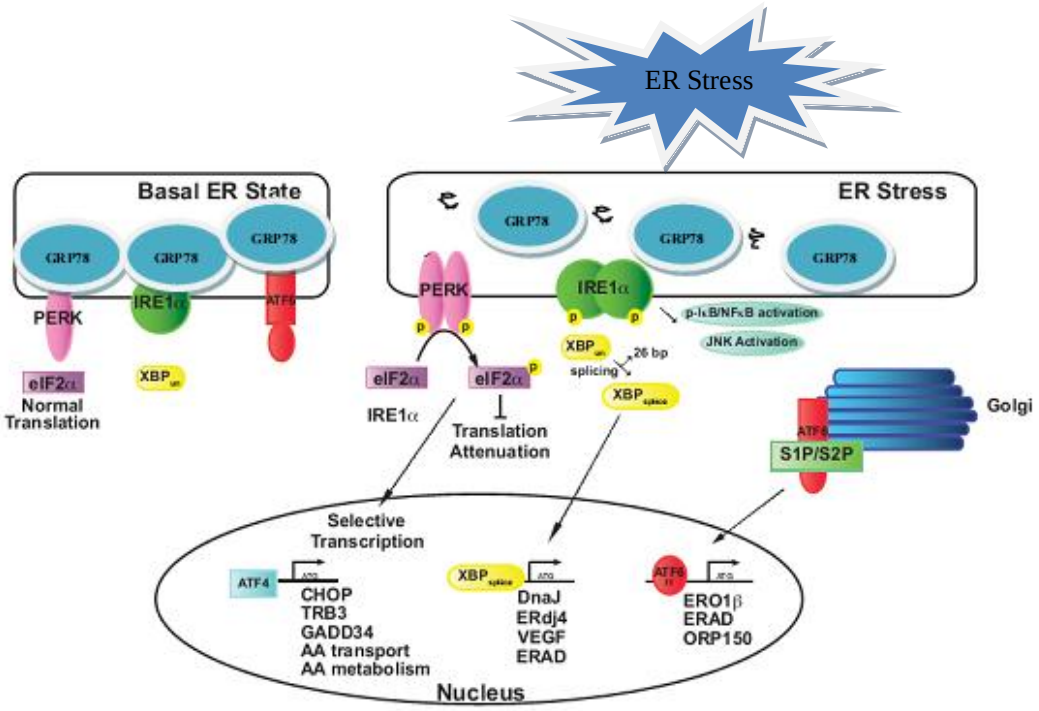


**Fig.2 ER stress signaling pathway**

When ER stats with its basal stage, GRP78 associate with PERK, IRE1α and ATF4, and maintain all of them inactive. When ER stress induced by certain disease condition

2) SREBP and ER stress:

When ER function is abnormal, a series of downstream signaling pathways can be triggered, including apoptosis and lipid dysregulation.[39404142] Research studying the relationship between ER stress and SREBP activation began with studies on homocysteine, a thiol-containing amino acid. It had been found in human vascular endothelial cells that homocysteine-induced ER stress could induce SREBP upregulation and increase the risk of cardiovascular disease. [4344] Furthermore, The activation of SREBP is associated with increased activation of genes responsible for cholesterol/triglyceride biosynthesis and uptake and with intracellular accumulation of cholesterol[45]. In order to investigate if ER stress has a direct effect on SREBP activation, the ER chaperone GRP78/BiP was overexpressed. It inhibited homocysteine-induced SREBP activation, which suggests a direct involvement of ER stress in SREBP activation.[45] In accordance with this, in vivo studies showed that cholesterol and triglycerides were significantly increased in the livers in animal models with diet-induced hyperhomocysteinemia, and this was associated with UPR and SREBP activation. [45]

There has been evidence that ER stress could induce SREBP-2 activation in HeLa and MCF7 cell lines, and such activation is dependent on the conventional S1P/S2P proteolytic pathway. [46] The exact mechanism of how ER stress induces SREBP activation remains unclear. However, potential hypotheses have been proposed. One is that GRP78 dissociation from the SCAP-SREBP complex upon ER stress allows its transportation to Golgi and further activation by the proteases S1P and S2P. Another possibility is that phosphorylation of eIF2α as a consequence of ER stress could degrade Insig1 protein levels, allowing the SCAP-SREBP complex to be transported to the Golgi. The third possibility is that caspases could cause the cleavage of SREBP from the ER membrane.[47]

## II STATEMENT OF HYPOTHESIS

Better understanding ANGII profibrotic signaling will be important in finding a potential target for the therapy of renal fibrosis. The hypothesis of this study is that SREBP-1 is an important mediator of ANGII-induced fibrogenic responses in MC, and PI3K/Akt signaling and ER stress have direct involvement **Fig 3**. These may thus be new potential targets for the treatment of renal fibrosis.

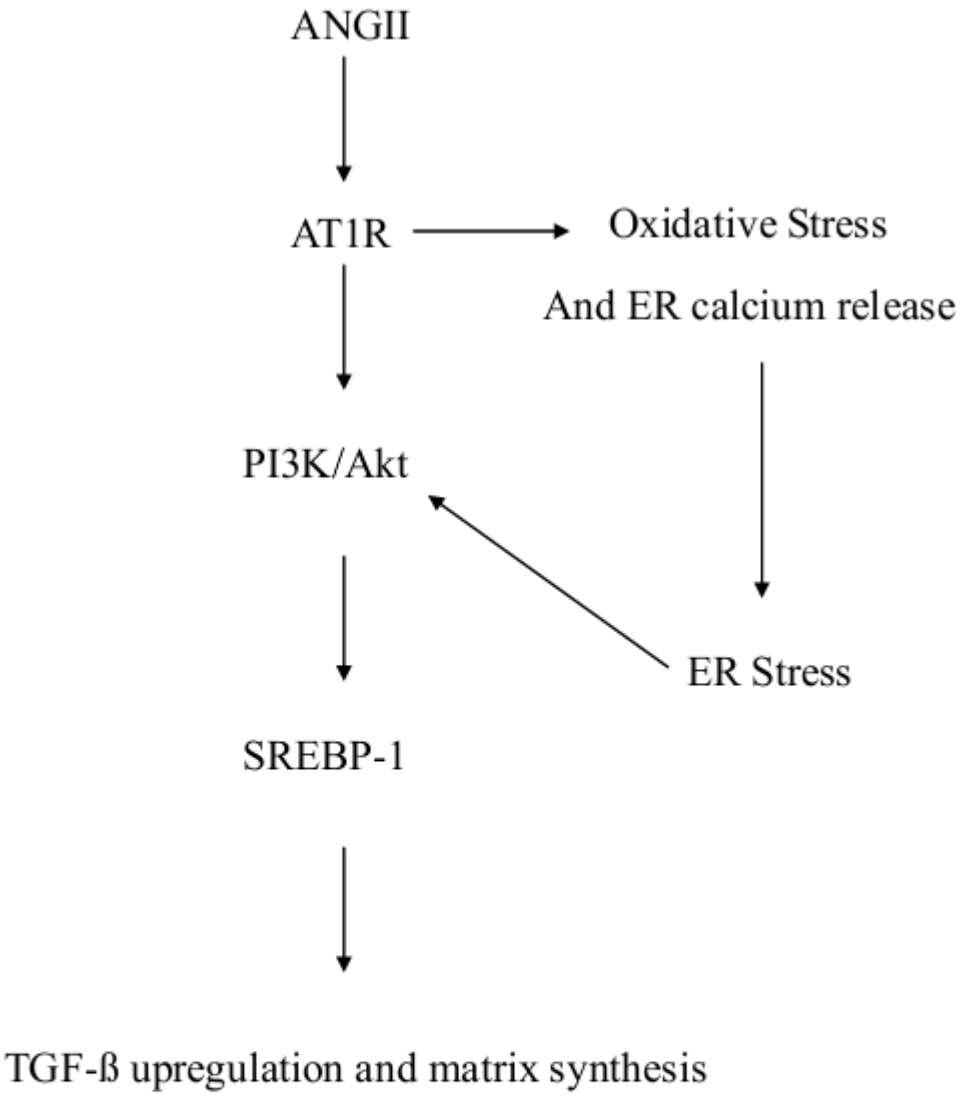


**Fig.3 Postulated mechanism of SREBP-1 activation by Ang II in MC**

In this study we demonstrated ANGII could induce TGF- ß upregulation through SREBP-1 activation, and AT1R is the key receptor. Through AT1R ANGII could induce oxidative stress and ER calcium release which can cause ER stress. ER stress could induce PI3K/Akt activativation which can also be induced directly by ANGII. This signalling pathway eventually leads to TGF- ß upregulation and matrix synthesis.

## III. METHODS

### *2.1. Mesangial Cell Culture*

Glomeruli were obtained from Sprague Dawley rats by differential sieving, and primary rat MCs obtained by selection of MC from glomerular outgrowths. MCs were characterized by their stellate appearance, rapid outgrowth in restricted medium and through PCR, the presence of vimentin and absence of Von Willenbrand factor and cytokeratins. MC were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum, streptomycin (100 μg/ml) change all ug and umol to the micro symbol and penicillin (100 units/ml) at 37 ℃ in 95% air, 5% CO2. Cells between passages 10 and 20 were used. For all experiments, cells were serum deprived at confluence 24hours before ANGII (100nM 3h) treatment.

### *2.2. Protein Extraction/Western Blots*

Cells were lysed and protein extracted using cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 2 mM DTT, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors added prior to use: 1 μg/ml leupeptin, and 2 ug/ml aprotinin and 25 μg/ml ALLN. Cell lysates were centrifuged at 4 ℃, 14,000 rpm for 10 min to pellet cell debris. Supernatant which contains soluble proteins was diluted in 5x reducing sample buffer, boiled for 5 min, and 50 μg was separated on SDS-PAGE and Western Blots were performed. Antibodies used were monoclonal SREBP-1 (1:500; Santa Cruz), monocolonal GRP78 (1:1000; BD Transduction), monoclonal phospho-eIF2α (1:1000; Cell Signaling), monoclonal CHOP (Santa Cruz 1:200), polyclonal phosphoAkt S473 (1:1000 Cell Signaling ) and monoclonal actin (1:5000; Sigma). After transfer, nitrocellulose membranes were blocked for 1 h at room temperature with TBST with 5% nonfat dry milk and incubated with the primary antibody overnight at 4 ℃ in

TBST with 5% bovine serum albumin. Membranes were washed three times with TBST and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase in TBST with 5% milk. Antibody was detected by enhanced chemiluminescence (Millipore).

### *2.3. SRE-GFP Transfection Assay*

MC between passages 10-13 were transiently transfected using Effectene (Qiagen). with green fluorescent protein (GFP) reporter plasmid under control of the sterol regulatory element (SRE) SRE-GFP (obtained from Dr. R. Austin) for 16 hours. Medium was then changed for 8 hours after which cells were serum deprived for 16 hours. MC were then treated with ANGII for 1 or 3 h and cells were lysed with lysis buffer (pH 7, 0.2N HCl with 200ul 2N NaOH). GFP was detected by fluorometer with excitation at 475 nm and emission at 510 nm. Readings were normalized to protein concentration.

### *2.4. Luciferase Assay:*

At 85% subconfluency, MC were transfected with 0.5 μg of TGFβ1 promoter-luciferase construct (kindly provided by Dr. N. Kato) and 0.05 μg pCMV-β-galactosidase (β-gal) (Clontech) using Effectene (Qiagen). MC were then serum deprived for 24 hours followed by drug treatment. Cells were lysed with Reporter Lysis Buffer (Promega, Madison, WI) using one freeze-thaw cycle, and luciferase and β-gal activities measured on clarified lysate using specific kits (Promega) with a Berthold luminometer and a plate reader (420nm) respectively. Beta-gal activity was used for normalizing transfection efficiency

### *2.5. Animal Studies:*

Animal studies were carried out in accordance with McMaster University and Canadian Council on Animal Care guidelines. Eight 7-8 week old C57BL/6 male mice underwent right uninephrectomy. After two weeks of recovery, four were implanted with a minipump (Alzet micro-osmotic pump 1007D) with ANGII (Sigma) at 1.5 ng/min/gram body weight for 7 days. The remainder received a minipump with

vehicle (saline). Mice were sacrificed after 7 days, kidneys extracted and analysed by immunohistochemistry.

### *2.6. Immuohistochemistry*

Kidney sections with paraffin were incubated with xylene for 10 min 3 times, 100% ethanol for 3 min 3 times and then incubated with endogenous peroxidase for 10min. Heat antigen retrieval was carried out, followed by incubation with blocking serum in TBS for 15 min. Sections were then incubated with primary antibodies SREBP-1 (Abcam ab44153 rabbit 5μg/ml) or GRP78 (BD Transduction 1:40) for 2 hours, followed by appropriate biotinylated secondary antibody for 30 mins. Slides were washed well with TBS 2 times and incubatedwith Streptavidin/Peroxidase for 10 min. After incubation they were washed once with TBS followed by one wash with water. Slides were then incubated with Nova Rad (Vector Labs) until a brown color appeared, after which slides were dipped into water. They were then stained with Gill hematoxylin for 30 seconds for nuclear staining, washed 3 times with water, then coverslipped with permount.

### *2.7. Statistical Analysis*

Statistical analysis was carried out using SPSS Statistics 17.0 for windows. We used one-way ANOVA and Tukey's honestly significant difference for protein expression analysis. A P value<0.05 (2-tailed) was considered significant. Data are presented as mean±SEM, and the number of repetitions is denoted as *n*.

## IV. Results

### *4.1. Define the time course and dose response of ANGII-induced SREBP-1 activation.*

First, we wanted to investigate the time course of SREBP-1 activation under the treatment of ANGII using the common dose of 100 nM, at the times 1, 3 and 6 hours. As shown in **Fig. 4,** SREBP-1 activation was significantly induced at 3 hours. In order to define the dose course of ANGII-induced SREBP-1 activation, I extended the ANGII treatment dose from 50nM to 1000nM. As shown in **Fig. 5,** SREBP-1 activation significantly increased at 50nM. Subsequent studies (not shown) showed a more reliable induction at 100nM, and this dose was used for further studies. These results confirmed that SREBP-1 activation could be induced by ANGII in both a time and dose dependent manner. ANGII 100nM treatment for 3hours was used in the subsequent experiments.

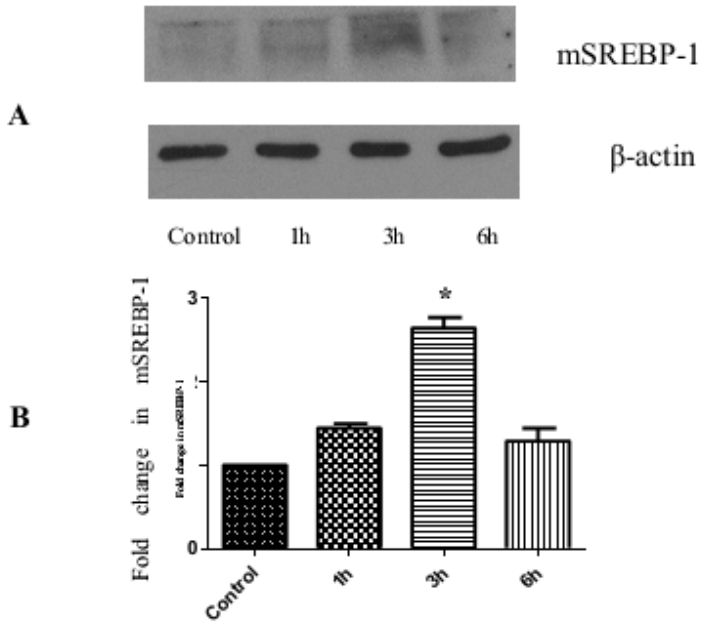


**Fig.4 Ang II induced SREBP-1 activation in a time dependent manner**
MC were treated with Ang II (100nM for 1h, 3h, and 6h). mSREBP-1 activation was tested by Western blot, with β-actin as a loading control (A). Quantitative result of (A) is shown in (B) * represents significant difference between 3hours and Control. *P<0.05*

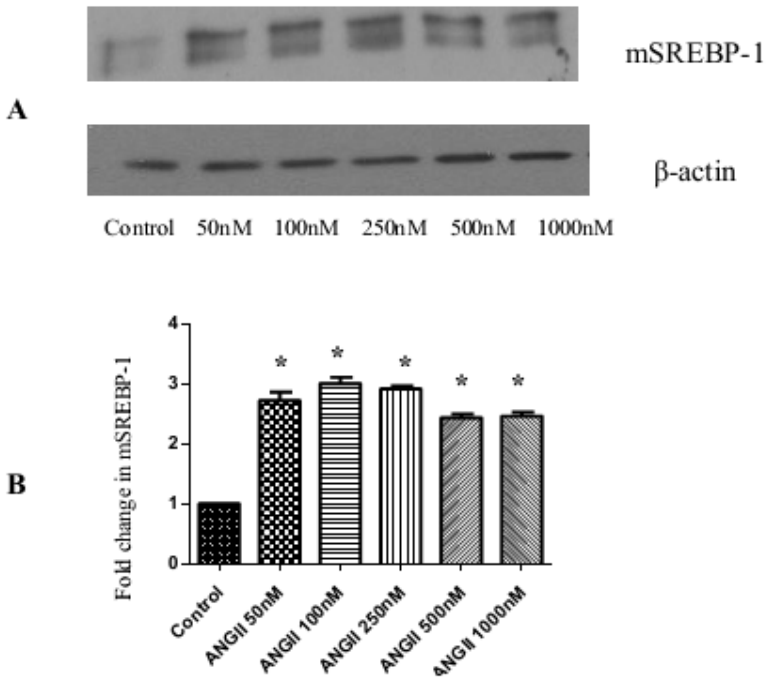


**Fig.5 Ang II induce SREBP-1 activation in dose dependent manner**

MC were treated with Ang II (50nM, 100nM, 250nM, 500nM, 1000nM for 3h). SREBP-1 activation was tested by Western blot with β-actin as a loading control (A) Quantitative result of (A) are shown in (B) * represents significant difference between drug treatment group and Control. *P<0.05 n=4*

To confirm SREBP-1 DNA binding functionality, We used a green fluorescent protein (GFP) reporter plasmid under control of the sterol regulatory element (SRE) (obtained from Dr. R. Austin). MC were transiently transfected with SRE-GFP for 16 hours, and after 24 hours of serum deprivation cells were treated with ANGII for 1 and 3 hours. The degree of fluorescence represents activated SREBP-1 driving GFP transcription. As shown in **Fig. 6** the fluorescence quantity increased close to 2 -fold after 1 hour of treatment with ANGII and 2.5-fold after 3 hours. This result suggests that in response to ANGII, mSREBP-1 is fully functional and able to bind to its SRE promoter regions. To confirm that GFP protein is expressed in SRE-GFP transfected MC, we performed a western blot with a GFP antibody to test its protein expression. As seen in **Fig.7**, MC from the SRE-GFP transfected group express green florescent protein whereasthe untransfected group do not.

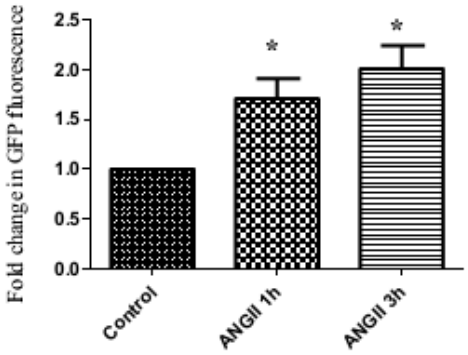


**Fig.6 ANGII-induced SREBP-1 DNA biding function**

MC were transiently transfected using Effectene with SRE-GFP, then treated with ANGII (100nM for 1h and 3h). GFP was detected by fluorometer with excitation at 475nm and emission at 510nm.*represents significant difference between ANGII 1h or 3h and Control. *P<0.05 n=3*

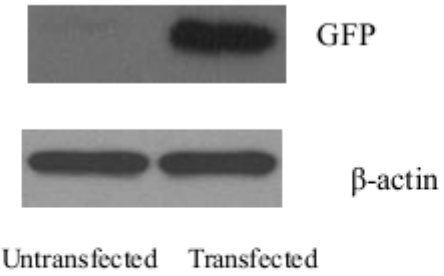


**Fig.7 ANGII induced SRE-GFP GFP protein expression**

MC were transiently transfected with or without SRE-GFP, and then treated with ANGII (100nm for 3h). GFP was detected by western blot with β-actin as a loading control.

### *4.2. (A) Identify signaling pathways by which ANGII induces SREBP-1 activation*

SCAP is one of the most important protein chaperones which assist in SREBP-1 transport to the Golgi apparatus in the classic SREBP-1 maturation process.[20] Fatostatin has been shown to efficiently block SCAP function. Here we asked if ANGII-induced SREBP-1 activation requires the classic SREBP maturation process. We pretreated MC with Fatostatin (20μM, 4hours) to inhibit SCAP, followed by ANGII treatment. We found that MC pretreated with Fatostatin express significantly less mSREBP-1 compared with the control group **Fig. 8,** suggesting that SCAP is required for SREBP-1 activation. We also performed the SRE-GFP assay to investigate if SCAP is required for SREBP-1 DNA binding activity. We repeated the

same SRE-GFP fluorescence quantification after pretreatment of MC with Fatostatin for 20μM, 4 hours followed by ANGII for 3 hours. As shown in **Fig. 9,** SREBP-1 DNA binding activity is significantly blocked by pretreatment with Fatostatin compared with the ANGII-treated group. This result further confirmed mSREBP-1 induced by ANGII requires the classic SREBP maturation process.

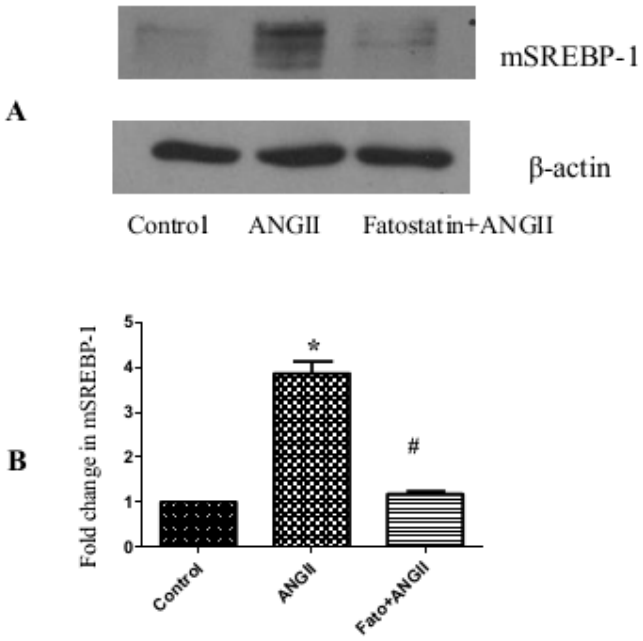


**Fig.8 ANGII-induced SREBP-1 activation was blocked by Fatostatin**

MC were treated with Ang II (100nM for 3h) with or without Fatostatin pretreatment (20μM 4h). mSREBP-1 activation was tested by Western blot with β-actin as a loading control (A) Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control, # represents significant difference between Fatostatin+ANGII and ANGII. *P<0.05 n=4*

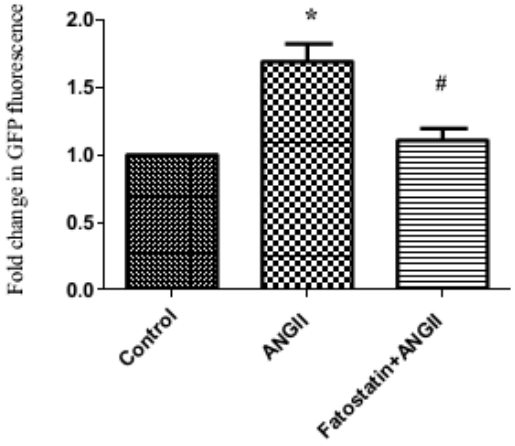


**Fig.9 Fatostatin blocks ANGII-induced SREBP-1 DNA biding Function**

MC were transiently transfected with Effectene with SRE-GFP, and then treated with ANGII (100nM for 3h) with or without Fatostatin pretreatment (20μM 4h). GFP was detected by fluorometer with excitation at 475nm and emission at 510nm. * represents significant difference between ANGII and Control, # represents significant difference between Fatostatin + ANGII and ANGII. $p<0.05$ $n=3$

AEBSF or 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride is a serine protease inhibitor which can significantly block the activation of proteases includingS1P. Because SREBP maturation requires the proteases S1P and S2P, here we used AEBSF 500 μM for 1hour pretreatment followed by ANGII 100nM 3h to determine whether S1P is required for ANGII-induced SREBP-1 activation. As can be seen from **Fig. 10,** AEBSF inhibited SREBP-1 activation. Thesedata suggest that S1P (and S2P) are required.

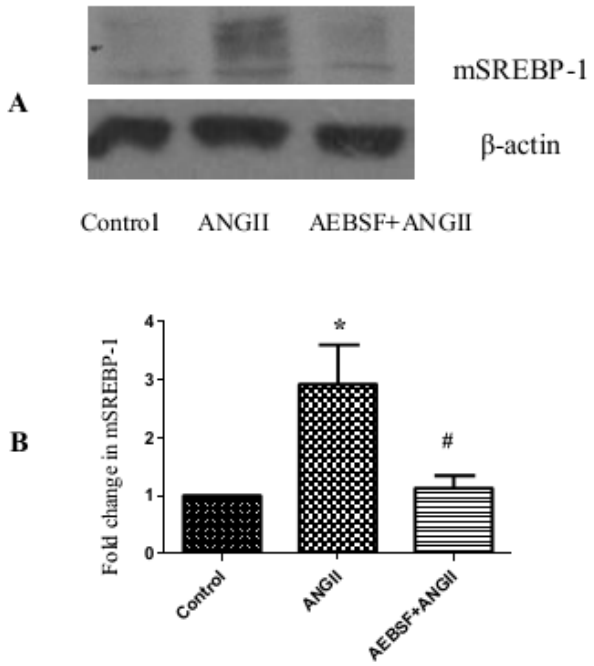


**Fig.10 ANGII-induced SREBP-1 activation is blocked by AEBSF**

MC were treated with Ang II (100nM for 3h) with or without AEBSF pretreatment (500μM for 1h). mSREBP-1 activation was tested by Western blot with β-actin as loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control, # represents significant difference between AEBSF+ ANGII and ANGII . *P<0.05 control n=4*

The AT1R transduces the physiological effects of ANGII. We used the AT1R inhibitor Losartan 10 μM 30min to pretreat MC followed by ANGII 100nM 3h treatment. As can be seen from **Fig.11,** Losartan blocks ANGII-induced SREBP-1 activation. This result suggested that ANGII-induced SREBP-1 activation is mediated by the AT1R.

As a very important mediator of ANGII responses, the EGFR has been investigated as a downstream mediator of AT1R signaling. We assessed if EGFR is also involved in ANGII-induced SREBP-1 activation. MC were treated with AG1478, an EGFR inhibitor, 1 μM for 30minutes, followed by ANGII treatment. As shown in **Fig. 12** AG1478 did not have a significant effect on ANGII-induced SREBP-1 activation, excluding a role for the EGFR in ANGII-induced SREBP1 activation.

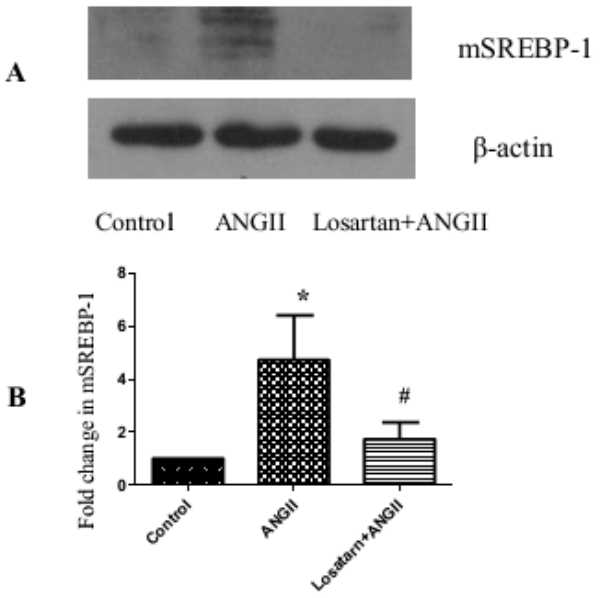


**Fig.11 ANGII-induced SREBP-1 activation is blocked by Losartan**

MC were treated with ANGII (100nM for 3h) with or without Losartan pretreatment (1μM 30min). mSREBP-1 activation was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control, # represents significant difference between Losartan +ANGII and ANGII. *P<0.05 n=3*

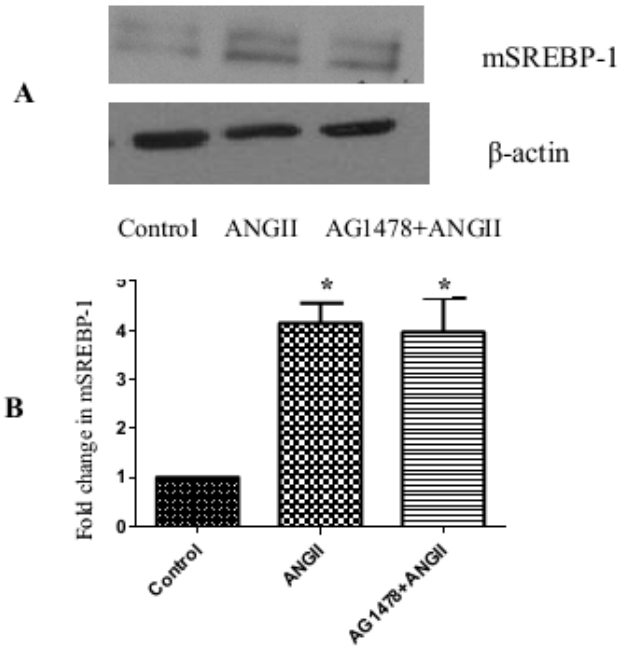


**Fig.12 ANGII-induced SREBP-1 activation is not blocked by AG1478**

MC were treated with ANGII (100nM for 3h) with or without AG1478 pretreatment (1μM 30min). mSREBP-1 activation was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII or AG1478+ANGII and Control. *P<0.05 n=3*

Another important signaling response to ANGII is PI3K/Akt activation. Because PI3K is upstream of Akt, we fist evaluated if PI3K is involved in ANGII-induced SREBP activation using two different inhibitors, LY294002 and wortmannin. MC were treated with LY294002, 20μM for 30 minutes or wortmannin 100nM for 1hour, followed by ANGII. As shown in **Fig.13** both LY294002 and wortmannin significantly suppressed ANGII-induced SREBP-1 activation which suggests that PI3K plays an important role.

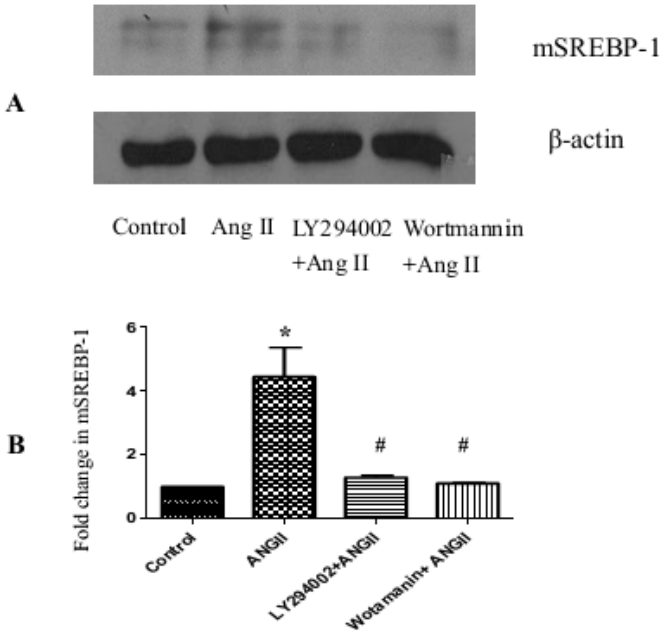


**Fig.13 ANGII-induced SREBP-1 activation is blocked by LY294002 and Wortmannin**
MC were treated with Ang II (100nM for 3h) with or without LY294002 (20μM 30min) or Wortmannin (100nM 1h) pretreatment. mSREBP-1 activation was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control, # represents significant difference between LY294002+ANGII or Wortmannin+ANGII and ANGII. *P<0.05 n=4*

We also used the Akt inhibitor VIII to block Akt activation to determine whether Akt is involved in ANGII-induced SREBP-1 activation. As can be seen from **Fig.14**, the Akt inhibitor significantly blocks ANGII-induced SREBP-1 activation, indicating that Akt is required for SREBP-1 activation by ANGII.

PI3K could also activate the mammalian Target of Rapamycin (mTOR) through Akt activation. To assess whether mTOR is also required for SREBP-1 activation, we pretreated MC with the mTOR inhibitor Rapamycin followed by ANGII treatment. As

seen in **Fig. 15** mTOR inhibition could not suppress ANGII-induced SREBP-1 activation, suggesting that mTOR is not involved in ANGII-induced SREBP activation.

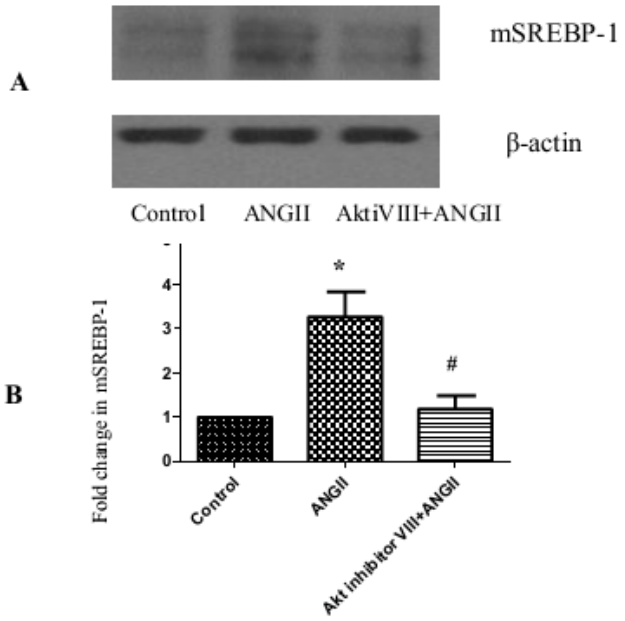


**Fig.14 ANGII-induced SREBP-1 activation is blocked by Akt inhibitor VIII**

MC were treated with Ang II (100nM for 3h) with or without Akt inhibitor VIII (10μM 1h) pretreatment. mSREBP-1 activation was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control, # represents significant difference between AKT inhibitor VIII and ANGII. *P<0.05 n=3*

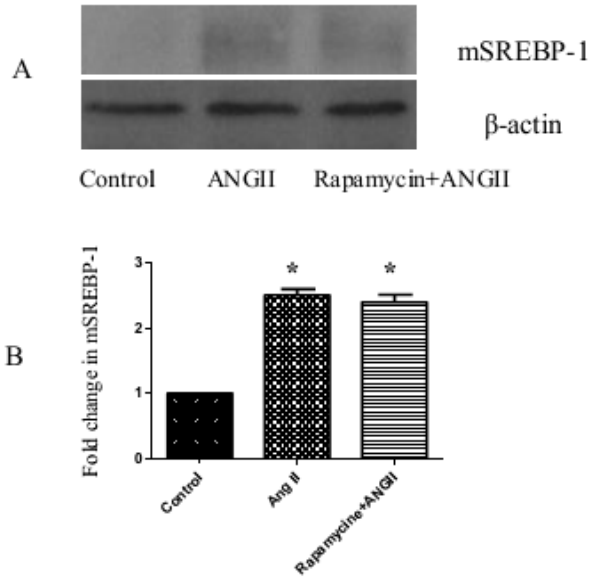


**Fig.15 ANGII-induced SREBP-1 activation is not blocked by Rapamycin**

MC were treated with Ang II (100nM for 3h) with or without Rapamycin (20ng/ml 1h) pretreatment. mSREBP-1 activation was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII or Rapamycin +ANGII and Control. *P<0.05 n=3*

### *4.2. (B) Investigate the role of ER stress in ANGII-induced SREBP-1 activation*

(1) ANGII induces ER stress

Previous studies have shown that ER stress could induce SREBP-1 activation.[48] Interestingly, there is also a study showing that ANGII could induces ER stress, potentially through increased protein synthesis, leading to cardiac hypertrophy, in Wistar-Kyoto rats. [55] Here we wanted to see if ANGII could similarly induce ER stress in MC. MC were treated with ANGII 100nM from 30min to 6 hours. As shown in **Fig. 16,** the ER stress chaperone glucose regulated protein 78 (GRP78) increased in a time dependent manner. As one of the consequences of ER stress, phosphorylation of eIF2α is also an important indicator of ER stress. As shown in **Fig 16,** phospho-eIF2α is also induced by ANGII treatment. Another target gene of ER stress is C/EBP homologous protein (CHOP). This was also increased in response to ANGII.

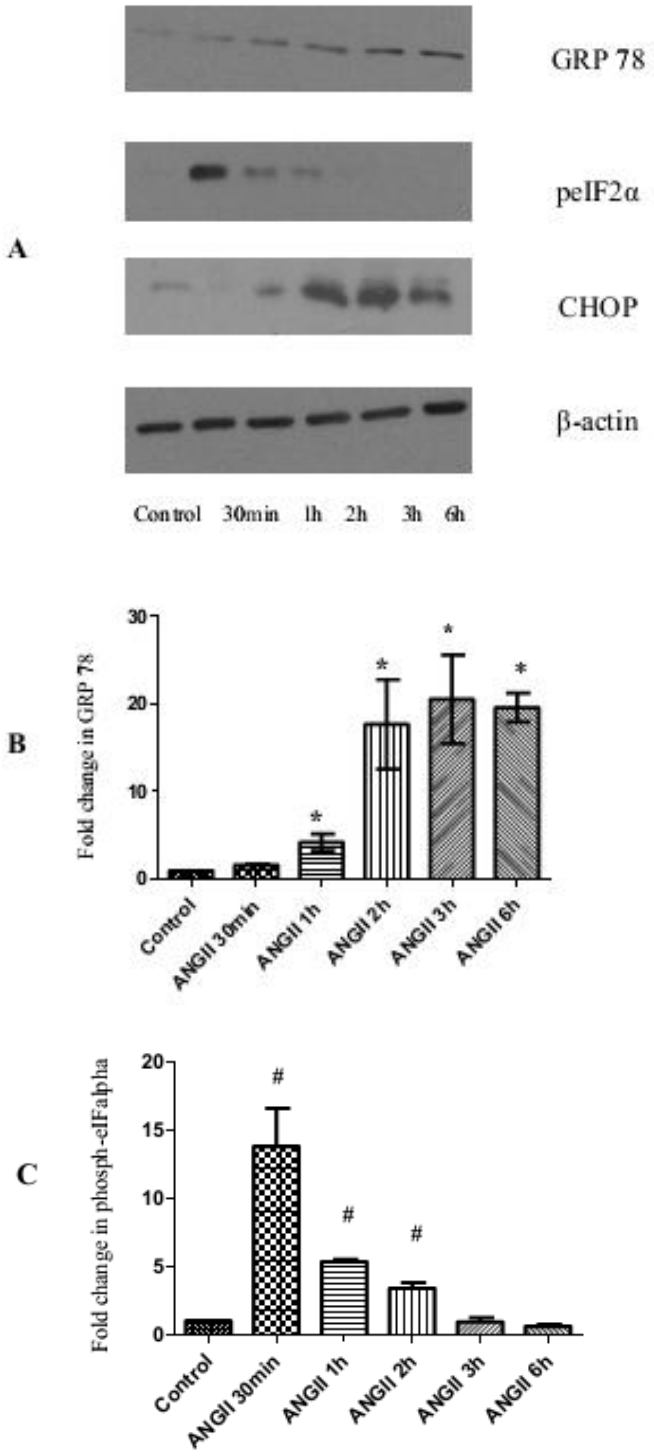


**Fig.16 Ang II induces ER stress in MC**

MC were treated with Ang II (100nM for 30mins, 1h, 3h, 6h.), GRP78, phospho-eIF2α, and CHOP were tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) and (C) * represents significant difference between ANGII 1h or 2h or 3h or 6h and Control, # represents significant difference between ANGII 30min or 1h or 2h and Control. $P<0.05$ $n=3$

(2) AT1R is required for ANGII-induced ER stress

In the previous result we showed that AT1R is required for ANGII-induced SREBP-1 activation. However, whether ANGII-induced ER stress is mediated by the AT1R is unknown. We thus pretreated MC with Losartan for 1hour followed by ANGII for 30 min (for peIF2α) or 3 hours (for GRP78). As shown in **Fig.17** and **Fig.18,** both GRP78 and pEIF2a induction by ANGII were blocked by AT1R inhibition, suggesting that the AT1R is necessary for ANGII-induced ER stress.

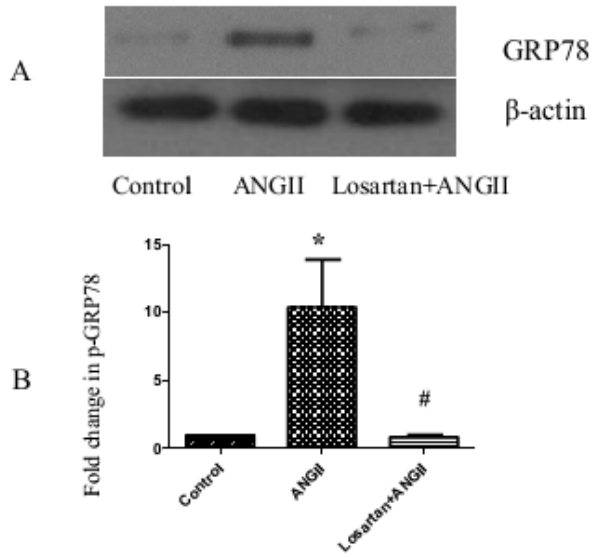


**Fig.17 ANGII-induced GRP78 upregulation is blocked by Losartan**

MC were treated with Ang II (100nM for 3h) with or without losartan pretreatment (1μM 30min). GRP78 was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control # represents significant difference between Losartan+ANGII and ANGII. *P<0.05 n=3*

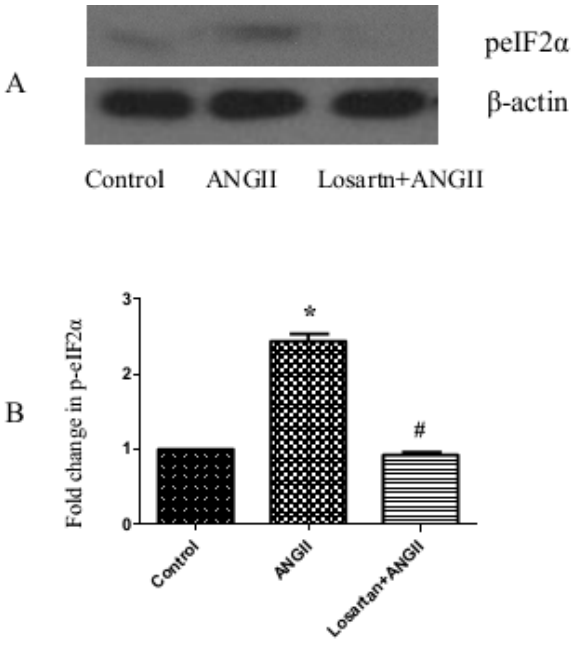


**Fig.18 ANGII-induced peIF2α upregulation is blocked by Losartan**

MC were treated with Ang II (100nM for 3h) with or without losartan pretreatment (1μM 30min). peαIF2a was tested by Western blot with β-actin as a loading control (A) Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control, # represents significant difference between Losartan+ANGII and ANGII. *P<0.05 n=3*

(3) ER stress is required for ANGII-induced SREBP-1 activation

Next we wanted to assess whether inhibiting ER stress could suppress the activation of SREBP-1 by ANGII. Here we used two different inhibitors of ER stress: chemical chaperones 4-phenyl butyric acid (PBA) and Salubrinal which have been shown to stabilize protein conformation and improve ER folding capacity, and to help transportation of mutant proteins to alleviate ER stress.[49] We used both chemical chaperones to see if ANGII- induced SREBP-1 activation could be blocked. As can be seen from **Fig.19** and **Fig.20,** 4-PBA significantly decreased SREBP-1 activation and Salubrinal had the same effect.

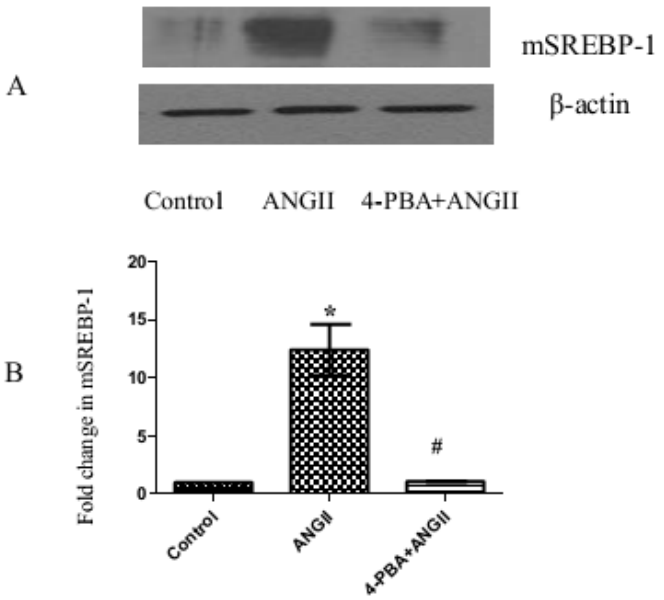


**Fig.19 ANGII-induced SREBP-1 activation is blocked by 4-PBA**

MC were treated with Ang II (100nM for 3h) with or without 4-PBA pretreatment (5mM 2h). mSREBP-1 activation was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B)* represents significant difference between ANGII and Control, # represents significant difference between 4-PBA+ANGII and ANGII. *$p<0.05$ $n=3$*

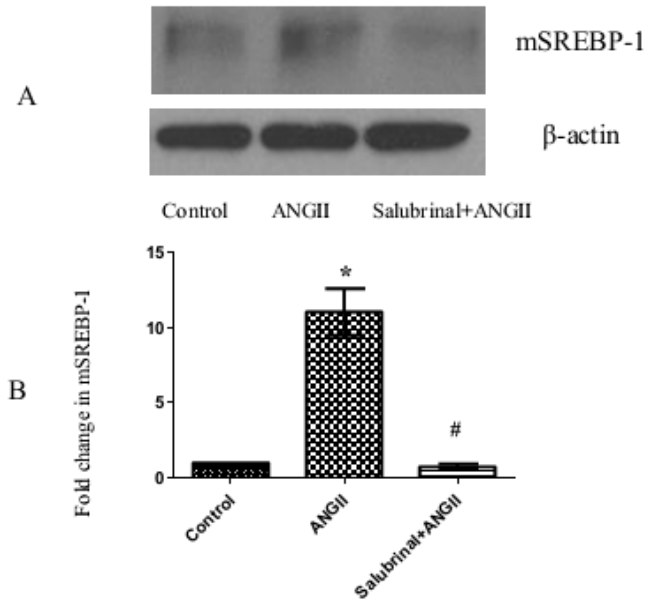


**Fig.20 ANGII-induced SREBP-1 activation is blocked by Salubrinal**

MC were treated with Ang II (100nM for 3h) with or without Salubrinal (100μg/ml, 1h)or Salubrinal (30μM, 1h) pretreatment. mSREBP-1 activation was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control, # represents significant difference between Salubrinal+ANGII and ANGII. *$p<0.05$ $n=3$*

(4) ER calcium release is important for ANGII-induced SREBP-1 activation

Studies have shown that ER calcium release could cause ER stress.[5051] In this study, we thus assessed if calcium release is required for ANGII-induced SREBP1 activation. We used TMB-8 [8-(NN-diethylamino)octyl-3,4,5-trimethoxybenzoate] which is an intracellular calcium antagonist and BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), a calcium-specific polyamino carboxylic acid that works as an intracellular calcium chelator and can protect cells against toxic calcium overload. MC were pretreated with these, followed by ANGII treatment. As can be seen from **Fig.21** they both significantly suppressed ANGII-induced SREBP activation, suggesting calcium release is needed to activate SREBP in response to ANGII.

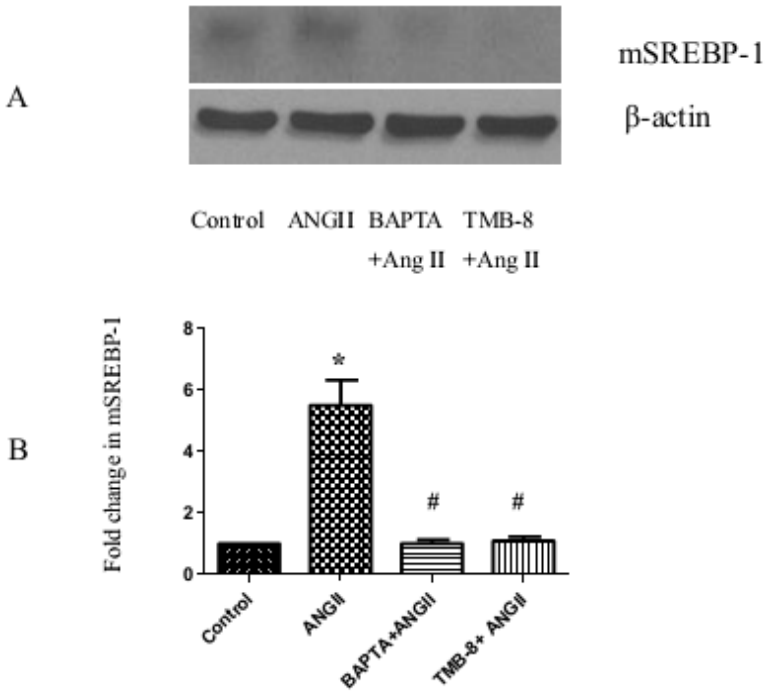


**Fig.21 ANGII-induced SREBP-1 expression is blocked by BAPTA and TMB-8**

MC were treated with Ang II (100nM for 3h) with or without BAPTA (30μM 1h)or TMB-8 (100μM 1h )pretreatment. mSREBP-1 activation was tested by Westem blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control,# represents significant difference between BAPTA+ANGII or TMB-8+ANGII and ANGII. $P<0.05$, $n=3$

Exposure of cells to oxidative stress could also activate ER stress,[52] and oxidative species can also release ER calcium. Here we used N-acetyl-L-cysteine (NAC), which is an antioxidant known for its ability to alleviate oxidative stress. We pretreated MC with NAC followed by ANGII treatment. As can be seen from **Fig.22** NAC significantly blocked ANGII-induced SREBP activation, indicating that oxidative stress is involved.

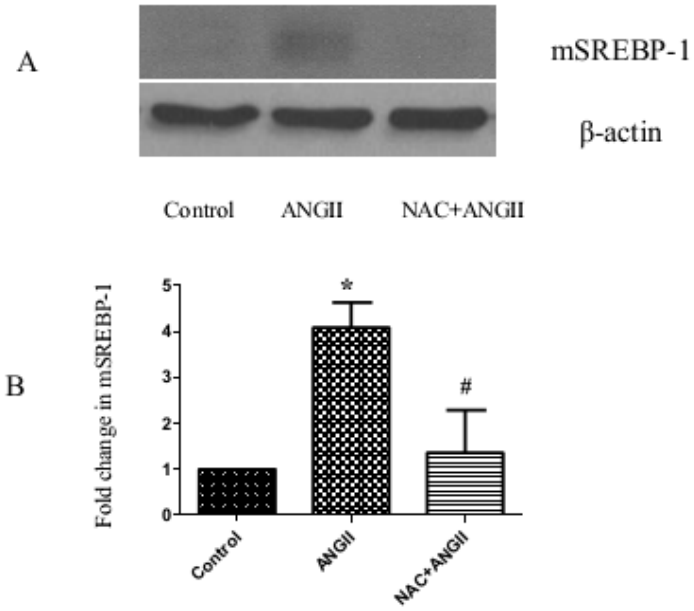


**Fig.22 ANGII-induced SREBP-1 expression is blocked by NAC**

MC were treated with Ang II 100nM for 3h with or without NAC (10μmM 10min) pretreatment. mSREBP-1 activation was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control, # represents significant difference between NAC+ANGII. $P<,0.05,n=3$

(5) ER stress is required for ANGII-induced Akt activation

Akt activation was shown in an earlier figure to be required for ANGII-induced SREBP-1 activation. However, the role of Akt in ER stress induction in response to ANGII is unknown. One study has shown that Akt is the downstream target of GRP78 in mediating cisplatin resistance in ER stress-tolerant human lung cancer cells.[53] Here, we used three chemical chaperones: Ursodeoxycholic acid taurine-conjugated derivative (TUDCA), 4-PBA and Salubrinal to pretreat MC followed by ANGII

treatment for 30min. As seen in **Fig.23** Akt activation could be blocked by ER stress attenuation, suggesting that ER stress induces Akt activation in response to ANGII.

(6) ER stress-induced Akt activation in MC downstream of PI3K

Since ER stress induced Akt activation in response to ANGII, we wanted to assess whether ER stress itself could induce Akt activation directly and if PI3K was required. We pretreated MC with PI3K inhibitors LY294002 and Wortmannin followed by ER stress induction with Tunicamycin (TM) or Thapsigargin (TG). As shown in **Fig.24** both TM and TG could induce Akt activation independently and PI3K/Akt inhibitors suppressed this, confirming that ER stress is an upstream activator of PI3K/Akt.

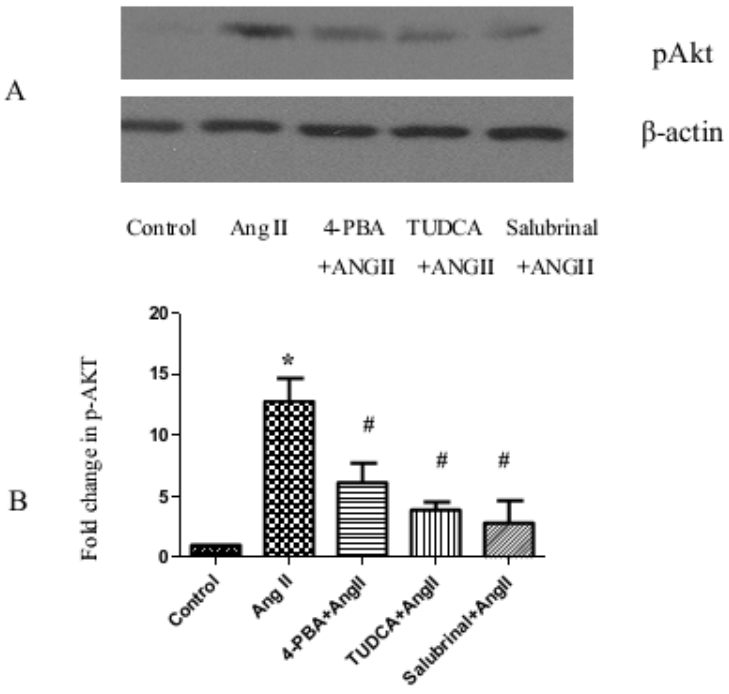


**Fig.23 ANGII-induced Akt actitivity is blocked by 4-PBA,TUDCA and Salubrinal**

MC were treated with Ang II (100nM for 3h) with or without 4-PBA (2.5mM), TUDCA (300μg/ml) or Salubrinal (30μM for 1h) pretreatment. Akt activation was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in (B) * represents significant difference between ANGII and Control, # represents significant difference between 4-PBA+ANGII or TUDCA+ANGII or Salubrina+ANGII and ANGII. *P<,0.05 n=3*

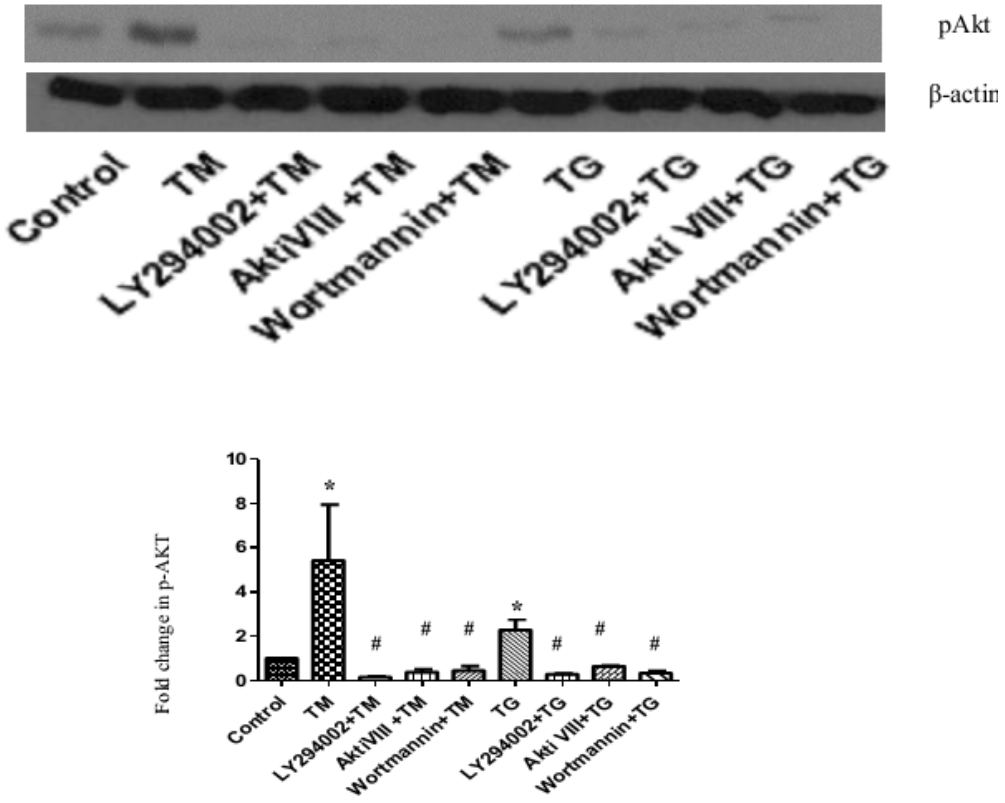


**Fig.24 ANGII-induced Akt activation is blocked by PI3K/pAkt inhibitor**

MC were treated with Tm (500ng/ml, 1h) or TG(200nM 2h) with or without LY294002 (20μM 1h), AKTiVIII 10μM 1h), or Wortmannin (100nM 1h) pretreatment. Akt activation was tested by Western blot with β-actin as a loading control (A). Quantitative result of (A) are shown in(B) * represents significant difference between TM or TG and Control. # represents significant difference between LY294002+TM or AKTiVIII+TM or Wortmannin+TM and TM; LY294002+TG or AKTiVIII+TG or Wortmannin+TG and TG $P<,0.05$, $n=3$

### *4.3. ANGII-induced SREBP-1 activation and TGF-ß1 promoter activation*

As discussed earlier, TGF-ß activation in glomerular diseases is a well-known cause of renal fibrosis. Here we wanted to confirm that ANGII could induce TGF-ß promoter activity and to assess whether it could be blocked by S1P inhibition with AEBSF and SCAP inhibition with Fatostatin. We used MC transfected with a TGF-ß1 promoter-luciferase construct. As can be seen from **Figs.25** and **26,** TGF-ß promoter activity was significantly increased by ANGII treatment and blocked by Fatostatin and AEBSF. These two experiments show that ANGII-induced TGFβ promoter activation requires SREBP-1 activation. Next we assessed if ER stress is involved, by using the chemical chaperones TUDCA and Salubrinal. TGF-ß promoter activation was significantly blocked by both treatments **Fig.27**, indicating that ER stress is

necessary for ANGII-induced TGF-ß promoter activation.

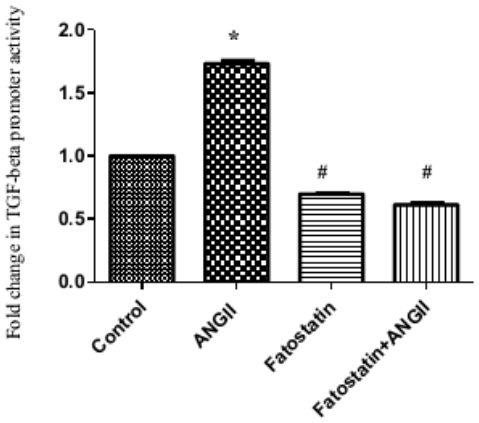


**Fig.25 ANGII-induced TGF-beta promoter activity is blocked by Fatostatin**

MC were transfected with 0.5ug of a TGFβ1 promoter (-1132to +11)-luciferase construct and 0.05μg pCMV-β-galactosidase (β-gal), then exposed to Ang II (100nM for 24h) with or without Fatostatin (20μM for 4 h). Luciferase and β-gal activities were measured on clarified lysate using specific kits (Promega) with a Berthold luminometer and a plate reader (420nm) respectively. β-gal activity was used to adjust for transfection efficiency. * represents significant difference between ANGII and Control, # represents significant difference between Fatostatin or Fatostatin+ANGII and ANGII . *P<,0.05 n=3*

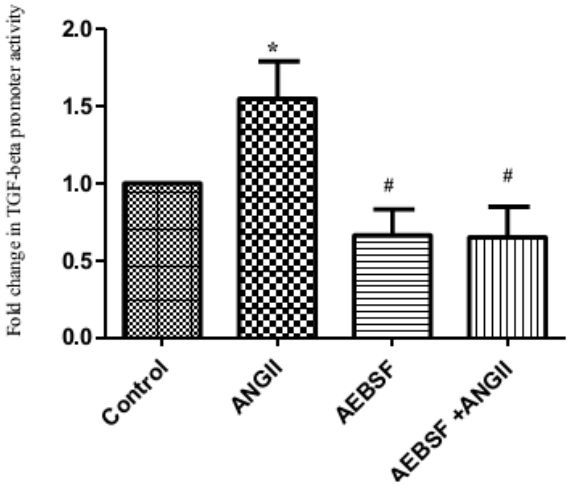


**Fig.26 ANGII-induced TGF-beta promoter activity is blocked by AEBSF**

MC were transfected with 0.5μg of a TGFβ1 promoter (-1132to +11)-luciferase construct and 0.05μg pCMV-β-galactosidase (β-gal), then exposed to Ang II (100nM for 24h)with or without AEBSF (500μM for 1h). Luciferase and β-gal activities were measured on clarified lysate using specific kits (Promega) with a Berthold luminometer and a plate reader (420nm) respectively. β-gal activity was used to adjust for transfection efficiency. * represents significant difference between ANGII and Control, # represents significant difference between AEBSF or AEBSF+ANGII and ANGII. *P<,0.05 n=3*

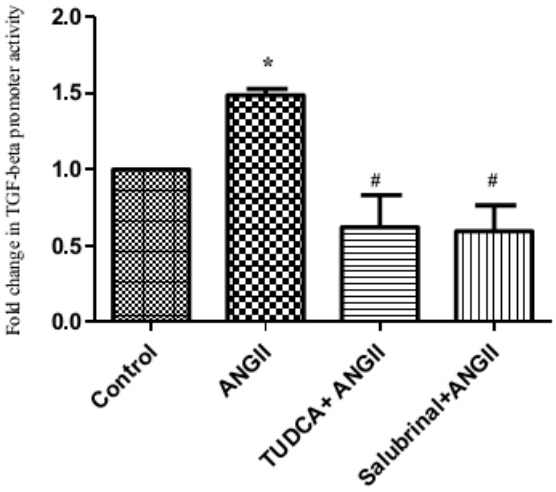


**Fig.27 ANGII-induced TGF-beta promoter activity are blocked by TUDCA and Salubrinal**

MC were transfected with 0.5μg of a TGFβ1 promoter (-1132to +11)-luciferase construct and 0.05μg pCMV-β-galactosidase (β-gal), then exposed to Ang II (100nM for 24h) with or without TUDCA (100μg/ml for 1 h) or Salubrinal (30μM 1h). Luciferase and β-gal activities were measured on clarified lysate using specific kit (Promega) with a Berthold luminometer and a plate reader (420nm) respectively. β-gal activity was used to adjust for transfection efficiency. * represents significant difference between ANGII and Control, # represents significant difference between TUDCA+ANGII or Salubrinal+ANGII and ANGII. *P<, 0.05 n=3*

### *4.4. GRP78 and SREBP-1 are significantly increased in mice treated with ANGII*

In order to see if ANGII could induce SREBP-1 in glomeruli in vivo, we used uninephrectomized C57BL/6 mice infused with ANGII as an in vivo model. Glomeruli from mice infused with ANGII showed more SREBP-1 staining compared with the placebo group **Fig 28**. More importantly, most of the SREBP-1 positivity is nuclear, suggesting the activation of SREBP-1. Similarly, GRP78 is also induced in glomeruli from ANGII-infused mice **Fig.28.**

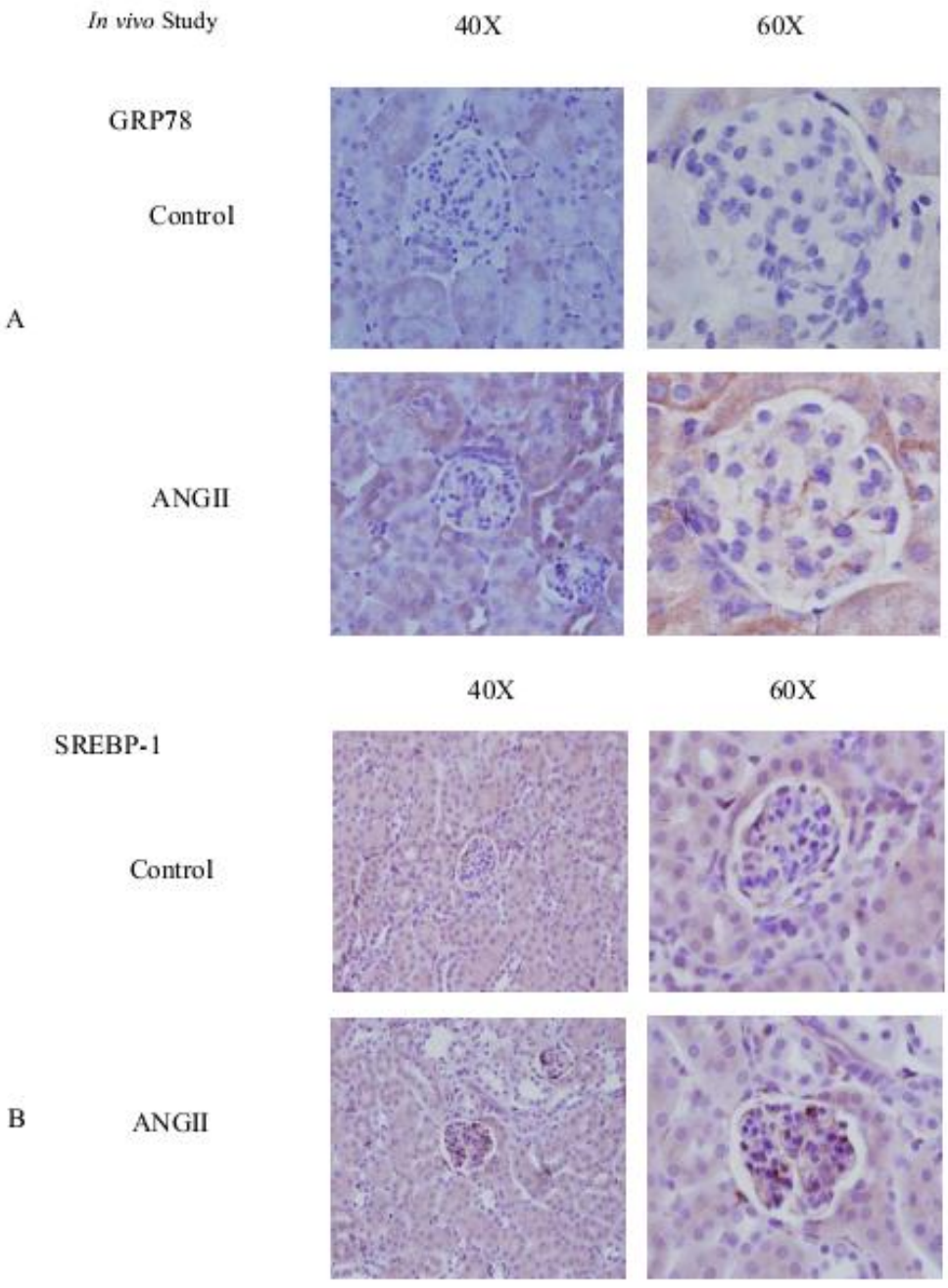


**Fig.28 IHC Staining for SREBP in C57 uninephrectomized mice with ANGII infusion**

C57 male mice received uninephrectomy surgery, and after 2 weeks received minipump with ANGII infusion of 1000nM/kg/day for 7 days. Kidney samples in paraffin were stained with GRP 78 (BD Transduction 1:40) antibody (A) or SREBP-1 (Abcam ab44153 rabbit 5ug/ml) antibody (B) for IHC

## V. Summary of Findings

- ANG II induces TGF-ß1 activity through SREBP-1 activation
- ANG II-induced SREBP-1 activation requires the AT1R and activation of the PI3K/AKT signaling pathway
- ER stress is required for both ANGII-induced TGF-ß1 upregulation and SREBP-1 activation
- AngII induces GRP78 and SREBP-1 in glomeruli in vivo

## VI. Discussion

The present results have identified that SREBP-1 has a potential role in ANGII-induced TGF-ß upregulation. We showed that ANGII could induce TGF-ß1 transcript upregulation and that this could be blocked by the SCAP inhibitor Fatostatin and S1P inhibitor AEBSF**.** Given the fact that ANGII could induce SREBP-1 activation in a time and dose dependent manner, and SREBP-1 has been identified as an important mediator in TGF-ß related renal fibrosis[21 22] , we propose that SREBP1 has an important role in ANGII-induced glomerular fibrosis.

The maturation of SREBP requires a series of processes, namely transportation to the Golgi, proteolytic cleavage of SREBP and post-translational modification.[54] We showed that SREBP-1 undergoes proteolytic cleavage in response to ANGII, and our results with SRE-GFP confirmed that this was associated with its activation. This indicates that ANGII-induced mSREBP could be successfully transported to the nucleus to be functionally active.

During this study we asked the question if ANGII-induced mSREBP activation is through the classic SREBP/SCAP maturation process. In order to answer this we blocked 2 important factors in this signaling pathway, SCAP and S1P, by using their respective inhibitors Fatostatin and AEBSF. As expected, both SCAP and S1P are required, indicating that ANGII-induced SREBP upregulation is through the classic SREBP/SCAP maturation process. However, the mechanism of how ANGII induces SREBP-1 activation remained unclear.

As one of the most important receptors for ANGII, the AT1R plays a very important role in maintaining ANGII physiological and pathologic function[55] and is widely distributed in all organs. It was thus important to know if it is involved in ANGII-induced SREBP upregulation. One of the most commonly used AT1R blockers is Losartan. It is a selective and competitive ATIR antagonist, reducing the end organ responses to ANGII. We treated MC with the AT1R inhibitor Losartan

followed by ANGII treatment and showed the AT1R mediates ANGII-induced SREBP-1 activation. Since the AT1R can transactivate the EGFR, [56] we assessed its role in this pathway. Our results show that EGFR is not required for ANGII-induced SREBP-1 activation. As a downstream factor of AT1R, EGFR signaling appears to be independent of SREBP-1 activation.

The other well studied signaling pathway downstream of ANGII is PI3K/Akt signaling.[57] The mechanism by which AT1R activates PI3K/Akt signaling is through activation of the G protein Gβγ. ANGII can directly activate Gβγ and mediate L-type calcium channel activation and this subunit can also activate PI3K[12]. PI3K/Akt have been identified as important signaling mediators in response to ANGII to induce TGF-ß upregulation in murine MC.[12] In MC, 3-phosphoinositide-dependent protein kinase-1 (PDK-1) activity plays an important role in ANGII-induced hypertrophy and fibronectin accumulation. It requires PDK-1 phosphorylation on tyrosine 9 and 373/376. Mutations in these tyrosine residues attenuate ANGII-induced hypertrophy and fibronectin accumulation. Transfection of small interfering RNA for Src inhibited ANGII-mediated PDK-1 activation and tyrosine phosphorylation, suggest that Src is an upstream mediator for PDK-1 activation. As downstream signaling molecules of PDK-1, PI3K/Akt are suggested to play a role in ANGII-induced SREBP-1 activation. By using PI3K inhibitors LY294002 and Wortmannin and Akt inhibitor VIII, we found that ANGII-induced SREBP-1 activation was significantly blocked by all three inhibitors. This strongly suggested that PI3K/Akt is required for ANGII induced-SREBP activation. However, SREBP-1 activity could not be blocked by Rapamycin, indicating that this pathway is mTOR-independent. The potential mechanism by which PI3K/Akt signaling mediates ANGII-induced SREBP-1 activation is suggested from a study showing phosphorylation-dependent degradation of the SREBP family of transcription factors by $SCF^{Fbw7}$. During SREBP activation, glycogen synthase kinase3 (GSK3) could significantly induce SREBP ubiquitination by phosphorylating its T426 and S430 which stimulates its interaction with ubiquitin ligase $SCF^{Fbw7}$. The activation of Akt could mediate phosphorylation of Ser-9 on

GSK-3 and negatively regulate the kinase activity of GSK-3. More importantly, there has been evidence showing that factors which can activate PI3K/Akt such as insulin could also enhance SREBP activation. In order to better understand the role of GSK3 in ANGII-induced SREBP-1 activation, we would need to investigate the effect of ANGII on GSK3, which remains unclear.

Evidence showed that ER stress could induce SREBP activation.[58] In order to understand the role of ER stress in ANGII-induced SREBP-1 activation, we first wanted to see if ANGII could induce ER stress. We found that ANGII could significantly induce ER stress and that AT1R was required. The chemical chaperones which alleviate ER stress also significantly blocked ANGII-induced SREBP-1 activation, suggesting that SREBP-1 activation is mediated by ER stress. Studies have shown that ER stress and ER calcium release could be caused by exposure of cells to oxidative injury.[59] Calcium release from the ER has been considered as one of the common links between oxidative stress and ER stress. In this study we showed that both oxidative stress and intracellular calcium are important in ANGII-induced SREBP activation.

Reactive oxygen species (ROS) are chemically reactive molecules that contain oxygen. They can be produced in almost all cellular compartments and eventually cause protein damage.[52] There has been evidence showing protein folding and production of ROS are related. Increased ROS production in response to ANGII has been demonstrated. Although a link between ER stress and oxidative stress, mediated by calcium leaking from the ER lumen, has been demonstrated, whether ROS is involved in ANGII-induced SREBP activation was unknown.

The relation between ER stress and PI3K/Akt signaling is still unclear. Several studies showed that ER stress could lead to Akt activation[60], with a suggestion that Akt may be a downstream target of GRP78. [53] However, there are also studies showing that Akt could mediate chondrocyte survival from ER stress.[61] We found that in a short time, both TM and TG could induce PI3K-mediated Akt activation. On the other hand, the inhibition of ANGII-induced Akt activation by the chemical

chaperones 4-PBA and Salubrinal indicates that ER stress in necessary in this signaling pathway and is upstream of PI3K/Akt activation. It thus seems that ANGII has two signaling pathways involved in inducing SREBP activation. One of them is through PI3K/Akt and the other is through oxidative stress and ER stress. Both pathways require the AT1R. However, whether there is any cross talk between these two pathways remains unknown, and the exact mechanism of how ANGII induces ER stress is still unclear.

TGF-β1 has been shown to be an important mediator of glomerulosclerosis in the progression of diabetic and hypertensive renal disease.[1] We showed that ANGII could induce TGF-β1 promoter activation and that this could be inhibited by both SCAP and SIP inhibition. Other results in the lab showed that TGFβ promoter activation was inhibited by dominant negative SREBP-1a and 1c (results not shown). All of these indicate that SREBP-1 is playing an important role in TGF-β1 upregulation in response to ANGII. However, treatment of MC with TM or TG could not induce TGF-β1 promoter activation (results not shown), indicating that ER stress alone is not enough for TGF-β1 upregulation. On the other hand, it seems ER stress is a necessary factor required for TGFβ upregulation, attested to by the inhibition of promoter activation by ER stress inhibitors TUDCA and Salubrinal.

Studies showed that inhibition of ANGII signaling could improve diabetic and hypertensive nephropathy. [55] However, there has not been any study showing whether ANGII could induce SREBP-1 in glomeruli in vivo. We showed that ANGII infusion for 1 week significantly increases glomerular SREBP-1 as well as GRP78 expression. More importantly, most of the SREBP expression was nuclear, suggesting that it has been activated. Thus, taken together, this data suggest that targeting SREBP-1, as well as ER stress, are potential novel therapeutic avenues for the treatment of chronic progressive renal disease.

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