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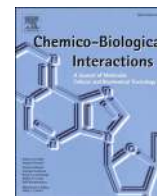
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Role of RAS/Wnt/ β -catenin axis activation in the pathogenesis of podocyte injury and tubulo-interstitial nephropathy



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ABSTRACT

Renin-angiotensin system (RAS) plays a key role in the development and progression of chronic kidney disease (CKD). Recent studies have demonstrated activation of Wnt/ β -catenin pathway by RAS in CKD. However, the underlying mechanisms of RAS and Wnt/ β -catenin signaling interaction and their contribution to the pathogenesis of CKD have not been fully elucidated. Present study is designed to investigate the role of RAS/Wnt/ β -catenin axis activation in tubulo-interstitial fibrosis and glomerulosclerosis by the cultured HK-2 and podocytes. HK-2 cells and podocytes are treated by angiotensin II (Ang II). Ang II up-regulates expression of various Wnt mRNA and active β -catenin protein in HK-2 cells and podocytes in the time- and dose-dependent manners. In addition, Ang II induces injury, oxidative stress and inflammation and impaired Nrf2 activation in HK-2 cells and podocytes. This was accompanied by up-regulations of RAS components as well as Wnt1, activated β -catenin and its target proteins. RAS/Wnt/ β -catenin axis activation results in epithelial-to-mesenchymal transition in HK-2 cells and injuries podocytes. The effect of Ang II is inhibited by losartan and ICG-001, a Wnt/ β -catenin inhibitor. We further found that treatment with natural products, ergone, alisol B 23-acetate and pachymic acid B inhibit extracellular matrix accumulation in HK-2 cells and attenuated podocyte injury, in part, by inhibiting Ang II induced RAS/Wnt/ β -catenin axis activation. In summary, activation of RAS/Wnt/ β -catenin axis results in podocytes and tubular epithelial cell, injury and up-regulations of oxidative, inflammatory and fibrotic pathways. These adverse effects are ameliorated by ergone, alisol B 23-acetate and pachymic acid B. Therefore, these natural products could be considered as novel Wnt/ β -catenin signaling inhibitors and anti-fibrotic agents.

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1. Introduction

Activation of intrarenal renin-angiotensin system (RAS) plays a key role in the development and progression of chronic kidney disease (CKD), hypertension and cardiovascular disease. RAS includes renin, angiotensinogen (AGT), angiotensin II (Ang II), angiotensin-converting enzyme (ACE), angiotensin II type 1 receptor (AT1) and angiotensin II type 2 receptor (AT2). RAS contributes to tubulo-interstitial fibrosis via induction of epithelial mesenchymal transition (EMT), up-regulation of pro-fibrotic factor,

extracellular matrix (ECM) accumulation and podocyte loss [1–3]. Numerous studies have demonstrated activation of intrarenal RAS after kidney injury and its contribution to kidney injury. *In vitro* studies have demonstrated that Ang II-stimulated production of pro-fibrotic factors by podocytes and proximal tubular epithelial cells and caused ECM accumulation [4]. Furthermore, *In vivo* studies have revealed the association of elevated renin and Ang II levels and tubulo-interstitial fibrosis in the remnant kidney subjected to subtotal nephrectomy [5]. In particular, abnormalities of prorenin receptor, ACE and AT1 contributed to the development of renal fibrosis [6]. Numerous studies have demonstrated that AT1 receptor blockers attenuated renal fibrosis in unilateral ureteral obstruction model. Increased pro-fibrotic factors caused tubulo-interstitial injury and glomerulosclerosis via excessive production and deposition of ECM [1], which are the main features of CKD.

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Effects of Ang II on the kidney tissue are mainly mediated via AT1 receptor. Additionally, Ang II accelerated renal inflammation and fibrosis by activating TGF- β 1 and NF- κ B signaling pathways [7,8]. In fact, tubulo-interstitial fibrosis was attenuated in AT1 receptor-deficient mice [7].

The canonical Wnt/ β -catenin pathway is an evolutionarily conserved developmental signaling cascade that plays an important role in embryonic development, organ development and tissue homeostasis [9,10]. Activation of Wnt signaling inhibits glycogen synthase kinase-3 activity, thereby causes accumulation of dephosphorylated- β -catenin in cytosol, and β -catenin translocation into the nucleus. Within the nucleus β -catenin binds to transcription factors, lymphoid enhancer-binding factor (LEF)/T-cell factor (TCF) family assembles a complex by recruiting the transcriptional coactivator, cAMP response element-binding protein (CREB)-binding protein (CBP), to *trans*-activate its target genes [11,12]. Under physiological conditions, the Wnt/ β -catenin signaling pathway is silent in normal adult kidneys, but becomes activated in diverse types of CKD such as adriamycin nephropathy, 5/6 nephrectomy, obstructive nephropathy, diabetic nephropathy, chronic allograft nephropathy and patients with advanced CKD [5,13–17].

RAS, Wnt/ β -catenin signaling and renal fibrosis are inseparably linked as they form a vicious cycle in which RAS provokes Wnt/ β -catenin signaling which causes renal fibrosis. Renal fibrosis, in turn, provokes RAS and elevates arterial blood pressure by limiting renal blood flow. Accordingly, blockade of this signaling cascade may ameliorate proteinuria, attenuate kidney injury and mitigate renal fibrosis in animal models of CKD. Intrarenal RAS activation has been shown to result in up-regulation of Wnt/ β -catenin signaling and its target genes such as *twist*, *snail1*, and *MMP-7* in the renal fibrosis model [14,18,19]. ANG II also exerts profibrotic effects in mesangial cells, podocytes, proximal tubular epithelial cells and collecting duct cells in the kidney [3,20]. Furthermore, increased β -catenin and Wnts up-regulate RAS components, which cause activation of RAS [21]. Although RAS, Wnt/ β -catenin signaling and renal fibrosis form a vicious cycle in CKD, it is not clear whether RAS interaction with Wnt/ β -catenin pathway in proximal tubular epithelial cells and podocyte cause fibrosis. The present study was performed to investigate the hypothesis that RAS over-activation leads to up-regulation of fibrosis-associated proteins via activation of the Wnt/ β -catenin-dependent signaling pathway in human proximal tubular epithelial cells (HK-2) and immortalized mouse podocyte cell lines (MPC5). In addition, currently available drugs i.e. ACE inhibitors or angiotensin II receptor blockers attenuate RAS activity but have limited efficacy, partly due to compensatory up-regulation of renin expression. Natural products *Polyporus umbellatus*, surface layer of *Poria cocos* and *Alisma orientale* are widely used for their diuretic and nephroprotective effects. Our previous studies have shown that administrations of ergone (ERG) from *Polyporus umbellatus* and the extract of the surface layer of *Poria cocos* were highly effective in promoting diuresis and attenuating renal injury and dysfunction in CKD animals [22–28]. In addition, we demonstrated that the extract of *Alisma orientale* had strong diuretic and anti-diuretic activities [29,30]. In this study, we investigated the effects of ERG, alisol B 23-acetate (ABA) and pachymic acid B (PAB) on activation of RAS/Wnt/ β -catenin axis and inflammatory pathway in Ang II-treated HK-2 cells and podocytes to determine their efficacy as novel candidate drugs for the treatment of kidney injury.

2. Materials and methods

2.1. Cell culture and treatment

Human kidney proximal epithelial cells (HK-2) and

conditionally immortalized mouse podocytes were purchased from the China Center for Type Culture Collection (CCTCC). HK-2 was cultured in DMEM-F12 supplemented with 10% fetal bovine serum (GIBCO, Carlsbad, CA, USA), and grown at 37 °C with 5% CO₂. MPC5 was cultured in DMEM containing 5.5 mM glucose with 10% fetal bovine serum and grown at 33 °C with 5% CO₂ and differentiated at 37 °C. HK-2 and podocytes were stimulated with Ang II at doses of 0, 0.01, 0.1, 0.5, 1.0, 2.0 μ M and 1.0 μ M for 0, 1, 3, 6, 12, 24 and 48 h.

Angiotensin II type 1 receptor blocker losartan (LOS, 1 μ M, Selleck Chemicals, Huston, USA) and Wnt/ β -catenin inhibitor ICG-001 (ICG, 10 μ M; Selleck Chemicals, Huston, USA) were used. The Wnt/ β -catenin inhibitor ICG-001 inhibits β -catenin-mediated gene transcription. To discover novel inhibitors of RAS and Wnt/ β -catenin signaling pathway and anti-fibrotic candidate drugs, ERG, ABA and PAB were isolated from natural products *Polyporus umbellatus* [31], *Alisma orientale* [32] and surface layer of *Poria cocos* [33]. In some experiments, cells were incubated with ERG, ABA and PAB at 10 mM. We further investigate the inhibition effects of these natural products on RAS and Wnt/ β -catenin signaling pathways. Whole-cell lysates were prepared and subjected to Western blot analysis, and total RNA was extracted for PCR analysis.

2.2. Gene expression studies by real-time polymerase chain reaction (RT-PCR)

Total RNA from cultured HK-2 cells or podocytes was extracted using a High Pure RNA Isolation Kit (RNAiso Plus, Takara Bio, Japan) according to the manufacturer's instructions, including DNase-free treatment. The quantity and purity of the RNA preparations were determined by measuring the optical densities at 260 and 280 nm. The housekeeping gene GAPDH was used as an internal standard. The primers were used to amplify the genes (Table 1). Total RNA was reverse transcribed by a Transcriptor First Strand cDNA Synthesis Kit according to the instructions of the manufacturer (Roche, Germany) using Bio-Rad T100™ system. RT-PCR reaction mixture in a 20- μ L volume contained 10 μ L of *Premix Taq*™ (Takara Bio, Japan), 1.0 μ L of diluted reverse transcription product (0.1 μ g cDNA), 0.4 μ L of sense and antisense primer sets and 8.2 μ L of double distilled water. PCR-cycling conditions were 94 °C for 3 min, followed by 30 cycles with denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 30 s finished with 1 cycle final elongation at 72 °C for 10 min. PCR product of annealing reactions were analyzed by nucleic acid gel electrophoresis using 0.75 mm thick agarose gel in the running buffers 1 \times TAE including 40 mM Tris, 2 mM Na₂EDTA and 20 mM acetic acid pH 8.5. The gels contained 100 mL 1 \times TAE, 1 g agarose and 10 μ L nucleic acid dye. The gels were run at approximately 70 V for 1 h.

Quantitative real-time RT-PCR (qRT-PCR) was performed on Bio-Rad CFX 96 Touch™ system (Bio-Rad, USA). The PCR reaction mixture in a 20- μ L volume contained 10 μ L of SYBR® *Premix Ex Taq*™ II (Takara Bio, Japan), 1.0 μ L of diluted reverse transcription product (0.1 μ g cDNA), 0.4 μ L of sense and antisense primer sets and 8.2 μ L of double distilled water. The mRNA levels of genes were calculated by normalizing with GAPDH or β -actin. The gels were visualized by Tanon 6600 Luminescent Imaging Workstation (Tanon Science & Technology Co., Ltd. Shanghai, China) and developed by autoluminography.

2.3. Western blot analysis

Cultured HK-2 cells and podocytes were lysed in RIPA lysis and protein was extracted for Western blot analysis. Protein expression was performed by Western blot analysis as described previously [34,35]. Protein concentration of total cell lysates was determined by Pierce™ BCA Protein Assay Kit (23227, Thermo Scientific, USA)

Table 1
Primers for real-time RT-PCR (5' → 3'). The thermal cycling program comprises an initial denature step at 94 °C for 3 min, followed by 94 °C for 30 s and 56 °C for 30 s for 30 cycles.

HK-2 cell Primers				
Gene	Species	Forward	Reverse	Product Size (bp)
Wnt1	<i>Homo sapiens</i>	CGGGCTTTATCTTCGCTATCA	GTAGTCACACGTGCAGGATT	95
Wnt2-1	<i>Homo sapiens</i>	CGGGAATCTGCCTTTGTTTATG	TTGGATCACAGGAACAGGATTT	103
Wnt2b-2	<i>Homo sapiens</i>	TGCAGTGACAACATCCACTAC	GCGACCACAGCGGTTATTA	117
Wnt3-2	<i>Homo sapiens</i>	CTGACTTCGGCGTGTTAGT	CCTCGTTGTTGTGCTTGTTTC	93
Wnt3a	<i>Homo sapiens</i>	CAAGATTGGCATCCAGGAGT	ATGAGCGTGTCACTGCAAAAG	173
Wnt4	<i>Homo sapiens</i>	ATGGAAGTACACCCTCTGG	CCTGGAAGGACCCACAGATA	201
Wnt5a	<i>Homo sapiens</i>	GGGTGGGAACCAAGAAAAAT	TGGAACCTACCCATCCATA	194
Wnt7a	<i>Homo sapiens</i>	CGTGCTCAAGGACAAGTACA	GTACGACAGTGCTTCTTGAT	103
Wnt7b	<i>Homo sapiens</i>	CCTGCTGAAGGAGAAGTACAA	GTCTCCATGGGCTTCTGATAG	120
Wnt8b	<i>Homo sapiens</i>	CCAATCGGGAGACAGCATT	AGTCATCACAGCCACAGTTATC	108
Wnt9a	<i>Homo sapiens</i>	CGGAGACAACCTTAAGTACAG	ACGAGGTTGTTGTGGAAGTC	103
Wnt9b	<i>Homo sapiens</i>	TGTAAGTGCCATGGCGTATC	CCGAGTCATAGCCGAGTTT	106
Wnt10b	<i>Homo sapiens</i>	TCTGACAAGGGGACAGAACC	TCATTGGCTTAGACCCGACT	240
Wnt11	<i>Homo sapiens</i>	CAGGCAGTGAACAAGACAT	TGAGGGTCTTGAGCAGAGT	230
Wnt16	<i>Homo sapiens</i>	CCAGTTCAGACACGAGAGATG	GCAGCCATCACAGCATAAATAA	138
β-catenin	<i>Homo sapiens</i>	ACAAGCCACAAGATTACAAG	ATCAGCAG TCTCATTCCAA	92
GAPDH	<i>Homo sapiens</i>	CCCTTCATTGACCTCAACTACA	ATGACAAGCTTCCCGTTCTC	101
Podocyte Primers				
Gene	Species	Forward	Reverse	Product Size (bp)
Wnt1	mouse	TGCAAAATGGCAATTCGAAAC	GGAGGTGATTGCGAAGATGAA	120
Wnt2	mouse	TACTGTATCAGGGACCGAGAG	GCCTTCCCACACACATATA	108
Wnt2b	mouse	CCGGACTGATCTGTCTACTTT	TCACCCCATCTGTTCTTTAG	123
Wnt3	mouse	CTGGACCACATGCACCTAAA	CGTACTGTCTTGAGGAAGTC	121
Wnt3a	mouse	CATGAACCGTCACAACAATGAG	CACITTCACAGCTGCCAGATA	97
Wnt4	mouse	CAGTGAGAACTGGAGAAAGTG	TCCACAAAAGGACTGTGAGAAG	126
Wnt5a	mouse	GTTCTGGACGCTAGAGAAAAG	GCCTGCTTCATTGTTGTGTAAG	94
Wnt6	mouse	TCAGTTCAGTTCCTGTTCC	GAAAGTGTCTCTCGGATGTC	94
Wnt7a	mouse	TCAGGCTCTGTACCACTAA	GGACGGCTCTGTTGATTT	94
Wnt7b	mouse	GGAGAAGCAAGGCTACTACAAC	CATCCACAAAAGCGACGAGAA	101
Wnt8a	mouse	GTGTCATGGCATCTCAGGAA	GCGGTCATCTTGGCCTTTA	100
Wnt8b	mouse	GCCCAGAGTGGTATTGAAGAA	AAATGCTGTCTCCCGTTAG	123
Wnt9a	mouse	AAGGTGATAAAGGCTGAGTG	CCTGAGCGAGGTTCTCATATT	145
Wnt10b	mouse	GCTGAGCAGAGCCATCTTTAT	CTCAAAGTAAACCAGCTCTCCA	103
Wnt11	mouse	ACAGGATCCCAAGCCAATAAA	GGCATTTCACACTCGTTTCCAG	94
Wnt16	mouse	CAGCATCCAGATCTCAGACAAA	CCGAGTAGTTGGGACTTATG	119
β-catenin	mouse	GCTGCTCATCCACTAATGT	CCGCGTCATCTGATAGTTAAT	94
β-actin	mouse	CAGCTGAGAGGGAAATCGTG	CGTTGCCAATAGTGATGACC	150

according to the manufacturer's instructions, and 30 µg of total protein was separated on 12% Tris-Glycine resolving gel including 1.6 mL ddH₂O, 2.0 mL 30% acryl-bisacrylamide mix, 1.3 mL 1.5 M Tris (pH 8.8), 0.05 mL 10% SDS, 0.05 mL 10% ammonium persulfate and 0.002 mL TEMED and 5% Tris-Glycine stacking gels including 1.4 mL ddH₂O, 0.33 mL 30% acryl-bisacrylamide mix, 0.25 mL 1.5 M Tris (pH 6.8), 0.02 mL 10% SDS, 0.02 mL 10% ammonium persulfate and 0.002 mL TEMED at 70 V for 30 min and then 120 V for 60 min in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to 0.45 µm polyvinylidene difluoride (PVDF) membrane (10600023, Amersham™ Hybond™, GE Healthcare, USA) at 230 mA for 60 min in the 100 mL, 200 mL CH₃OH and 700 mL ddH₂O. The membrane was washed three times for 10 min in 1 × TBS and 0.1% Tween-20. The membranes were incubated for 1 h in 1 × TBS, 0.1% Tween-20 and 5% non-fat milk blocking buffer. The membrane was washed three times for 10 min in 1 × TBS and 0.1% Tween-20 and then overnight at 4 °C in the same buffer containing the primary antibodies. The following primary antibodies were employed [dilution]: angiotensinogen (AGT, 1:200, sc-7419, Santa Cruz Biotechnology, CA), renin (1:2000, 14291-1-AP, Proteintech, China), ACE (1:1000, ab77990, Abcam, USA), AT1 (1:800, ab124505, Abcam, USA), Wnt1 (1:1000, ab85060, Abcam, USA), dephosphorylated active β-catenin (1:2000, no. 05–665, Millipore, USA), β-catenin (1:1000, 610154, BD

Transduction Laboratories, USA), collagen I (col I, 1:5000, ab34710, Abcam, USA), collagen I (col III, 1:5000, ab7778, Abcam, USA), alpha smooth muscle actin (α-SMA, 1:300, ab7817, Abcam, USA), fibronectin (1:1000, ab2413, Abcam, USA), vimentin (1:200, ab92547, Abcam, USA), E-cadherin (1:500, ab76055, Abcam, USA), snail1 (1:1000, ab180714, Abcam, USA), matrix metalloproteinase-7 (MMP-7, 1:400, ab5706, Abcam, USA), twist (1:2000, ab50581, Abcam, USA), fibroblast-specific protein 1 (S100A4) (FSP1, 1:1000, ab197896, Abcam, USA), nuclear factor-kappa B (NF-κB, 1:2000, ab16502; Abcam, USA), monocyte chemoattractant protein-1 (MCP-1, 1:1000, ab7202; Abcam, USA), cyclooxygenase-2 (COX-2, 1:1000, ab62331; Abcam, USA), nuclear factor-erythroid-2-related factor 2 (Nrf2, 1:1000, ab31163, Abcam, USA), Heme oxygenase-1 (HO-1, 1:2000, ab68477, Abcam, USA), podocin (1:2000, ab50339; Abcam, USA), nephrin (1:1000, ab58968; Abcam, USA), podocalyxin (1:2000, ab154305; Abcam, USA), synaptopodin (1:500, sc-21536, Santa Cruz Biotechnology, CA), desmin (1:10000, ab32362, Abcam, USA), Wilms' tumor 1 (WT1, 1:1000, ab180840, Abcam, USA) and AKT2 (1:1000, ab38513, Abcam, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-tubulin were purchased from Proteintech Company (China). The membrane was washed three times for 10 min in 1 × TBS and 0.1% Tween-20 before a 2 h incubation in 1 × TBS and 0.1% Tween-20 buffer containing horseradish peroxidase-conjugated goat anti-rabbit (1:5000)

(ab6721, Abcam, USA) and goat anti-mouse (1:5000, A21010, Abbkine, USA) secondary antibodies. The membrane was washed 6 times in $1 \times$ TBS and 0.1% Tween-20, then visualized by the Amersham ECL prime western blotting detection reagent (RPN2232, GE Healthcare, USA) in Tanon 6600 Luminescent Imaging Workstation (Tanon Science & Technology Co., Ltd. Shanghai, China) and developed by autoluminography. The ratio of each protein was calculated by densitometry using free ImageJ software (version 1.48v, NIH, Bethesda, MD, USA) and Band densities were normalized by α -tubulin or GAPDH expression levels. Each experiment was repeated at least three times in the study.

2.4. Immunofluorescence staining

Indirect immunofluorescence staining was carried out using an established procedure [36]. Briefly, cultured HK-2 cells and podocytes on coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature and blocked with 10% normal goat serum in PBS buffer for 30 min. Then, HK-2 cells and podocytes were incubated with the specific primary antibodies against active β -catenin (1:500, no. 05–665, Millipore, USA) at 4 °C for overnight. The cells were then rinsed with PBS 3 times and then incubated with Alexa Fluor[®] 488-conjugated goat anti-rabbit IgG H&L (1:200, ab150077, Abcam, USA). DAPI was incubated at room temperature for 10 min to localize nuclear. Thereafter, the stained HK-2 cells and podocytes were washed with PBS and mounted with 80% glycerol/PBS for subsequent examination using a laser-scanning confocal microscope (FV1000, Olympus, Japan) equipped with FV10-ASW 4.0 VIEW (Olympus).

2.5. Statistics analysis

All data are expressed as means \pm SD. Comparison between groups were assessed by one-way ANOVA using the GraphPad Prism software v 5.0 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Ang II activates Wnt/ β -catenin signaling pathway and induces HK-2 cell injury

To evaluate whether Ang II treatment induces Wnt/ β -catenin signaling pathway, HK-2 cells are treated by Ang II in the different time and doses. As shown in Fig. 1, Significant increase in Wnt1, Wnt2, Wnt2b, Wnt4, Wnt5a, Wnt9b and Wnt11 mRNA expression are observed in HK-2 cells after Ang II (1 μ M) treatment in the time-dependent manner within 24 h. The mRNA expression of Wnt3, Wnt3a, Wnt7a, Wnt8a and Wnt10b are also increased at 0, 1, 3, 6, 12, 24 and 48 h particularly at later time points. However, Wnt7a and Wnt8b show only a slight expression. Notably, compared with the 12 h and 24 h expression of all Wnt mRNA expression are most significantly increased at 48 h exposure to Ang II (1 μ M). Similarly, Wnt1, Wnt2, Wnt2b, Wnt5a, Wnt7a, Wnt9 and Wnt10b mRNA expression are significantly increased in a dose-dependent manner from 0 μ M to 1.0 μ M at 12 h. Several other Wnts including Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt7a and Wnt16 are also increased at different dose and time points. These results demonstrated that Ang II is capable to induce various Wnt mRNA expression in HK-2 cells in the time- and dose-dependent manners. We next evaluated whether Ang II treatment could increase β -catenin protein expression in HK-2 cells. We quantify the protein concentration of active β -catenin by Western blot analysis. Fig. 2 shows that Ang II up-regulates protein expression of active β -catenin in HK-2 cells in the time- and dose-dependent manners,

especially in 1 μ M in 12 h. Increasing evidence demonstrated that Wnt/ β -catenin signaling pathway is activated by Ang II treatment in HK-2 cells. Additionally, immunofluorescent staining showed that active β -catenin expression is dramatically increased with 1.0 μ M Ang II in HK-2 cells particularly in their nuclei at 12 h (Fig. 2C).

3.2. Ang II activates RAS/Wnt/ β -catenin axis and causes interaction of activated RAS and Wnt/ β -catenin signaling

To further investigate the interaction with RAS and Wnt/ β -catenin signaling, we treat HK-2 cells with Ang II in the presence or absence of losartan and ICG-001. Ang II treatment substantially induced up-regulation of protein expressions of AGT, rennin, ACE and AT1 in HK-2 cells, which could be inhibited by losartan and ICG-001 (Fig. 3A). These results demonstrated that RAS components are activated after Ang II treatment, and up-regulated expression of RAS components are attenuated by RAS and Wnt/ β -catenin inhibitors. In addition, Ang II treatment up-regulated the protein expression of Wnt1, active β -catenin and β -catenin in HK-2 cells, which demonstrate the activation of Wnt/ β -catenin signaling pathway. Ang II-induced up-regulation of Wnt1, active β -catenin and β -catenin protein expression were prevented by losartan (Fig. 3B). Additionally, Ang II treatment significantly increased the Wnt1 and β -catenin mRNA levels in HK-2 cells, which are inhibited by losartan and ICG-001 (Fig. 3C and D). Immunofluorescence staining shows a clear nuclear localization of active β -catenin and its dramatically increased expression in Ang II-induced HK-2 cells. These results demonstrated that Wnt/ β -catenin signaling pathway is activated by Ang II treatment and these changes are ameliorated by losartan and ICG-001 treatment (Fig. 3E). Additionally, our study show that Wnt/ β -catenin inhibitor ICG-001 not only inhibits up-regulation of Wnt/ β -catenin signaling proteins including Wnt1, active β -catenin and β -catenin, but also inhibits up-regulation of RAS proteins including AGT, rennin, ACE and AT1 in HK-2 cells (Fig. 3). Blockade of RAS could inhibit Wnt/ β -catenin signaling pathway, and Wnt/ β -catenin inhibitor blocks RAS. RAS and Wnt/ β -catenin signaling are inseparably linked as they form a vicious cycle in which RAS activates Wnt/ β -catenin signaling, in turn, Wnt/ β -catenin signaling provokes RAS which causes elevated blood pressure. Taken together, these results demonstrated interaction of RAS and Wnt/ β -catenin signaling in HK-2 cells.

3.3. Ang II activates β -catenin target proteins and induces ECM accumulation in HK-2 cells

We assessed whether Ang II-induced ECM accumulation is mediated by Wnt/ β -catenin signaling and examines the expression of β -catenin target proteins in HK-2 cells. The β -catenin target proteins sail1, MMP-7, twist and FSP-1 are dramatically increased by Ang II treatment in HK-2 cells (Fig. 4A). The activation of Wnt/ β -catenin signaling is accompanied by ECM accumulation including collagen I, collagen III, α -SMA and vimentin as well as down-regulating E-cadherin (Fig. 4C), which contributes to accumulation of EMT. Consistently, Ang II-mediated up-regulation of sail1, MMP-7, twist and FSP-1 were inhibited by losartan and ICG-001. Notably, the combination of losartan and ICG-001 showed stronger inhibitory effect than losartan or ICG-001 alone on β -catenin target proteins sail1 and MMP-7 (Fig. 4A). To discover novel inhibitors of Wnt/ β -catenin signaling pathway, we next evaluate the effects of ERG, ABA and PAB on the Ang II-induced β -catenin target proteins. Ang II-mediated up-regulation of sail1, MMP-7, twist and FSP-1 are repressed by ERG, ABA and PAB treatment (Fig. 4B). Up-regulation of collagen I, collagen III, α -SMA

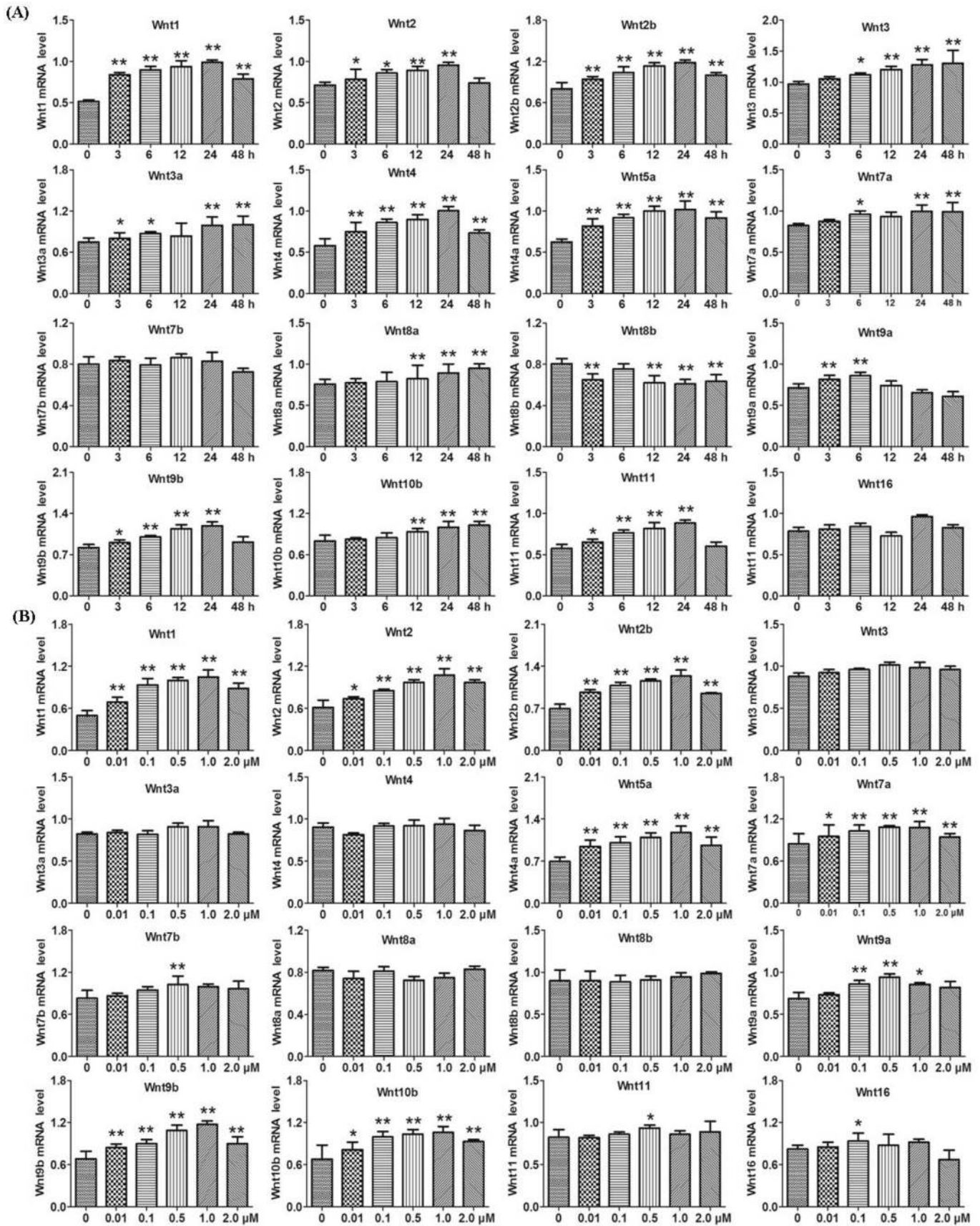


Fig. 1. RT-PCR demonstrates time-dependent (A) and dose-dependent (B) mRNA expressions of various Wnts in AngII-treated in HK-2 cells.

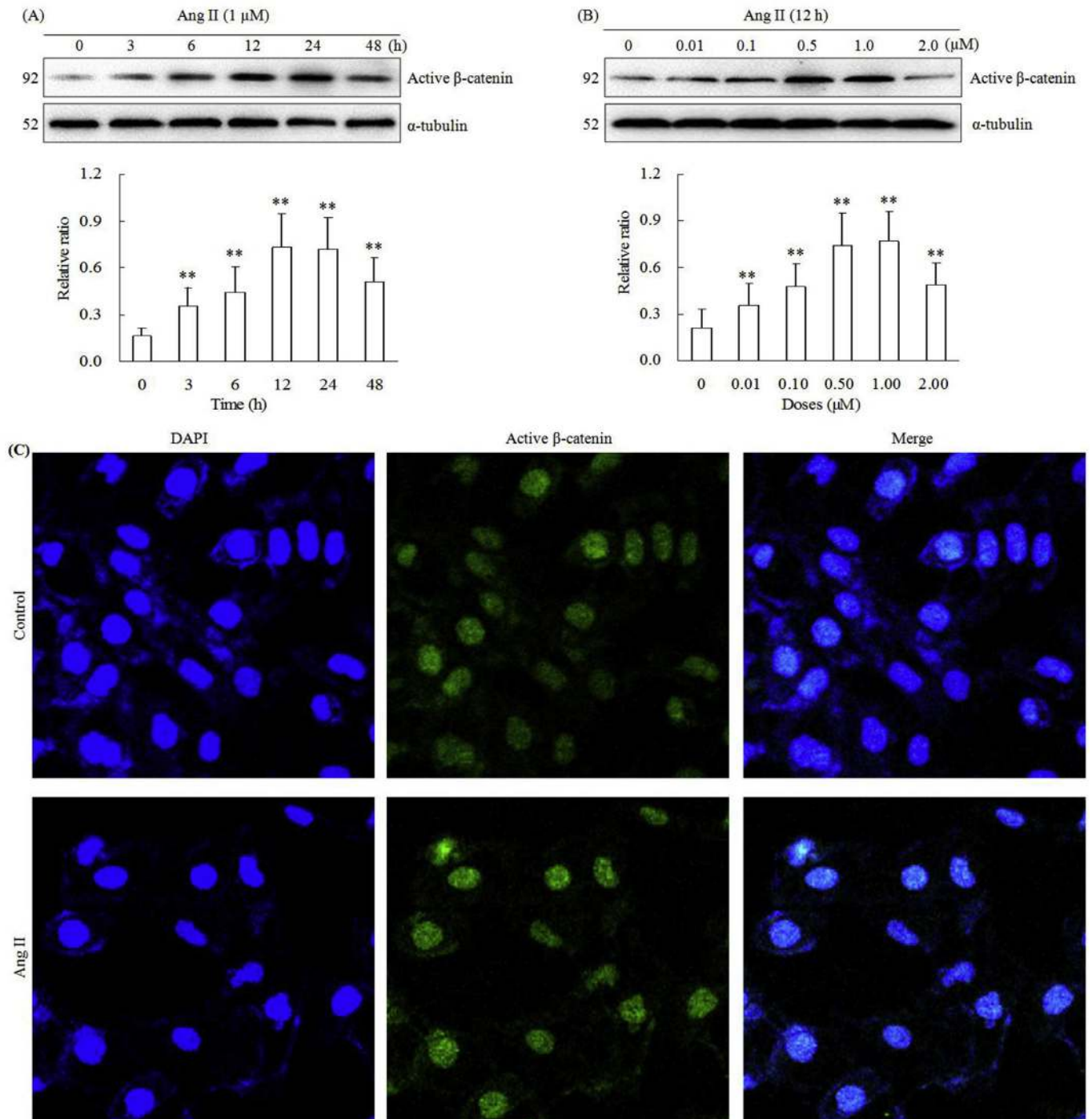


Fig. 2. Time (A) and dose (B) courses of active β -catenin expression levels in the HK-2 cells induced by Ang II. Ang II (1 μ M) increased active β -catenin at 3, 6, 12, 24 and 48 h. Ang II (12 h) also increased active β -catenin at 0.01, 0.1, 0.5, 1.0 and 2.0 μ M * P < 0.05, ** P < 0.01 compared with untreated cell. (C) Representative immunofluorescent staining images indicating β -catenin underwent nuclear translocation in Ang II-induced HK-2 cells. Cultured HK-2 cells were counterstained with DAPI for nuclear visualization.

and vimentin as well as down-regulation of E-cadherin by Ang II are inhibited by ERG, ABA and PAB treatment (Fig. 4D). These results indicated that ERG, ABA and PAB can ameliorate ECM accumulation by Wnt/ β -catenin signaling pathway *in vitro*. These findings demonstrate the efficacy of these natural products as novel Wnt/ β -catenin signaling inhibitor and anti-fibrotic agents.

3.4. Activation of RAS/Wnt/ β -catenin axis is accompanied by oxidative stress and inflammation in HK-2 cells

Oxidative stress and inflammation play a central part in the pathogenesis and progression of kidney injury [37–39]. Under physiological conditions, oxidative stress provokes up-regulation of endogenous anti-oxidant and cytoprotective proteins and enzymes

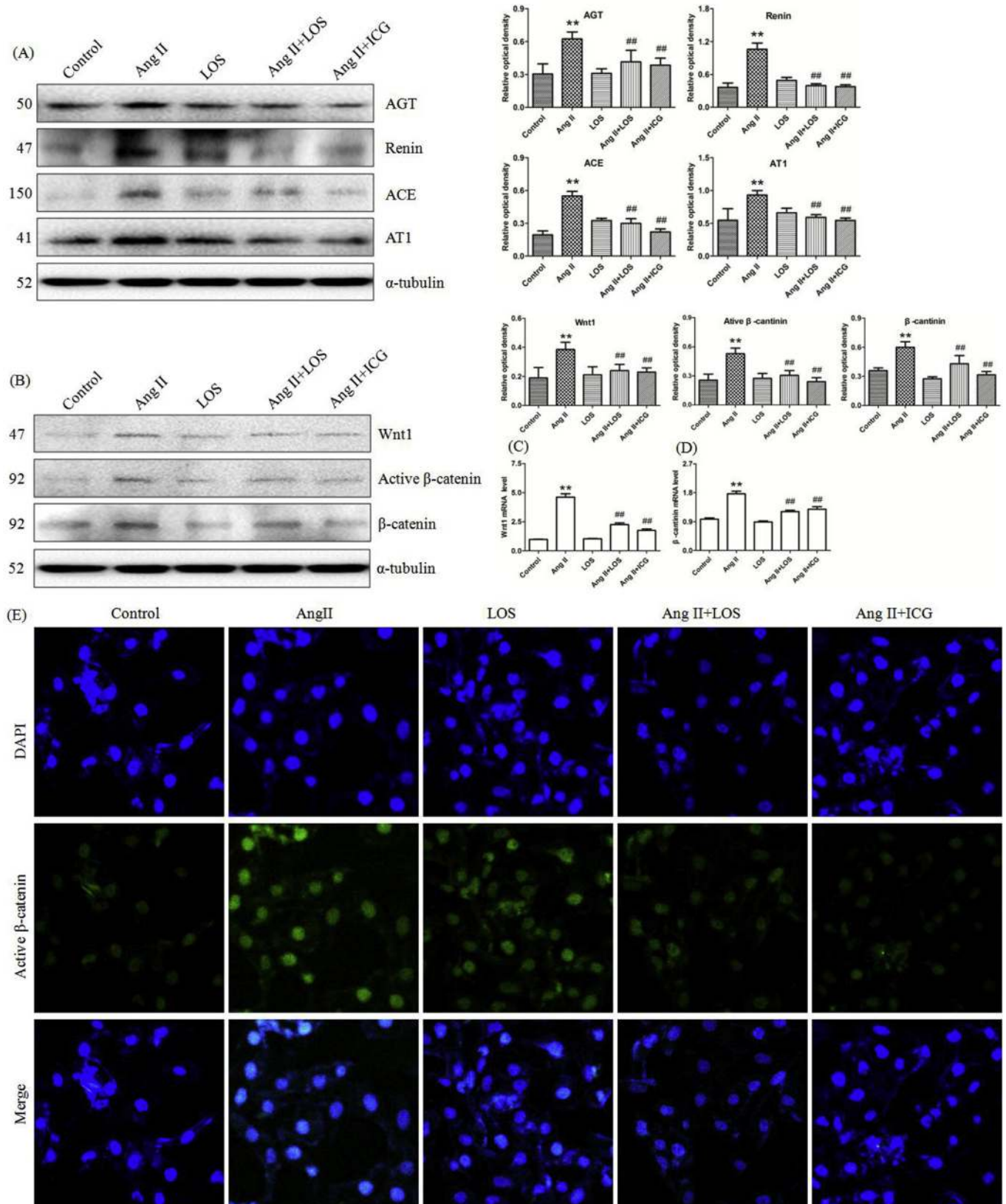


Fig. 3. Ang II (1 μ M) increases RAS protein levels including AGT, rennin, ACE and AT1 (A) and up-regulates Wnt1 and active β -catenin protein levels (B) in HK-2 cells and this effect was ameliorated by angiotensin II type 1 receptor blocker losartan (1 μ M) and β -catenin inhibitor ICG-001 (10 μ M) in 12 h. qRT-PCR analysis shows that Ang II (1 μ M) increases Wnt1 (C) and β -catenin (D) mRNA levels and this effect was blocked by losartan and ICG-001. * p < 0.05, ** p < 0.01 as compared with untreated control; # p < 0.05, ## p < 0.01 as compared with Ang II-treated cells individually. (E) Representative immunofluorescent staining images indicating β -catenin underwent nuclear translocation in Ang II-induced HK-2 cells and these changes were ameliorated by losartan and ICG-001 treatment. Cultured HK-2 cells were counterstained with DAPI for nuclear visualization.

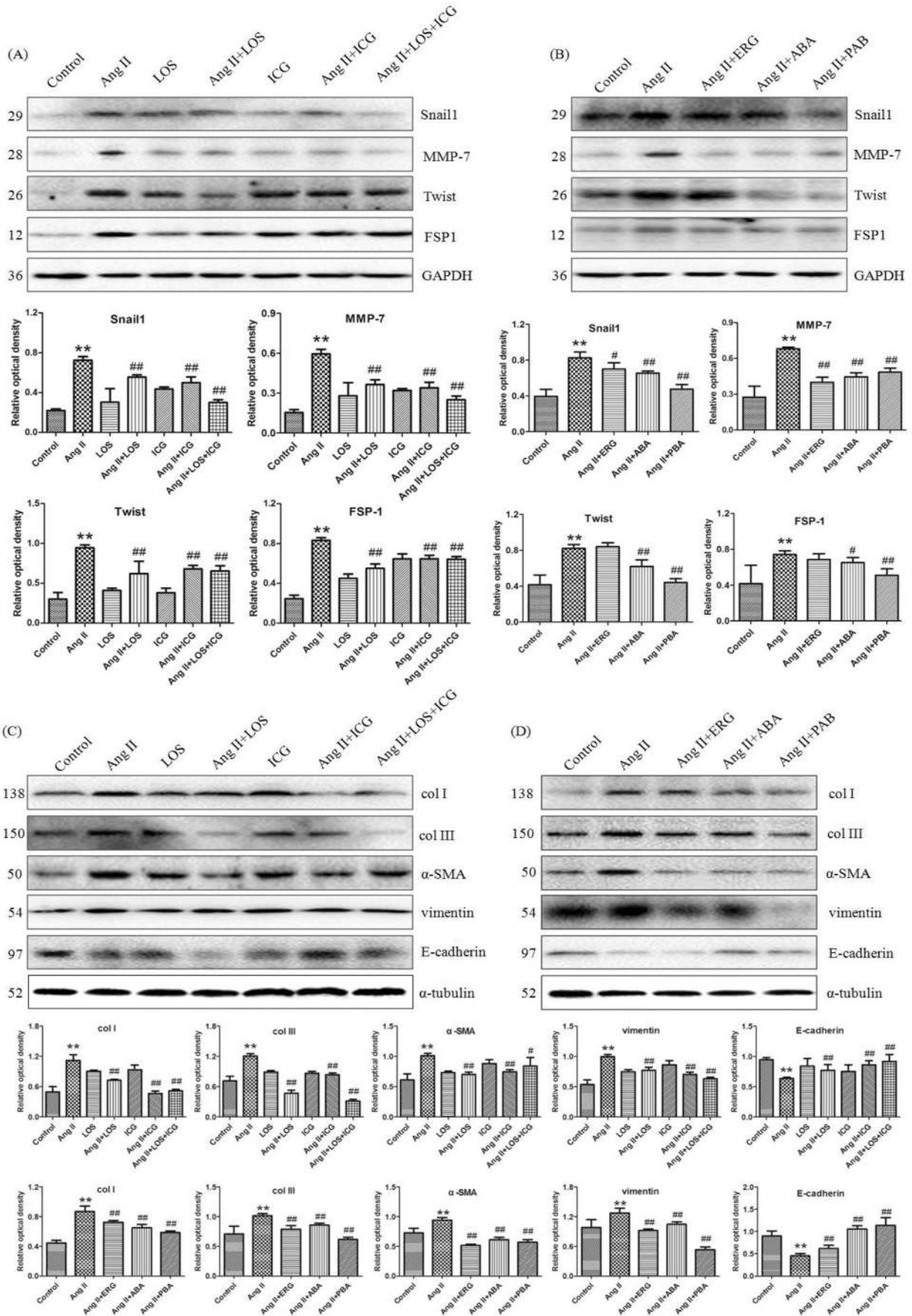


Fig. 4. Expression of β -catenin target proteins and ECM proteins was induced by Ang II (1 μ M) in HK-2 cells in 12 h. (A) Losartan and ICG-001 inhibit β -catenin target proteins sail1, MMP-7, twist and FSP-1 expression. (B) Ergone ABA and PAB inhibit sail1, MMP-7, twist and FSP-1 protein expression. (C) Losartan and ICG-001 inhibit fibrotic proteins col I, col III, α -SMA, fibronectin and vimentin expression and restore E-cadherin expression. (D) Ergone ABA and PAB inhibit fibrotic proteins col I, col III, α -SMA, fibronectin and vimentin expression and restore E-cadherin expression. Cell lysates were analyzed by specific antibodies against sail1, MMP-7, twist, FSP-1, col I, col III, α -SMA, fibronectin, vimentin, α -tubulin and GAPDH.

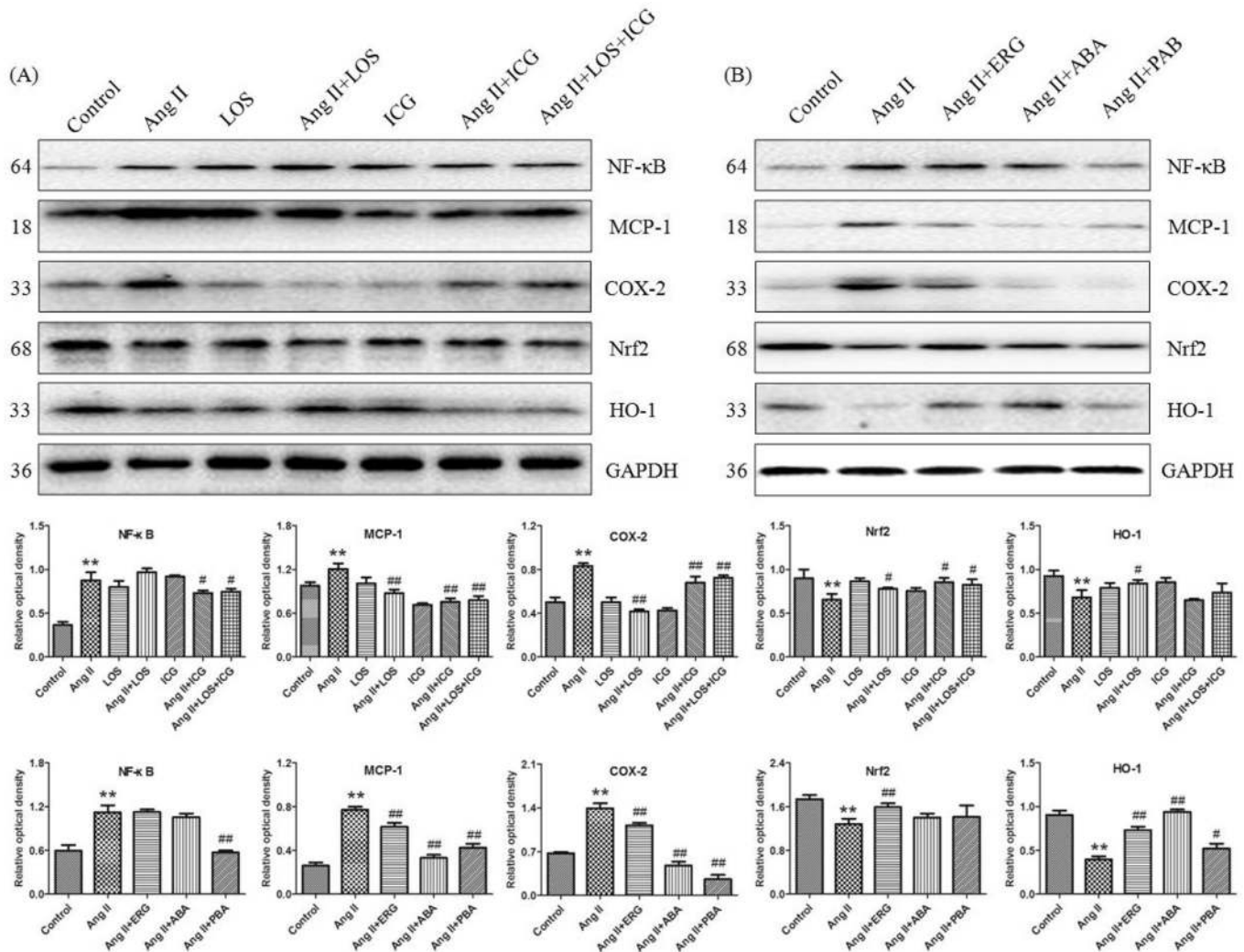


Fig. 5. Activated RAS/Wnt/ β -catenin axis was accompanied by oxidative stress and inflammation in cultured HK-2 cells. (A) Losartan and ICG-001 inhibit NF- κ B, MCP-1 and COX-2 expression and restore Nrf2 and HO-1 expression. (B) Ergone, ABA and PBA inhibit NF- κ B, MCP-1 and COX-2 expression and restore Nrf2 and HO-1 expression. Cell lysates were analyzed by specific antibodies against NF- κ B, MCP-1, COX-2, Nrf2, HO-1 and GAPDH.

to prevent kidney injury and dysregulation. This process is mediated by activation of Nrf2 which regulates and coordinates induction of many genes that encode different anti-oxidants and phase 2 detoxifying enzymes and proteins. We treat HK-2 cells with Ang II in the presence or absence of losartan, ICG-001, ERG, ABA and PBA. The results show a dramatical activation of NF- κ B in Ang II treated cells. Activation of NF- κ B is accompanied by significant up-regulation of inflammatory proteins MCP-1 and COX-2 and down-regulation of anti-oxidant system including Nrf2 and HO-1 (Fig. 5). Treatment with losartan, ICG-001, ERG, ABA and PBA inhibit Ang II-induced up-regulation of pro-inflammatory proteins NF- κ B, MCP-1 and COX-2 expression and restore Nrf2 and HO-1 expression in Ang II-treated HK-2 cells (Fig. 5).

3.5. Induction of Wnt/ β -catenin signaling pathway by Ang II in podocytes

As shown in Fig. 6, among several Wnts, a significantly increase of Wnt1, Wnt2, Wnt4, Wnt9a and Wnt11 mRNA expression are observed in podocytes after Ang II (1 μ M) treatment in the time-dependent manner in 12 h. The mRNA expression of Wnt7b, Wnt8a and Wnt10b are also increased at different time points.

However, Wnt2b and Wnt3 expressions are not altered in 48 h. Similarly, the mRNA expression showed a significantly increase of Wnt1, Wnt2, Wnt3a, Wnt7b, Wnt8a and Wnt10b mRNA expression in podocytes treated with Ang II in the dose-dependent manner from 0 μ M to 1.0 μ M at 6 h. Several other Wnts including Wnt4, Wnt5a, Wnt9a and Wnt16 are also significantly increased at later time points. These results demonstrate the ability of Ang II to induce Wnt mRNA expression in podocytes in time and dose-dependent manner. As shown in Fig. 7, Western blot analysis demonstrates the ability of Ang II to up-regulate protein expression of Wnt1 and active β -catenin in podocytes, especially in 1 μ M in 6 h. Immunofluorescent staining further shows that active β -catenin expression is dramatically increased in 1.0 μ M Ang II-treated podocytes at 6 h (Fig. 7E). Taken together, these findings demonstrate that Wnt/ β -catenin signaling pathway is activated by Ang II treatment in podocytes.

3.6. Wnt/ β -catenin signaling is involved in the Ang II-induced RAS and induces podocyte injury

To further determine whether activated RAS interacts with Wnt/ β -catenin signaling, podocytes are treated by Ang II (1 μ M) in the

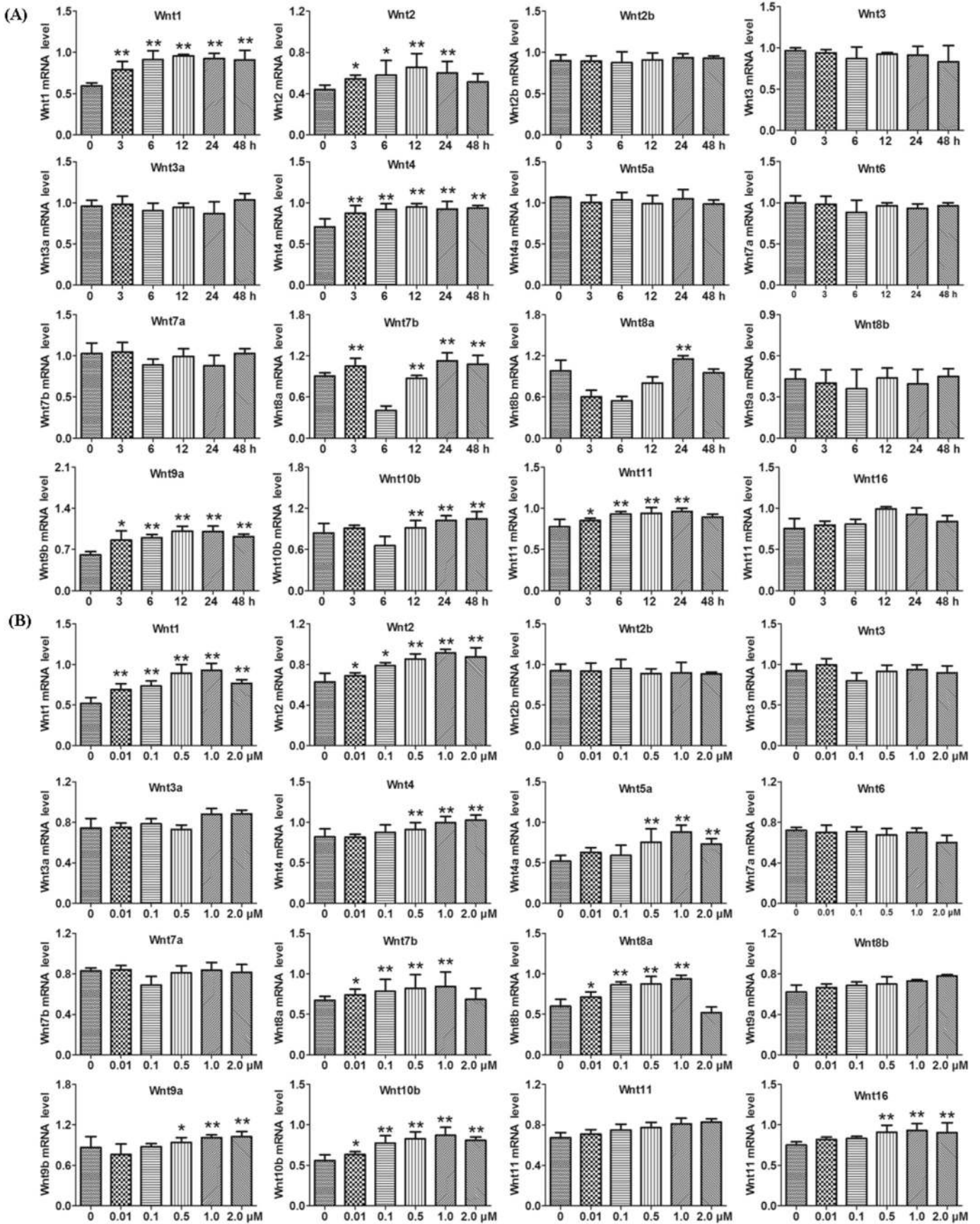


Fig. 6. Real time PCR demonstrated a time-dependent (A) and dose-dependent (B) change in expression of various Wnt mRNAs in podocytes.

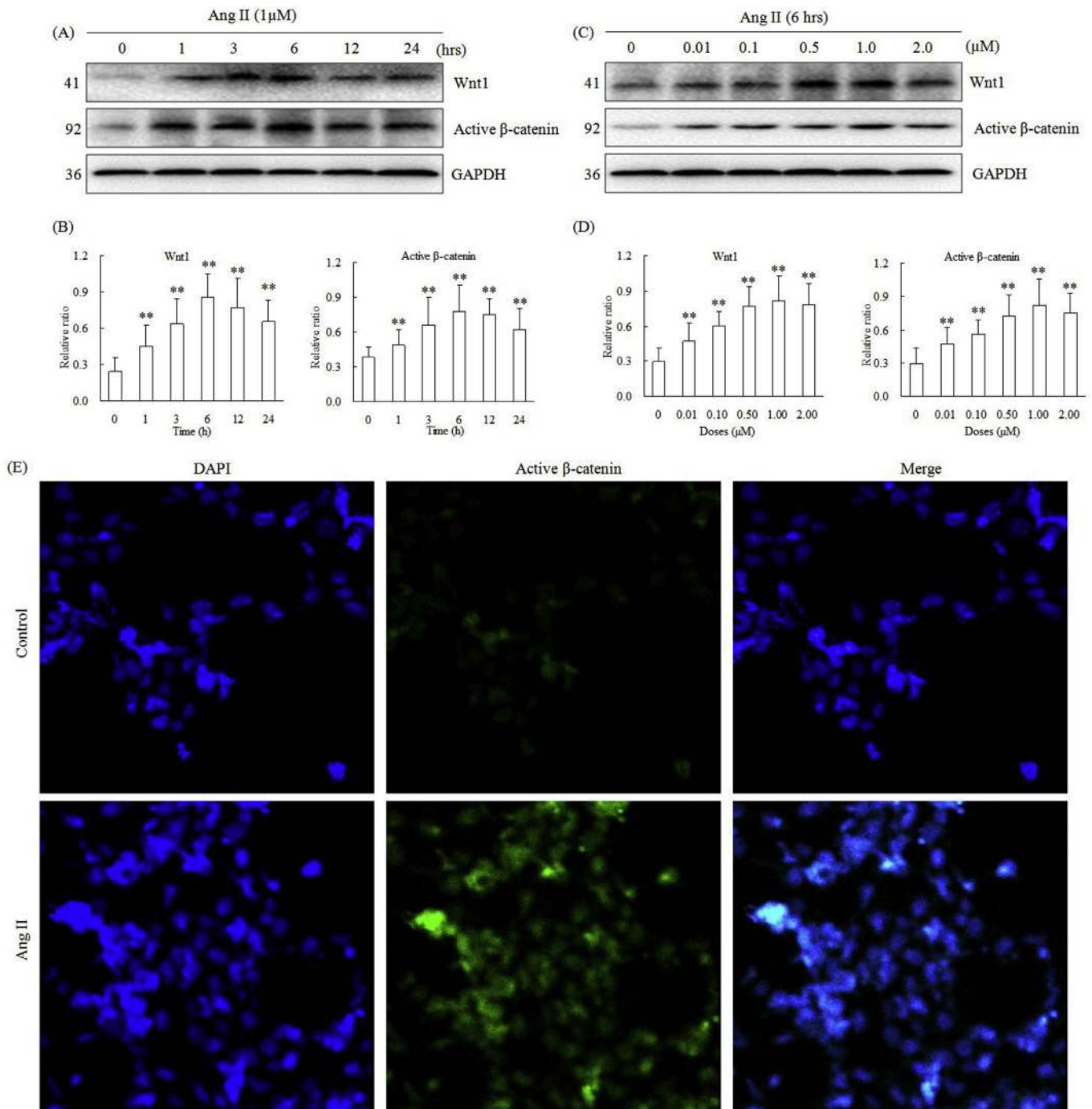


Fig. 7. Time (A and B) and dose (C and D) courses of wnt1 and active β -catenin expression levels in the podocytes incubated with Ang II. Ang II increased wnt1 and active β -catenin at 1, 3, 6, 12, 24 and 48 h, and Ang II also increased wnt1 and active β -catenin at 0.01, 0.1, 0.5, 1.0 and 2.0 μ M $^*P < 0.05$, $^{**}P < 0.01$ compared to the control (n = 6). (E) Representative immunofluorescent staining images indicating β -catenin underwent nuclear translocation in Ang II-induced podocytes. Podocytes were counterstained with DAPI for nuclear visualization.

presence or absence of losartan and ICG-001. Ang II treatment substantially induces the increased expression of AGT, rennin, ACE and AT1 proteins in podocytes and losartan inhibits expressions of these proteins in podocytes (Fig. 8A). The results demonstrated that RAS is activated by Ang II treatment. Ang II treatment also induces up-regulation of Wnt1, active β -catenin and β -catenin in podocytes. Ang II-mediated up-regulations of these proteins are inhibited by losartan and ICG-001 (Fig. 8B). qRT-PCR results revealed that Wnt1

and β -catenin mRNA levels are induced at 6 h after Ang II treatment in podocytes and Ang II-induced Wnt1 and β -catenin mRNA expression are inhibited by losartan and ICG-001 (Fig. 8C and D). Immunofluorescence staining showed a clear nuclear translocation of active β -catenin and its dramatically increased expression in Ang II-treated podocytes. These changes are ameliorated by losartan and ICG-001. Taken together these results demonstrated that Wnt/ β -catenin signaling pathway is activated by Ang II treatment and

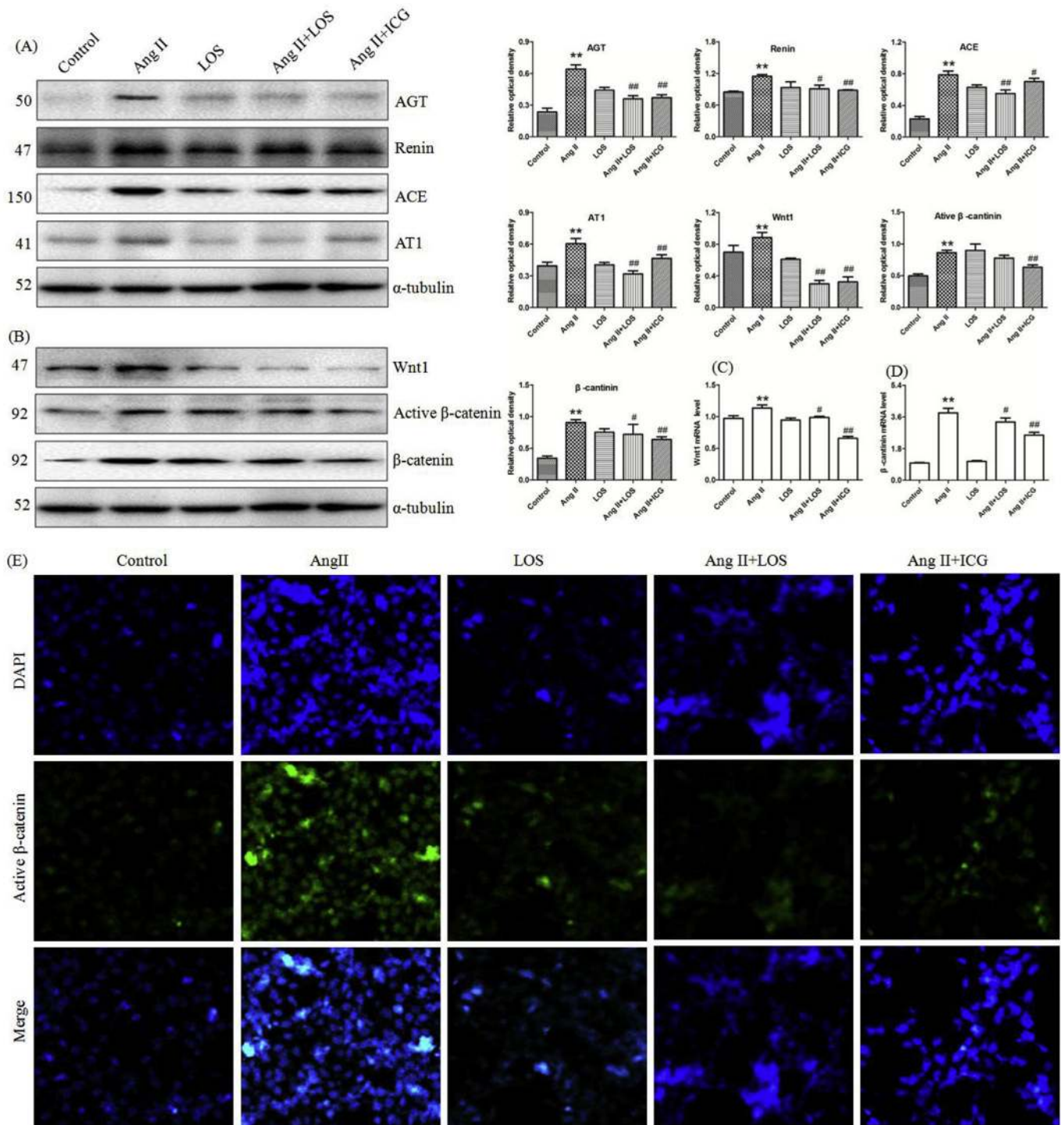


Fig. 8. Ang II (1 μ M) increases RAS protein levels including AGT, rennin, ACE and AT1 (A) and up-regulates Wnt1 active β -catenin and β -catenin protein levels (B) in podocytes and this effect was ameliorated by losartan (1 μ M) and ICG-001 (10 μ M) in 6 h qRT-PCR analysis shows that Ang II (1 μ M) increases Wnt1 (C) and β -catenin (D) mRNA levels and this effect was blocked by losartan and ICG-001. * $P < 0.05$, ** $P < 0.01$ as compared with untreated control; # $P < 0.05$, ## $P < 0.01$ as compared with Ang II-treated cells individually. (E) Representative immunofluorescent staining images indicating β -catenin underwent nuclear translocation in Ang II-induced podocytes and these changes were ameliorated by losartan and ICG-001 treatment. Cultured cells were counterstained with DAPI for nuclear visualization.

blockade of RAS could abolish activation of Wnt/ β -catenin signaling pathway. Additionally, our study showed that Wnt/ β -catenin inhibitor ICG-001 not only inhibits up-regulating Wnt/ β -catenin signaling, but also inhibits expression of RAS protein including AGT, rennin, ACE and AT1 in podocytes (Fig. 8). Thus, RAS and Wnt/ β -catenin signaling are inseparably linked as they form a vicious cycle

in which RAS activates Wnt/ β -catenin signaling and Wnt/ β -catenin signaling provokes up-regulation of RAS which causes arterial blood pressure. Taken together, these findings demonstrate the interaction of RAS and Wnt/ β -catenin signaling in promoting podocyte injury.

3.7. Ang II induces podocyte injury by activating Wnt/ β -catenin signaling

To investigate whether Ang II-induced podocyte injury is mediated by Wnt/ β -catenin signaling, we examined the expression of β -catenin target proteins in podocyte. The β -catenin target proteins sail1, MMP-7, twist and FSP-1 are dramatically increased in podocytes by Ang II treatment (Fig. 9A). Podocyte-specific proteins, such as podocin, nephrin, podocalyxin, synaptopodin and WT1 are dramatically reduced and desmin and AKT2 were dramatically elevated in Ang II-treated podocyte (Fig. 9C). Next, we determine the effects of losartan and ICG-001 on β -catenin target proteins in podocyte. Ang II-mediated up-regulation of sail1, MMP-7, twist and FSP-1 are inhibited by losartan and ICG-001 (Fig. 9A). We evaluate the effects of ERG, ABA and PBA on the Ang II-induced β -catenin target proteins. Ang II-mediated up-regulation of sail1, MMP-7, twist and FSP-1 are inhibited by ERG, ABA and PBA (Fig. 9B). Abnormal up-regulating and down-regulating podocyte-specific proteins by Ang II are inhibited by ERG, ABA and PBA (Fig. 9D). Taken together these findings indicated that ERG, ABA and PBA can ameliorate podocyte injury by Wnt/ β -catenin signaling pathway.

3.8. Activation of RAS/Wnt/ β -catenin axis is accompanied by oxidative stress and inflammation in podocytes

We treat podocytes with Ang II in the presence or absence of losartan, ICG-001, ERG, ABA and PBA. Activation of NF- κ B is accompanied by significant up-regulation of inflammatory proteins MCP-1 and COX-2 and down-regulation of anti-oxidant system including Nrf2 and HO-1 (Fig. 10). In contrast, losartan, ICG-001, ERG, ABA and PBA down-regulate inflammatory proteins; NF- κ B, MCP-1 and COX-2 expression and restore Nrf2 and HO-1 expressions in Ang II-treated podocytes (Fig. 10).

4. Discussion

In the present study, we demonstrate that Ang II promotes canonical Wnt/ β -catenin signaling activation and causes HK-2 cell and podocyte injury. Ang II induces activation of RAS in the cultured HK-2 cells and podocytes. Various Wnts mRNA levels and β -catenin target protein expressions including sail1, MMP-7, twist and FSP-1 are up-regulated by activated RAS. Ang II activates RAS/Wnt/ β -catenin axis and causes interaction of activated RAS and Wnt/ β -catenin signaling, which leads to HK-2 cell and podocyte injury. Inhibition of these signaling cascades by losartan, ICG-001, ERG, ABA and PBA ameliorate the Ang II-triggered EMT in HK-2 cell and podocyte injury *in vitro*. Interestingly, the combination of RAS and β -catenin inhibitors is more effective in suppressing abnormal proteins expressions than each one of them alone. This study provides a mechanistic insight into the RAS/Wnt/ β -catenin signaling in Ang II-induced EMT in HK-2 cell and podocyte injury.

RAS activation by Ang II accelerates renal injury by various mechanisms. In addition to causing hypertension, Ang II was demonstrated to induce TGF- β expression and provoke oxidative stress and inflammation, which are main factors in the initiation, development and progression of CKD [40]. Many investigations suggest that local intrarenal RAS activation contributes to kidney tissue injury [41,42]. Although activated RAS has an important effect on the pathogenesis of tubulo-interstitial fibrosis and podocyte injury, the mechanism by which Ang II induces EMT and podocyte injury remains complex and poorly understood. The present study

demonstrate that RAS activation causes EMT and podocyte injury by Wnt/ β -catenin signaling, a pathway that plays a fundamental role in kidney development and injury repair [10]. Although it is silent in the normal adult kidneys, Wnt/ β -catenin is re-activated in CKD. Our results indicate that activation of RAS/Wnt/ β -catenin axis causes expression of collagen I, collagen III, α -SMA, vimentin and E-cadherin in HK-2 cells and the expression of podocin, nephrin, podocalyxin, synaptopodin, WT1, AKT2 and desmin in podocytes. Previous study demonstrated that Ang II is the key fibrogenic factor in kidney injury and its effects are associated with cell cycle dysregulation [43]. Chronic infusion of Ang II induces ECM accumulation including fibronectin over-expression and collagen I deposition in renal interstitium [44]. Renal biopsies from adult patients with CKD have shown up-regulation of α -SMA and vimentin protein expression in tubular epithelial cells [45,46]. The mechanism may involve Akt and GSK-3 phosphorylation associated with early disruption of E-cadherin and β -catenin membrane colocalization with E-cadherin translocation to endosomes and β -catenin translocation to the nucleus [47]. Because the dysregulation of the evolutionarily conserved Wnt/ β -catenin signaling pathway is implicated in the development of fibrotic processes [47,48], we further demonstrated that Ang II induces profibrotic protein expression via Wnt/ β -catenin signaling pathway activation. The inhibition of RAS by losartan can inhibit Wnt/ β -catenin signaling and inhibition of Wnt/ β -catenin signaling by ICG-001 can, in turn, inhibit RAS and ameliorate Ang II-induced expression of profibrotic proteins and β -catenin target proteins. Notably, expression of β -catenin target proteins, sail1, MMP-7, twist and FSP-1, which are implicated in regulating cell proliferation correlated with β -catenin stabilization and are inhibited by ERG, ABA and PBA isolated from natural products.

Podocytes undergo phenotypic alterations including hypertrophy, dedifferentiation, apoptosis and detachment from the glomerular basement membrane. Podocytes can be detected in urine of diabetic patients. Alteration of podocyte phenotype causes podocyte dysfunction and albuminuria. Podocytes possess a local RAS that includes AT1 receptor [49,50]. Ang II decreases the ultra-filtration coefficient and increases glomerular capillary perm selectivity thereby causes albuminuria. Previous studies have shown that Ang II has a direct effect on podocyte functions via the AT1 receptor [51,52]. Accumulated evidence indicates that RAS repression can reduce the extent renal injury, in part, by alleviating podocyte injury. The present study demonstrated that Ang II causes significant reductions of podocin, nephrin, podocalyxin, synaptopodin and WT1 expression as well as elevation of desmin and AKT2 expression in podocytes. These findings indicated that Ang II could trigger phenotypic alteration and dysfunction in podocytes, which are consistent with the reported findings in experimental animal [53,54]. Inhibition of RAS with losartan and blockade of Wnt/ β -catenin with ICG-001 largely restored Ang II-mediated podocyte injury. These observations elucidated the role of RAS/Wnt/ β -catenin signaling cascade in mediating the Ang II-induced podocyte injury. Our study revealed that activation of Wnt/ β -catenin signaling regulates its downstream target genes expression, such as sail1, MMP-7, twist and FSP-1. Targeting the Wnt signaling pathway with ICG-001, a small molecule inhibitor, can block Ang II-induced podocyte injury and albuminuria [55]. Here, our findings showed that Ang II could activate canonical Wnt/ β -catenin signaling, and blocking this signaling can mitigate Ang II-induced phenotypic alterations of podocytes. ICG-001 and natural products ERG, ABA and PBA abolished Ang II-induced up-regulation of β -catenin downstream target proteins, and mitigated podocyte injury. Furthermore, we

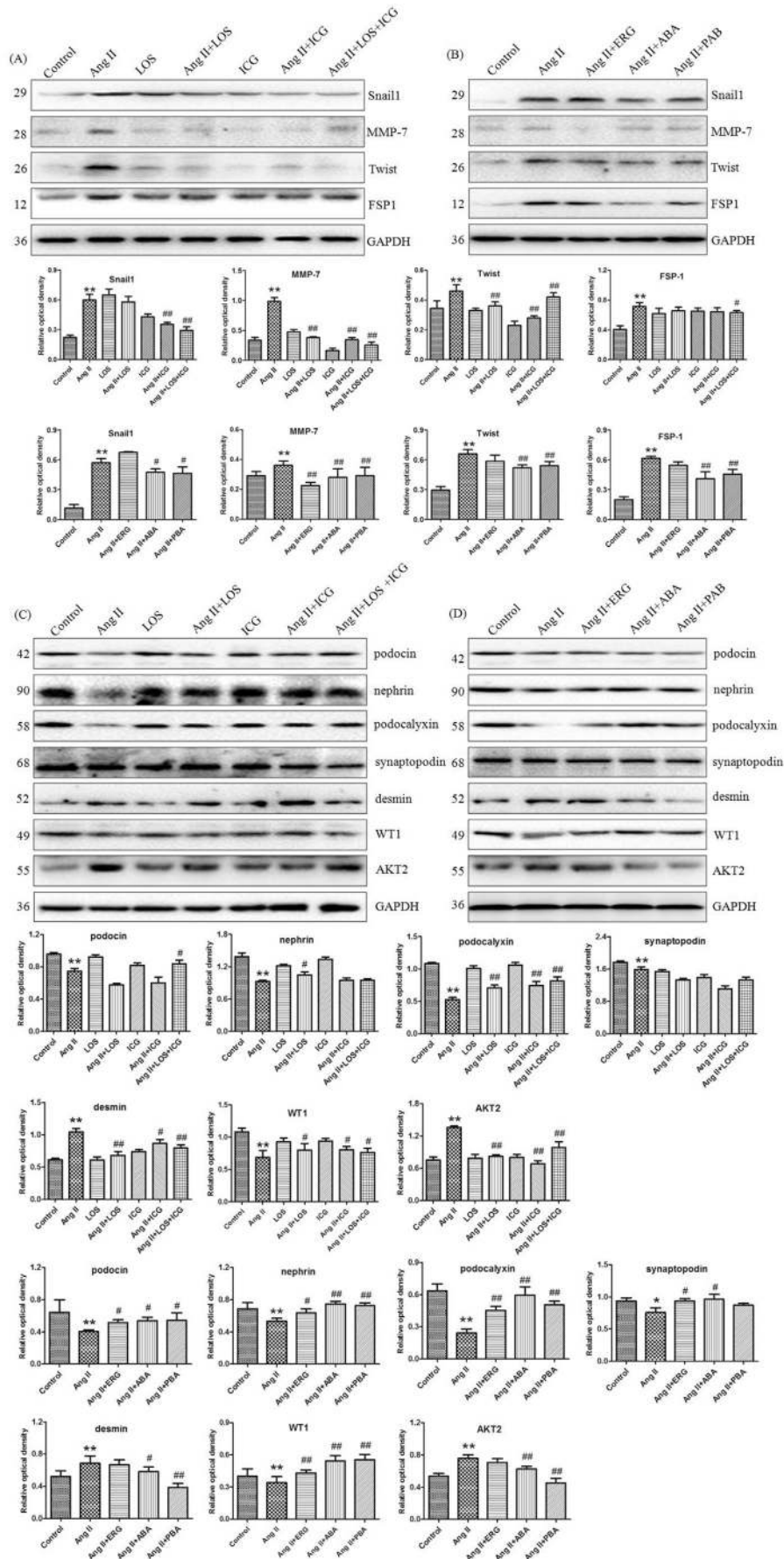


Fig. 9. Expression of β -catenin target proteins and podocyte-specific proteins was induced by Ang II (1 μ M) in podocytes in 6 h. (A) Losartan and ICG-001 inhibit β -catenin target proteins sail1, MMP-7, twist and FSP-1 expression. (B) Ergone ABA and PAB inhibit sail1, MMP-7, twist and FSP-1 protein expression. (C) Losartan and ICG-001 restores podocyte-specific proteins and inhibits desmin expression. (D) Ergone ABA and PAB restore podocyte-specific proteins and inhibit desmin expression. Cell lysates were analyzed by specific antibodies against sail1, MMP-7, twist, FSP-1, podocin, nephrin, podocalyxin, synaptopodin, WT1, AKT2, desmin and GAPDH.

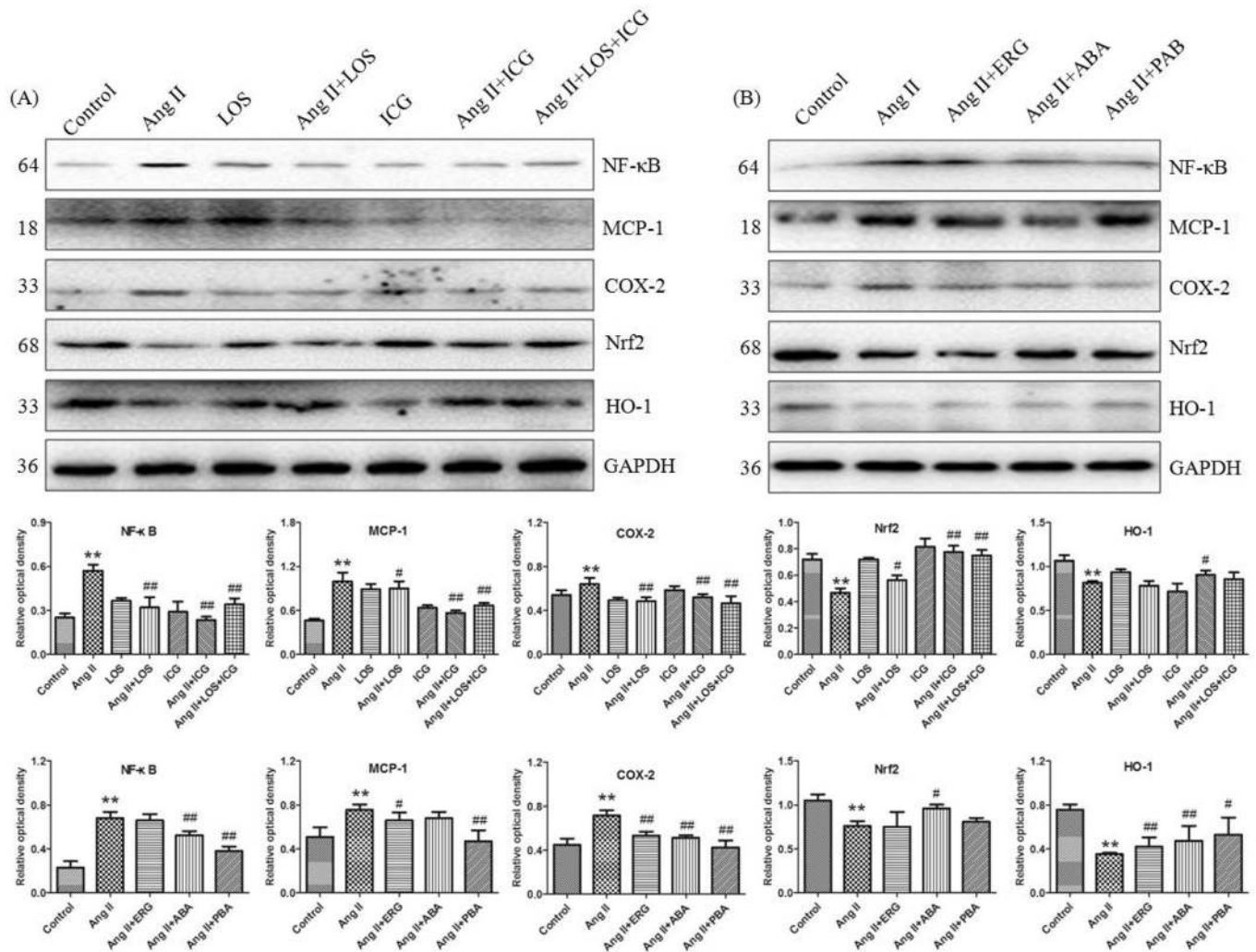


Fig. 10. Activated RAS/Wnt/ β -catenin axis was accompanied by oxidative stress and inflammation in podocytes. (A) Losartan and ICG-001 inhibit NF- κ B, MCP-1 and COX-2 expression and restore Nrf2 and HO-1 expression. (B) Ergone, ABA and PAB inhibit NF- κ B, MCP-1 and COX-2 expression and restore Nrf2 and HO-1 expression. Cell lysates were analyzed by specific antibodies against NF- κ B, MCP-1, COX-2, Nrf2, HO-1 and GAPDH.

found that inhibiting RAS with losartan block Ang II-induced activation of Wnt/ β -catenin signaling. Taken together these findings indicate that RAS/Wnt/ β -catenin axis is involved in the Ang II-induced podocyte injury.

Currently, ACE inhibitors and AT1 receptor blockers are the main RAS inhibitors used for the treatment of patients with CKD. However, they can cause compensatory up-regulation of renin. Many reports show that renin not only enzymatically converts angiotensinogen to angiotensin I but also binds to prorenin or renin receptor, which provokes many signaling events that cause induction of fibrosis-related proteins [56]. Therefore, the inhibition of RAS/Wnt/ β -catenin axis can be more effective for treatment of CKD. In this context, inhibition of the RAS/Wnt/ β -catenin signaling cascade with the natural products, ERG, ABA and PBA could be a potentially feasible strategy for CKD treatment.

In conclusion, Ang II-induced activation of RAS signaling cascade triggers activation of the canonical Wnt/ β -catenin signaling pathway which can cause tubule-interstitial fibrosis and podocyte injury. The reciprocal inhibition of RAS and Wnt/ β -catenin signaling pathway provides a novel way to find more effective therapy for renal injury, by targeting the RAS/Wnt/ β -catenin signaling cascades.

Conflict of interest statement

All authors state that they have no conflicts of interest to declare.

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Transparency document

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