Full Length Research Paper

Aldosterone induces apoptosis via the Wnt signalling pathway

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Evidence suggests that aldosterone (ALD) is involved in glomerular damage; however, it is rarely known whether ALD exerts a direct injurious effect on mesangial cells (MC). The objectives of this study were to determine the relationship between ALD and apoptosis, and investigate the cell signalling pathway by which ALD induces apoptosis. Rat MC were treated with ALD (10^{-8}, 10^{-7} or 10^{-6} M) for 24 h. In some experiments, MC were pretreated with 10^{-7} M spironolactone or 10^{-2} M LiCl for 1 h. Apoptosis was quantified using Annexin V-propidium iodide staining and flow cytometry, and caspase 3 activity was analysed. Gene and protein expression were quantified using quantitative real-time PCR and Western blotting, respectively. ALD directly induced apoptosis in MC in a caspase dependent manner. More importantly, Wnt signalling was involved in ALD-induced cell apoptosis. ALD suppressed the Wnt signalling pathway in MC, leading to downregulation of *Wnt4* and *Wnt5a* mRNA expression, increased GSK-3β protein expression and reduced β-catenin protein expression. A competitive antagonist of ALD, spironolactone (SPI), attenuated the ability of ALD to inhibit Wnt signalling. The Wnt signalling agonist, LiCl, inhibited ALD-induced apoptosis. This study suggests that ALD may directly induce apoptosis in MC via the Wnt signalling pathway. Modulation of Wnt signal transduction may be beneficial for enhancing mesangial cell survival in renal injury.

Key words: Aldosterone, apoptosis, β-catenin, GSK-3β, mesangial cells.

INTRODUCTION

Aldosterone (ALD) is an important mediator of the renin-angiotensin-ALD system (RAAS) and plays a pivotal role in the regulation of salt and extracellular fluid metabolism (Chen et al., 2009). Accumulating evidence suggests that ALD is a key factor which mediates renal injury (Ando et al., 2010; Diah et al., 2008; Fan et al., 2011). Moreover, inhibitors of ALD, such as spironolactone (SPI), can attenuate renal injury in human nephropathies and various experimental animal model of nephropathy (Aldigier et al., 2005; Rocha et al., 2000; Zhu et al., 2009). Several experimental reports suggest that ALD, in addition to exerting hemodynamic effects, may also directly contribute to the occurrence of mesangial cell

(MC) apoptosis (Mathew et al., 2008).The Wnt signalling pathway is essential during development and acts as a regulator of embryonic cell patterning, proliferation, differentiation, cell adhesion, cell survival and apoptosis (Bridgewater et al., 2011; Merkel et al., 2007; Vinas et al., 2010; Zeilstra et al., 2011). Wnt signalling regulates the early and late stages of apoptosis in neurons, endothelial cells, vascular smooth muscle cells and cardiomyocyte cell populations during both development and cellular injury (Li et al., 2006). Recently, Lin et al. (2006) suggested that Wnt signalling also modulates the survival of high glucose-stressed MC.

We hypothesized that Wnt signalling may be involved in regulating the fate of MC exposed to ALD. The aims of this study were to investigate whether ALD can induce apoptosis and alter Wnt signalling in MC, and determine whether modulation of Wnt signalling affects ALD-induced apoptosis in MC. This may reveal new, promising

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targets for intervention in renal disease.

MATERIALS AND METHODS

Cell culture

Rat MC (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Rockford ,LL, USA) containing 10% foetal bovine serum (FBS, Gibco by Invitrogen, Carlsbad, CA,USA) in a 5% CO_2, 37°C incubator. All experiments were performed on cells in the logarithmic phase of growth. The cells were harvested by trypsinisation and resuspended in DMEM for further studies. Cell viability was determined using trypan blue exclusion.

Experimental treatment of rat MC

MC were cultured in 6-well plates (1 ×106 cells/well) in DMEM containing 10% FBS with or without various concentrations of ALD (10^{-8}, 10^{-7} or 10^{-6} M) for 24 h. In some experiments, sub-confluent cell cultures were pre-treated with 10^{-7} M SPI or 10^{-2} M LiCl for 1 h prior to stimulation with ALD (10^{-7} M).

Real-time PCR analysis

Total RNA was extracted and purified from 1×106 cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and 500 ng total RNA was reverse-transcribed to cDNA. PCR was performed in 20 µl reactions containing cDNA template (equivalent to 100 ng total RNA), 0.4 µM of forward and reverse primers and 2× SYBR Green PCR Master Mix (Takara Bio Inc, Japan) using the STRATAGENE MX3005P Real-time PCR Detection System (Agilent Technologies, Palo Alto, CA, USA). DNA was denatured at 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The following primer oligonucleotide sequences were used: *Wnt5a* (forward: 5'-AGC CGA GAG ACA GCC TTC AC-3'; reverse: 5'-TCC TGC GAC CTG CTT CAT TG-3'; 289 bp product expected); *Wnt4* (forward: 5'-GCC ACG CAC TAA AGG AGA AG-3'; reverse: 5'-GGC CTT AGA CGT CTT GTT GC-3'; 215 bp product expected); and β-actin (forward: 5' -CGC CAA CCG CGA GAA GAT-3'; reverse: 5'-CGT CAC CGG AGT CCA TCA-3'; 168 bp product expected). The number of amplification steps required to reach an arbitrary intensity threshold (Ct) was computed. The relative gene expression levels were presented as $2^{-\Delta Ct}$, where ΔCt = Cttarget-Ctβ-actin. Fold change for the treatment was defined as the relative expression compared to the buffer (control) and was calculated as $2^{-\Delta\Delta Ct}$, where ΔΔCt = ΔCttreatment-ΔCtbuffer (Wang et al., 2005).

Protein extraction and Western blot analysis

Cultured MC were pelleted by centrifugation, lysed in cell lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1 mM PMSF) and incubated for 30 min on ice. The cell lysates were centrifuged at 18,000 g for 10 min at 4°C and the protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, LL, USA). Equal amounts of protein (50 µg) were subjected to 8% sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes using the Bio-Rad Western blot analysis apparatus (Bio-Rad Inc., Hercules, CA, USA). The membranes were blocked in tris-buffered saline containing 0.05% Tween20 (TBS-T) and 5% non-fat milk for 1 h at room temperature, incubated overnight at 4°C with the appropriate primary antibodies against GSK-3β, β-catenin and PARP (Cell Signaling Technology Inc., Beverly, MA, USA) or β-actin (Santa Cruz Biotechnology, CA, USA) as a normalized reference, and then incubated with near-infrared (NIR) fluorophore-conjugated secondary antibodies. The membranes were scanned and analyzed using an Odyssey IR scanner and Odyssey imaging software 2.1 (LI-COR Inc., Lincoln, NE, USA). The scan settings were high image quality, 169 mm resolution, at an intensity of 3.0 to 5.0 for both channels with no offset. The signals were analyzed as the integrated intensities of the defined regions around the bands of interest in either channel.

Analysis of apoptosis using flow cytometry

MC apoptosis was quantified by flow cytometry using Annexin V-FITC and propidium iodide (PI) double staining with the Annexin V-FITC apoptosis detection kit I (Biosea Biotechnology, Haimen, China), following the manufacturer's instructions. Briefly, MC (1×106 in each treatment group) were trypsinized, washed twice with cold PBS and re-suspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl and 2.5 mM $CaCl_2$). FITC-conjugated Annexin V was added at a final concentration of 0.5 µg/ml and incubated for 20 min at room temperature in the dark; then, PI was added at 1 µg/ml and the samples were immediately analyzed by flow cytometry (BD FACSAria, Franklin Lakes, NJ, USA).

### Analysis of caspase 3 activity

Caspase-3 activity was determined using the caspase-3 activity kit (Beyotime Institute of Biotechnology, Haimen, China), based on the ability of caspase-3 to covert acetyl-Asp-Glu-Val-Asp (DEVD) p-nitroanilide into the yellow formazan product, p-nitroaniline. Following the manufacturer's protocol, treated MC (2×106) were trypsinized, washed twice with pre-cold PBS and harvested in lysis buffer (10 mM Tris HCl, pH 7.5, 10 mM Na_2HPO_4/NaH_2PO_4, 130 mM NaCl, 1% Triton-X100 and 1 mM PMSF). The cell lysates were clarified by centrifugation at 18,000 g for 20 min at 4°C, and 15 µg protein was incubated at 37°C in buffer containing 25 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.1% CHAPS and 2 mM caspase-3 substrate. Caspase-3 activity was quantified using a nucleic acid protein analyzer (DU-640, Beckman,Brea,CA, USA) at an absorbance of 405 nm and expressed as the fold-change in enzyme activity compared to the control group. Each sample was analyzed in triplicates.

Statistical analysis

Results are presented as mean ± SE. Statistical comparisons were performed using one-way ANOVA followed by the least squares differences (LSD) test using SPSS 11.0 (Chicago, IL, USA). Statistical significance was defined as $p < 0.05$.

RESULTS

Aldosterone induces apoptosis in cultured MC. To confirm the effect of ALD on MC, equal numbers of MC were incubated with either buffer (control) or various concentrations of ALD (10^{-8}, 10^{-7} or 10^{-6} M) for 24 h, then the number of apoptotic cells were quantified using flow cytometry. ALD promoted apoptosis in MC in a dose-dependent manner (Figure 1).

A

B
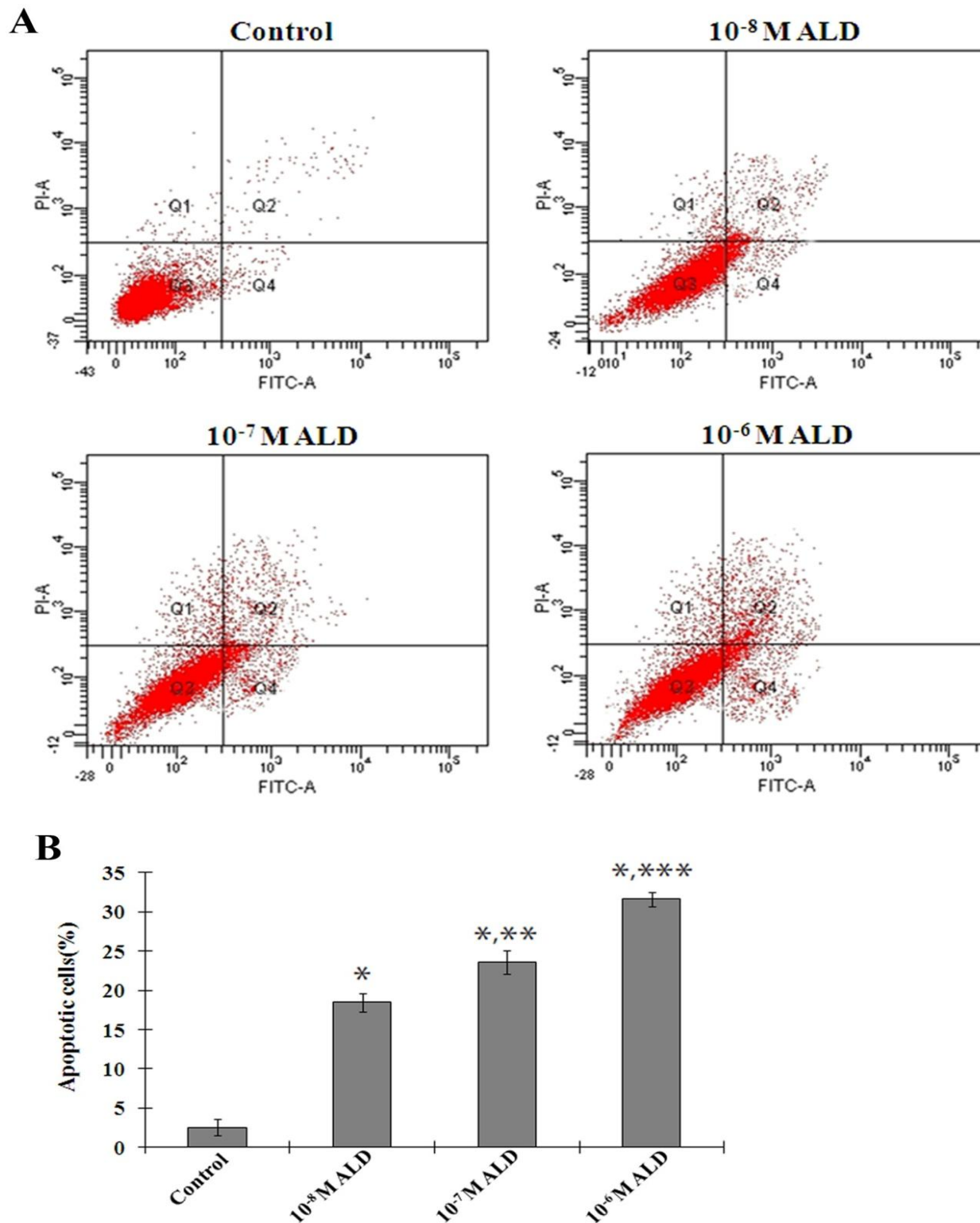
Figure 1. ALD induces dose-dependent apoptosis in MC. Equal numbers of subconfluent MC were incubated in media containing either buffer (control) or various concentrations of ALD for 24 h, and apoptosis was quantified using flow cytometry. (A) Representative Annexin V-PI staining flow cytometry plots. The X-axes represent FITC staining and the y-axes represent PI staining. The left lower quadrant (Q3) indicates Annexin V-FITC and PI negative viable cells, the right lower quadrant (Q4) indicates Annexin-V FITC positive and PI negative early-stage apoptotic cells, the right upper quadrant (Q2) indicates Annexin V-FITC and PI positive late stage apoptotic cells and the left upper quadrant (Q1) indicates PI positive dead cells. (B) Quantification of apoptosis in ALD-treated MC. Values are mean ± SE of three experiments, each carried out in triplicate; *, $p < 0.05$ vs. control; **, $p < 0.05$ vs. 10^{-8} M ALD; ***, $p < 0.05$ vs. 10^{-7} M ALD.
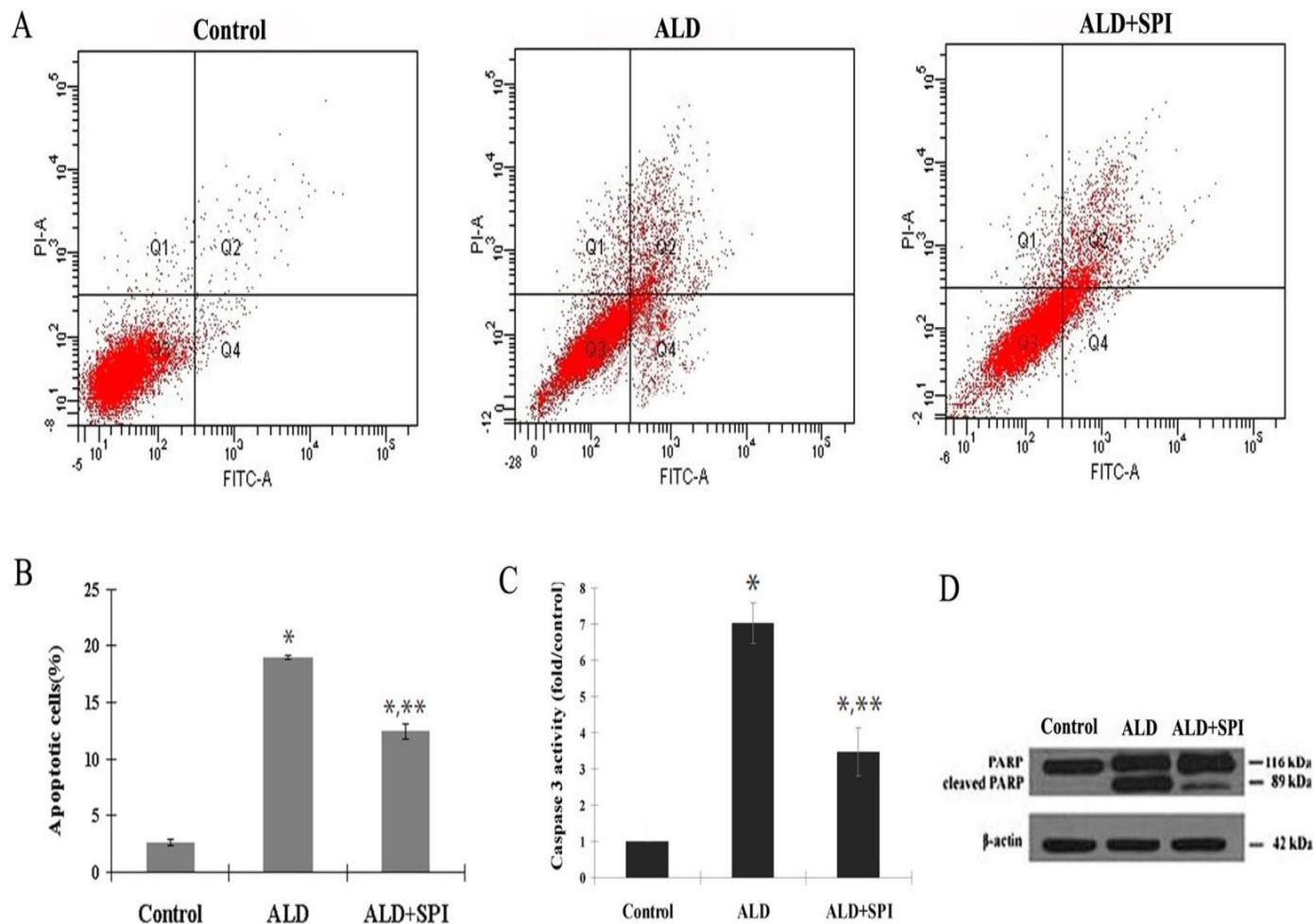
Figure 2. SPI inhibits aldosterone-induced caspase-dependent apoptosis in MC. Equal numbers of cells were pretreated for 1 h with SPI(10^{-7} M) before treatment with ALD (10^{-7} M) for 24 h. (A) Representative Annexin V-PI staining flow cytometry plots of ALD and ADL+SPI treated cells. (B) Quantification of apoptosis in ALD and ADL+SPI treated cells. (C) Caspase 3 activity assay of ALD and ADL+SPI treated cell lysates. (D) Western blot of PARP cleavage in ALD and ADL+SPI treated cells. All values are mean ± SE of three experiments, each carried out in triplicate; *, p<0.05 vs. control; **, p<0.05 vs. ALD.

SPI inhibits aldosterone-induced caspase-dependent apoptosis in MC

SPI is a steroid analogue with structural similarity to ALD; therefore, SPI functions as a competitive antagonist of ALD. To determine the role of SPI in ALD-induced MC apoptosis, equal numbers of cells were incubated in medium containing either buffer (control) or ALD (10^{-7} M) for 24 h. Alternatively, sub-confluent cell cultures were pre-treated with 10^{-7} M SPI for 1 h prior to stimulation with ALD (10^{-7} M) for 24 h. The number of apoptotic cells was quantified using flow cytometry. As shown in Figure 2a and b, ALD promoted apoptosis in MC; however, the ability for ALD to promote apoptosis MC was inhibited by SPI.

During apoptosis, the activation of effector caspases, such as caspase 3 and 7, is responsible for the proteolytic cleavage of a diverse range of structural and regulatory proteins (Salvesen and Dixit, 1997). To determine whether ALD-induced apoptosis was related to caspase-3-mediated proteolysis, we analyzed the activation of caspase-3 in ALD and ALD+SPI treated cells. The DEVD cleavage assay is a quantitative method used to detect caspase-3-like activity (Rehm et al., 2002). As shown in Figure 2c, the caspase 3 activity of lysates from ALD-treated cells was nearly seven times higher than control cell lysates. However, SPI inhibited the ability of ALD to induce caspase-3 activation. As the proteolytic cleavage of specific substrates by activated caspases is responsible for cellular dysfunction and structural destruction during apoptosis (Thornberry and Lazebnik, 1998), we analyzed the cleavage of PARP, as representative substrate, in control, ALD or ALD+SPI treated cells. PARP cleavage was observed in both ALD and ALD+SPI treated cells, leading to presence of the 89 kDa fragment which is a classical feature of apoptosis.
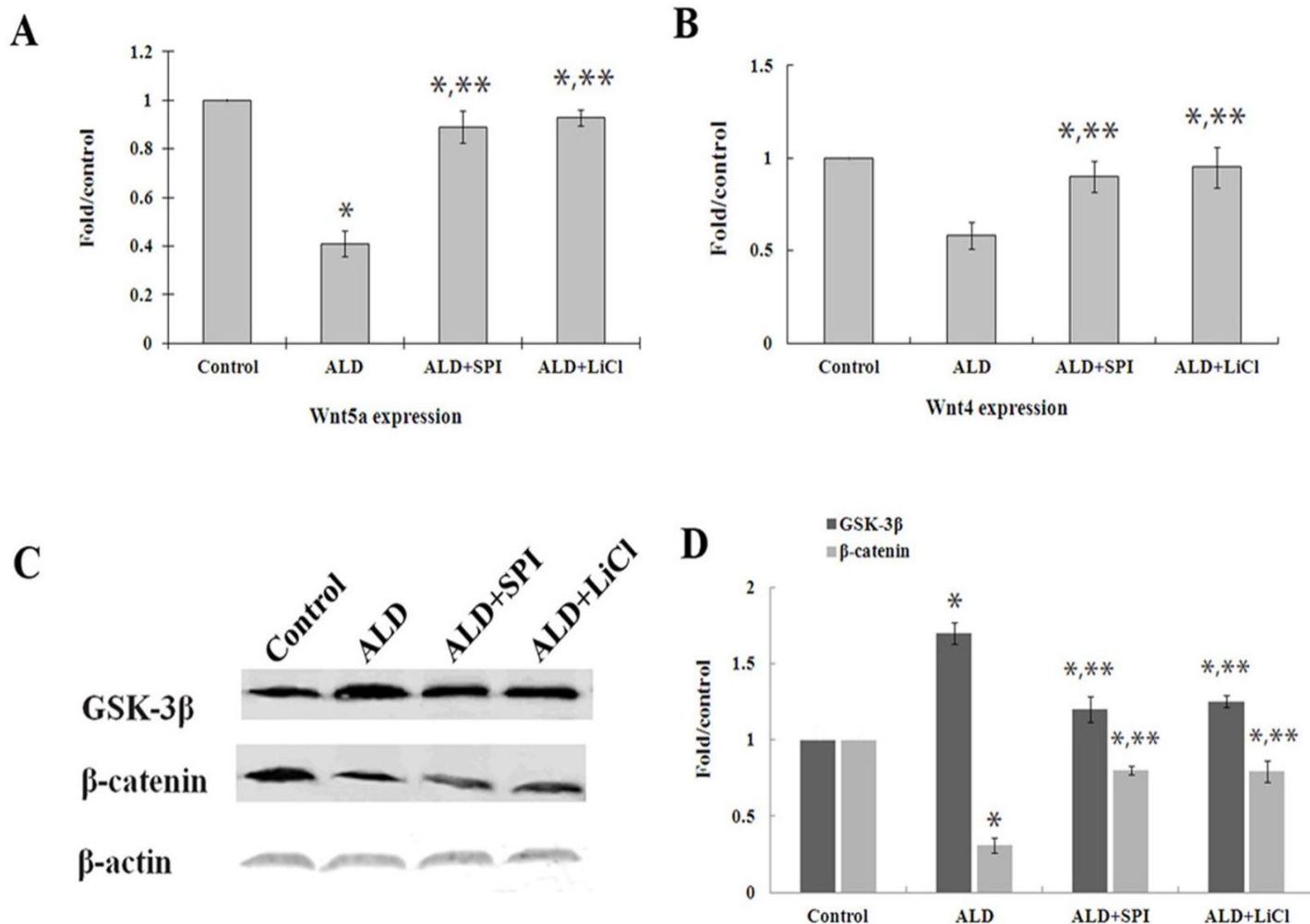
Figure 3. ALD Suppresses activation Of Wnt/B-Catenin Signalling In MC. Equal numbers of MC were pretreated for 1 h with SPI(10^{-7} M) or LiCl (10^{-2} M) before treatment with ALD (10^{-7} M) for 24 h. (A, B) Quantitative real-time PCR analysis of Wnt5a (A) and Wnt4 (B) mRNA expression in control, ALD, ALD+SPI, ALD+LiCl treated MC. (C, D) Western blot analysis of GSK-3β (C) and β-catenin (D) expression in control, ALD, ALD+SPI, ALD+LiCl treated MC. All values are mean ± SE of three experiments, each carried out in triplicate; *, $p<0.05$ vs. control; **, $p<0.05$ vs. ALD.

However, SPI significantly inhibited PARP cleavage in ALD treated MC (Figure 2d). These results strongly suggest that the caspase cascade was activated during ALD-induced cell apoptosis, and that SPI inhibited ALD-induced apoptosis in a caspase-dependent manner.

ALD suppresses activation of Wnt signalling

Next, we investigated whether ALD could alter Wnt signalling in MC. LiCl is an inhibitor of GSK-3β and can activate Wnt signalling. MC treated with LiCl served as the positive control. Real-time PCR showed that ALD significantly reduced *Wnt4* and *Wnt5a* mRNA expression in MC. SPI restored *Wnt4* and *Wnt5a* mRNA expression in ALD-treated MC, to the similar levels as control LiCL-

treated MC (Figure 3a and b). Moreover, ALD lead to a significant increase in GSK-3β protein expression and reduction in β-catenin protein expression (Figure 3c and d). SPI suppressed the ALD-mediated upregulation of GSK-3β and downregulation of β-catenin in MC (Figure 3c and d), leading to similar expression levels as control LiCL-treated MC. These results indicated that ALD inhibited the Wnt/β-catenin signalling pathway in MC, whereas the ALD inhibitor SPI attenuated the ability of ALD to inhibit Wnt signalling.

Wnt signalling agonists inhibit ALD-induced MC apoptosis; LiCl is an inhibitor of GSK-3β and activates Wnt signalling. Therefore, LiCl can be considered to be an agonist of the Wnt signalling pathway. To determine whether activation of Wnt signalling can inhibit ALD-induced MC apoptosis, equal numbers of cells were
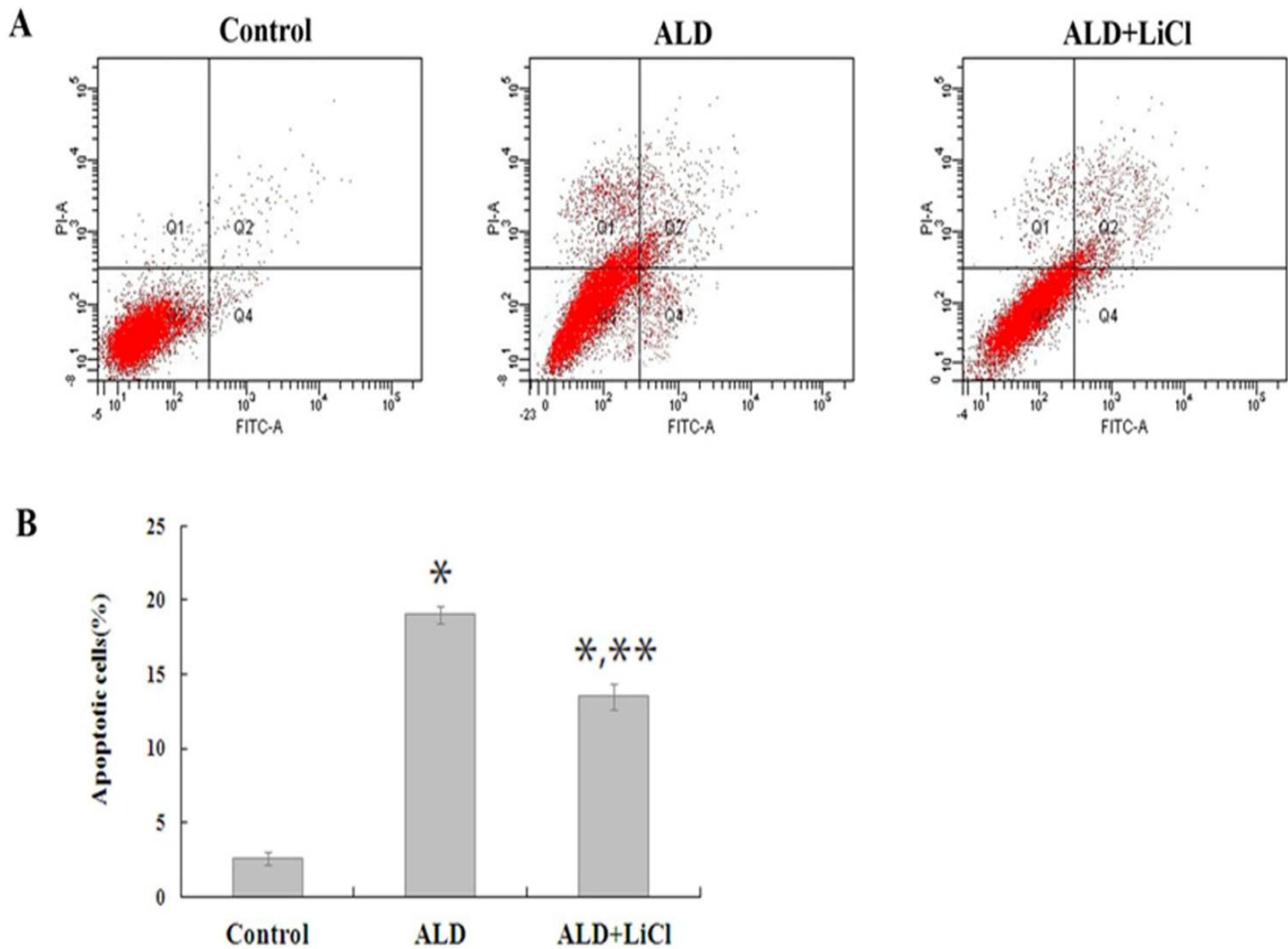
Figure 4. The Wnt signalling agonist Licl inhibits ALD-induced apoptosis in MC. Equal numbers of cells were pre-treated for 1 h with LiCl (10^{-2} M) before treatment with ALD (10^{-7} M) for 24 h. (A) Representative Annexin V-PI staining flow cytometry plots of ALD and ADL + LiCl treated cells. (B) Quantification of apoptosis in ALD and ADL + LiCl treated cells. All values are mean ± SE of three experiments, each carried out in triplicate; *, p<0.05 vs. control; **, p<0.05 vs. ALD.

incubated in medium containing either buffer, ALD (10^{-7} M) or ALD (10^{-7} M) + LiCl (10^{-2} M) for 24 h, and apoptosis was quantified by flow cytometry. As shown in Figure 4a and b, ALD-induced MC apoptosis was inhibited by the Wnt signalling agonist LiCl.

DISCUSSION

Plasma and tissue ALD levels are elevated in diabetic and other progressive nephropathies (Boldyreff and Wehling, 2003), and elevated plasma ALD may contribute to the progression of renal disease via direct actions on tubulointerstitial fibroblasts, glomerular MC and podocytes (Brilla et al., 1993; Chrysostomou and Becker, 2001; Lai et al., 2006). Molecular mechanisms including reactive oxygen species (ROS), MAPKs and Rho-kinase signalling may be involved in ALD-induced

renal injury (Huang et al., 2009; Sun et al., 2006; Toyonaga et al., 2011) however, to our knowledge, it is not known whether ALD can induce apoptosis via the Wnt signalling pathway.

This study provides the first indication that ALD induces apoptosis in MC by activation of GSK-3β and subsequent destabilization of β-catenin-responsive cell survival activity. We suggest that ALD alters the Wnt signal transduction pathway in MC, subsequently modulating molecules which regulate apoptosis or survival, leading to apoptosis. We demonstrated that ALD could directly induce apoptosis in cultured rat MC via a caspase-dependent manner. In contrast, the competitive antagonist of ALD, SPI, significantly inhibited ALD-induced MC apoptosis (Figure 2). Additionally, ALD suppressed the Wnt signalling pathway, leading to down-regulation of *Wnt4* and *Wnt5a* mRNA expression, and increased GSK-3β protein expression and reduced β-

catenin protein expression, while SPI prevented ALD-induced inhibition of Wnt signalling (Figure 3). The Wnt signalling agonist, LiCl, also inhibited ALD-induced apoptosis in MC.

Taken together, this study provides evidence to demonstrate that Wnt signalling is involved in the induction of apoptosis in MC by ALD. Modulation of Wnt signal transduction may be beneficial for enhancing the survival of MC during renal injury.

ACKNOWLEDGEMENT

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