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Aldosterone promotes fibronectin production through a Smad2-dependent TGF-β1 pathway in mesangial cells

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Abstract

Accumulating evidence demonstrates that aldosterone can cause extra-cellular matrix (ECM) accumulation, in addition to regulating sodium and potassium homeostasis. Increased extra-cellular matrix production by renal glomerular mesangial cells has been suggested to be involved in pathogenesis of glomerular sclerosis. The present studies examine whether aldosterone is also produced in renal mesangial cells, and the effect of aldosterone on ECM accumulation in these cells. In cultured renal mesangial cells, aldosterone synthase (CYP11B2), mineralocorticoid receptor (MR), and 11β-HSD2 mRNA expressions were detected by RT-PCR. The ability of renal mesangial cells to produce aldosterone was confirmed by directly detecting aldosterone in culture medium via radioimmunoassay. Real-time RT-PCR showed that the expression of CYP11B2 mRNA in mesangial cells was significantly enhanced by AngII ($P < 0.001$) and by potassium ($P < 0.05$). Exposure of the cultured mesangial cells to aldosterone significantly increased fibronectin production from 12.4 ± 1.9 to 74.6 ± 16.8 ng/ml ($P < 0.05$). The aldosterone induced fibronectin production was abolished by aldosterone receptor antagonist spironolactone. Aldosterone also increased the TGF-β1 reporter luciferase activity from 0.8 ± 0.1 to 1.7 ± 0.1 ($P < 0.05$). Immunoblot showed TGF-β1 protein expression was increased following aldosterone treatment. Blocking TGF-β1 signaling pathway by knocking down Smad2 significantly blunted the aldosterone induced fibronectin production. The present studies indicate that renal mesangial cell is a target of local aldosterone action, which promotes ECM protein fibronectin production via TGF-β1/Smad2 signaling pathway.

Keywords: Extracellular matrix; Mineralocorticoid receptor; Angiotensin II; Chronic kidney disease

Aldosterone is originally identified in the adrenal gland, and has been shown to play an important role in regulation of sodium and potassium excretion. Recent studies indicate that aldosterone is also produced in other peripheral tissues or cells, including endothelial cells, vascular smooth muscle cells, and the heart [1]. Studies suggest that the locally produced aldosterone plays an important role in promoting vascular and myocardial fibrosis, independent of its effects on hemodynamic regulation and fluid homeostasis [2–4]. Several lines of evidence also support a potential role of aldosterone in kidney damage in chronic kidney disease [5]. Animal studies show that selective blockade of aldosterone with a mineralocorticoid receptor antagonist markedly reduces glomerulosclerosis in diabetic or subtotally nephrectomized rats [6,7]. Consistent with animal studies, aldosterone receptor antagonist was reported to reduce proteinuria in patients with chronic kidney disease and diabetic nephropathies [8,9]. ACEI or angiotensin II receptor inhibitor (ARB), which also inhibits aldosterone, significantly reduced progressive decline of renal function and reduced urinary protein excretion [10–12]. However, the mechanism underlying the pathophysiologic action of aldosterone on the kidney is not completely elucidated. Renal mesangial cells are suggested to be actively involved in glomerular extra-cellular matrix (ECM) accumulation, an important pathologic process in chronic renal damage [13]. The present study, therefore, is to investigate whether glomerular mesangial cells are target of local

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aldosterone action, and the role of aldosterone in ECM production in mesangial cells.

Materials and methods

Culture of renal mesangial cells. Rat glomerular mesangial cells were obtained from glomeruli isolated from male Sprague–Dawley rats using differential sieving methods as previously described [14]. Cells were cultured in RPMI 1640 medium supplemented with 10% calf serum (Invitrogen, Carlsbad, California), penicillin (100 U/ml), and streptomycin (100 μg/ml). After serum starvation for 12 h, cells were treated with (1) various doses of angiotensin II (Sigma, St. Louis, MO) as indicated with or without 10^{-5} M losartan (Merck, Sharp and Dohme, USA) for 48 h; (2) 7 mM potassium for 48 h; or (3) 10^{-7} M aldosterone (Sigma, St. Louis, MO) with or without 10^{-9} M spironolactone (Sigma, St. Louis, MO) for 24 h.

RT-PCR. Total cellular RNA was extracted from cultured mesangial cells using Trizol (Invitrogen, Carlsbad, California) and treated with DNase (Takara Bio Inc, Otsu, Shiga, Japan) according to the manufacturer's protocol. RNA was reverse-transcribed using the reverse transcription system kit (Promega, Madison, WI) as described in the manufacturer's protocol. The PCR primers for rat CYP11B2, MR, and 11β-HSD2 were synthesized according to published literatures [15–17]. 2 μl of the single-stranded cDNA in the 30 μl reaction mixture was amplified using 25 pmol each of sense and antisense primers and 1 μl *Taq* DNA polymerase (3 U/μl) in 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, with 0.2 mM of each dNTP. The reaction mixture without cDNA under similar conditions served as controls. The PCR products were analyzed on a 1.5% agarose gel and sequenced.

Real-time RT-PCR. The cultured renal mesangial cells grown on a 24-well plate were treated with 10^{-9}, 10^{-8}, and 10^{-7} M AngII (with or without the specific AngII type 1 receptor antagonist, losartan), or with 7 mM potassium ($n = 4$) for 48 h. Total cellular RNA was extracted and reverse transcribed as above. PCR was performed with 2 μl cDNA in a final volume of 50 μl containing 1× Ex Taq HS buffer (Takara Bio Inc, Otsu, Shiga, Japan), 10 μM of sense primer 5′-CTG ATT GTC TGG AAA TTT CTT AAA CAG T-3′, and antisense primer 5′-AGA GCT ATG GCT CGT TTT TGA TAG A-3′, 1 mM dAGCU, 250 mM UNG, 10 μM probe 5′-(FAM)-CCC TCA TCC TGG CTG ACT TGC ATA CTT-3′(TAMRA), and 5 U/μl Ex Taq HS (Takara Bio Inc, Otsu, Shiga, Japan). To normalize the samples for absolute RNA amount, a GAPDH-PCR was performed with primers G1, 5′-GCC TGG ATC CCT AAA GAG ACA A-3′; G2, 5′-CGC GAT ATT CAA TTG GAT ACA CA-3′ and the probe, 5′-(FAM)-CCA TTT CCA AGA CTG ACA GCC CCA GA-3′(TAMRA). Real-time PCR was carried out in an iCycler (Bio-Rad, Hercules, CA) using the following thermal cycling profile: 37 °C for 5 min, 95 °C for 3 min 30 s, 94 °C for 20 s, followed by 42 cycles of amplification (94 °C for 20 s, 56 °C for 40 s). All samples were run in triplicate.

Radioimmunoassay analysis. To determine aldosterone production by renal mesangial cells, the renal mesangial cells were cultured in serum free medium for 48 h. Aldosterone in the culture medium was measured by radioimmunoassay according to manufacturer's protocol.

Immunofluorescence staining. Mesangial cells were grown on glass cover-slips in a 12-well plate at a density of 10^{-4} cells per well for 24 h. Cells were fixed in acetone for 20 min at room temperature and washed three times (5 min each time) with PBS. The cells were then incubated with anti-MR antibody (1:100, Santa Cruz Bio Inc, Santa Cruz, CA) or anti-11β-HSD2 antibody (1:100, Chemicon Int., Temecula, CA) overnight at 4 °C. After washing three times (5 min each time) with PBS, the cells were incubated with secondary antibodies. The MR primary antibody was detected using FITC labeled anti-goat IgG antibody (Cedarlane, Hornby, Ontario, Canada) at dilution 1:40 for 60 min at 37 °C. The 11β-HSD2 primary antibody was detected by incubating for 45 min at 37 °C with rabbit anti-sheep biotinylated (KPL, Gaithersburg, Maryland) secondary antibody in a 1:100 dilution in PBS and detected by FITC labeled streptavidin-ABC kit (Boster, Biotechnology, Wuhan, China). After washings in PBS, the expressions of MR and 11β-HSD2 were visualized under a fluorescence microscope. Images were captured using an Olympus camera at equal exposure time for all panels.

ELISA analysis. The amount of fibronectin (FN) released in cell culture medium was measured by ELISA (Sun Biotechnology, Shanghai, China) as described in the manufacturer's protocol.

Luciferase assay. The reporter construct p3TP-Lux was provided by Dr. Kinzler (Johns Hopkins Hospital). Mesangial cells were transfected with p3TP-Lux (1.0 μg). A fixed amount (50 ng) of *Renilla* luciferase driven by thymidine kinase (TK) promoter (pRL-TK; Promega, Madison, WI) was co-transfected as an internal control. After transfection with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California), the cells were exposed to aldosterone (10^{-7} M) or vehicle for 24 h. Luciferase assay was performed using the dual luciferase assay system kit according to the manufacturer's protocols (Promega, Madison, WI). The Smad2 reporter luciferase activity was adjusted by TK promoter driven *Renilla* luciferase activity and was displayed as fold of induction by aldosterone.

shRNA. To knock-down rat Smad2 gene expression, three separate hairpin sequences were synthesized. The annealed DNA fragments corresponding to the shRNA-sequences were cloned into the unique *Bam*HI-*Hind*III site downstream of human U6 promoter (Kangchen, Shanghai, China). This plasmid also encodes an enhanced green fluorescent protein, so that the transfection efficiency can be monitored by fluorescence microscopy. The shRNA expression plasmids or a control vector were transfected into rat mesangial cells using Lipofectamine 2000 reagent according to the manufacturer's instructions. The efficiency of knocking down Smad2 by these shRNA constructs was assessed by Smad2 immunoblot. The Smad2 shRNA construct with the highest efficiency was used for the present study. The sequence is 5′-GGTGTTCAATCGCATATTA-3′.

Western blot analysis. The expressions of TGF-β1 and Smad2 were detected by Western blot with rabbit polyclonal antibody raised against the rat (1:100 Santa Cruz Bio Inc, Santa Cruz, CA and 1:250 Invitrogen, Carlsbad, California, respectively) and peroxidase-labeled goat anti-rabbit IgG (1:600 CALBIOCHEM, Darmstadt, Germany).

Results

Aldosterone is synthesized in cultured renal mesangial cells

To determine whether cultured rat mesangial cells produce aldosterone, expression of aldosterone synthase (CYP11B2), a rate-limiting enzyme in aldosterone synthesis, was examined. Using primers derived from the rat aldosterone synthase cDNA, a PCR product of expected size was obtained from total RNA extracted from cultured mesangial cells (Fig. 1). Nucleotide sequencing indicated the PCR product was identical to mRNA of aldosterone synthase. Production of aldosterone in mesangial cells was further confirmed by detecting aldosterone in culture medium incubating mesangial cells (1.61 pg/10^6 cells), but not in medium without cells.

Mesangial cells express MR and 11β-HSD2

To examine whether renal mesangial cells were target for aldosterone, MR and 11β-HSD2 expressions were determined using RT-PCR. The 11β-HSD2 is required in aldosterone target cells to convert active glucocorticoid cortisol to inactive cortisone, therefore the cells can efficiently respond to aldosterone. By using primers derived from the MR and 11β-HSD2 sequences, a single PCR product with the predicted size was obtained. The PCR products

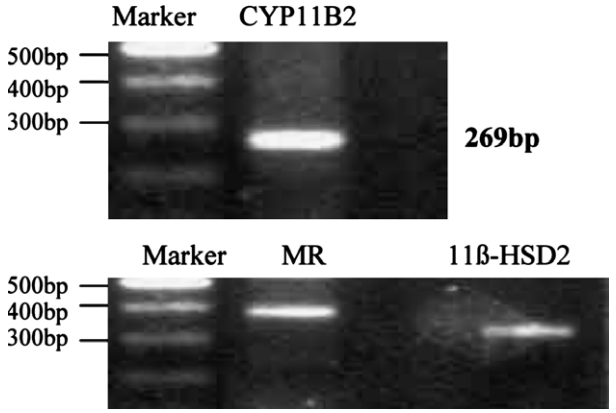

Fig. 1. Expression of CYP11B2, MR, and 11β-HSD2 in cultured renal mesangial cells. Renal mesangial cells were cultured. Total RNA was extracted as described in Materials and methods. CYP11B2, MR, and 11β-HSD2 mRNA expressions were determined by RT-PCR. The PCR products were verified by nucleotide sequencing.

were confirmed to be identical to MR or 11β-HSD2 mRNA by nucleotide sequencing. Immunofluorescence of MR and 11β-HSD2 showed that MR and 11β-HSD2 were distributed throughout the cytoplasm of mesangial cells (Fig. 2). No staining was detected when the MR or 11β-HSD2 primary antibodies were replaced by normal serum (Fig. 2).

AngII and potassium up-regulate CYP11B2 mRNA expression in renal mesangial cells

To examine whether CYP11B2 expression is regulated by angiotensin II or potassium as in adrenal gland, the cultured rat renal mesangial cells were exposed to angiotensin II or high potassium, and CYP11B2 mRNA expression was determined using real-time PCR. As shown in Fig. 3, angiotensin II significantly increased CYP11B2 mRNA expression in a dose dependent manner (46.26 ± 10.67 copies/GAPDH at 10^{-8} M AngII, 47.74 ± 6.67 copies/GAPDH at 10^{-7} M versus 21.86 ± 5.79 copies/GAPDH at baseline, $P < 0.001$, respectively). The angiotensin II induced CYP11B2 expression was abolished by angiotensin II AT1 receptor antagonist, losartan (Fig. 3). The CYP11B2 mRNA expression was also substantially enhanced when potassium concentration was increased from 5.5 to 7 mM (39.10 ± 3.17 copies/GAPDH at 7 mM, versus 21.77 ± 4.31 copies/GAPDH at 5.5 mM, $P < 0.05$, Fig. 3).

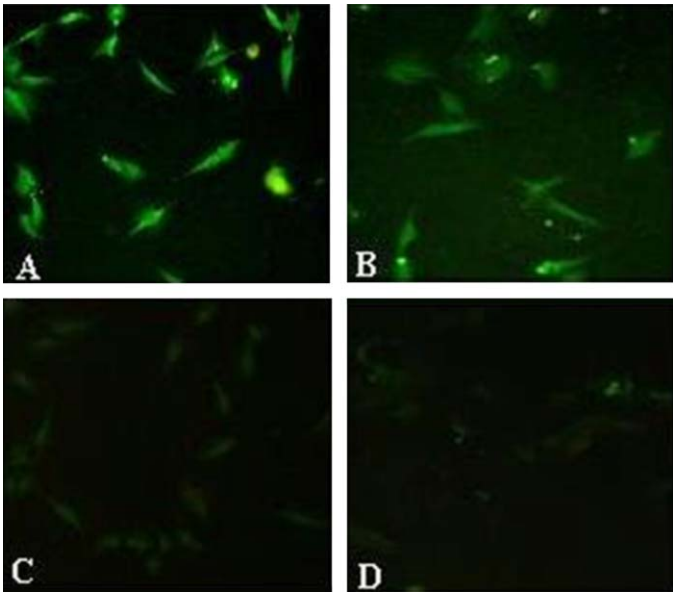

Fig. 2. Expression of MR (A) and 11β-HSD2 (B) proteins in cultured renal mesangial cells. Cultured mesangial cells were fixed with acetone. The expressions of MR and 11β-HSD2 were examined by immunofluorescence. Normal serum was used as negative control (C,D).

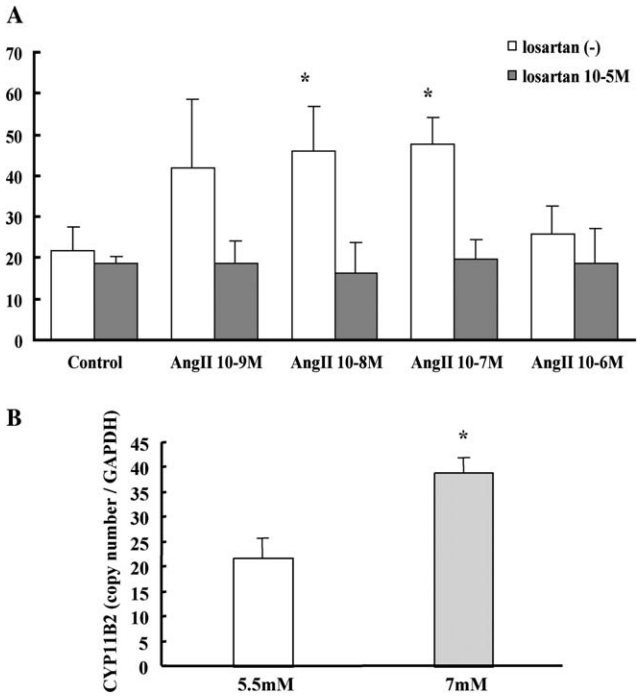

Fig. 3. Effects of AngII and potassium on CYP11B2 mRNA expression in cultured rat mesangial cells. (A) Cultured mesangial cells were treated with angiotensin II at indicated concentration for 48 h. CYP11B2 mRNA was determined by real-time PCR. (B) Cultured mesangial cells were incubated in a medium with normal or high potassium concentration for 48 h. CYP11B2 determined by real-time PCR. Values are means ± SE, $n = 4$ per group. $^{*}P < 0.05$, versus control.

Aldosterone increases fibronectin production in cultured renal mesangial cells

The above studies indicated that the renal mesangial cells express all the components of local aldosterone action. To determine the potential role of aldosterone on renal mesangial cell ECM production, the effect of aldosterone on fibronectin expression was examined. When cultured mesangial cells were exposed to aldosterone for 24 h, fibronectin production significantly increased determined by ELISA (74.6 ± 16.8 ng/ml versus control

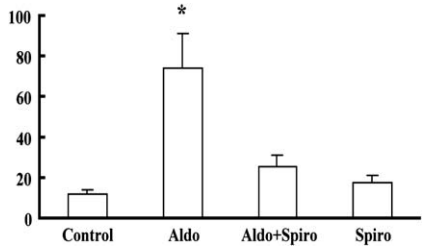

Fig. 4. Effect of aldosterone on fibronectin synthesis. Cultured renal mesangial cells were exposed to aldosterone (10^{-7} M) in the presence or absence of spironolectone (10^{-9} M) for 24 h. Culture medium fibronectin concentration was determined as described in Materials and methods. $^{*}P < 0.05$, versus control.

12.4 ± 1.9 ng/ml $P < 0.05$). The effect of aldosterone on fibronectin production was blocked by MR antagonist spironolactone (25.9 ± 5.3 ng/ml, $P < 0.05$) (Fig. 4).

Aldosterone increased TGF-β1 signaling

To determine the mechanisms by which aldosterone induced fibronectin production, we examined the effect of aldosterone on TGF-β1/Smad signaling pathway, which has been reported to play an important role in fibronectin production [18]. A Smad2-mediated TGF-β-responsive reporter system, 3TP-Lux, was used to examine the endogenous TGF-β1/Smad activity. Cultured renal mesangial cells were transfected with p3TP-LUX and pRL-TK, and exposed to aldosterone for 24 h. As shown in Fig. 5, aldosterone significantly increased the luciferase activity of p3TP-LUX by 2.1 ± 0.1-fold ($P < 0.05$). These results are consistence with the activation of TGF-β1 signal by aldosterone. Immunoblot showed that aldosterone (10^{-7} M) significantly increased the TGF-β1 protein expression (densitometry analysis: $134.2 \pm 13.9\%$, $P < 0.05$, Fig. 6). MR antagonist, spironolactone (10^{-9} M), abolished aldosterone enhanced TGF-β1 expression ($116.6 \pm 28.3\%$, Fig. 6).

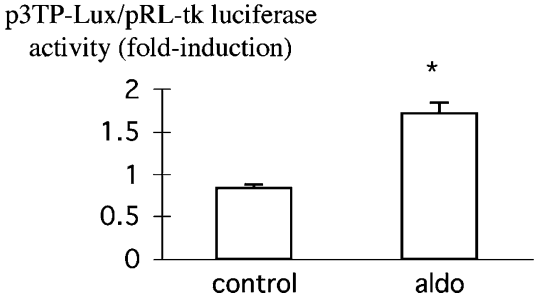

Fig. 5. Effect of aldosterone on TGF-β signaling activity. Rat mesangial cells were co-transfected with p3TP-LUX reporter plasmid and TK promoter-driven *Renilla* luciferase plasmid. The transfected cells were incubated for 24 h in the absence or presence of 10^{-7} M aldosterone. Luciferase activity was determined as described in the Materials and methods. The 3TP luciferase activity was adjusted with *Renilla* luciferase activity, and the fold of induction over control was displayed ($n = 5$). $^{*}P < 0.05$ versus control without aldosterone.

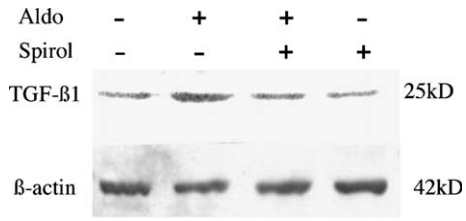

Fig. 6. Effect of aldosterone on TGF-β expression. Cultured renal mesangial cells were treated with aldosterone in the presence or absence of spironolectone for 24 h. TGF-β expression was determined by immunoblot. The data shown are representative of four independent experiments.

Smad2 knock-down abolishes aldosterone mediated fibronectin expression

Since Smad2 is a down stream molecule of TGF-β1 signaling pathway, the effect of Smad2 knock-down on aldosterone mediated fibronectin expression was examined. The Smad2 was knocked down using a shRNA approach. As shown in Fig. 7, Smad2 shRNA remarkably suppressed Smad2 protein expression. The suppressive effect of shRNA on Smad2 was specific, since Smad2 shRNA did not reduce β-actin expression. Importantly, Smad2 knock-down by Samd2 shRNA significantly reduced aldosterone induced fibronectin accumulation in cultured rat renal mesangial cells (27.5 ± 1.4 ng/ml, Fig. 8), further supporting that TGF-β1/Smad2 signaling pathway was involved in aldosterone induced fibronectin expression.

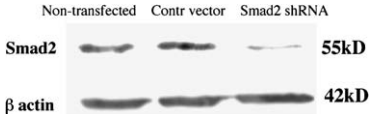

Fig. 7. Effect of Smad2 shRNA on Smad2 expression. Rat mesangial cells were transfected with Smad2 shRNA. Twenty-four hours later, Smad2 expression was analyzed by immunoblot. These are representative data of three independent experiments.

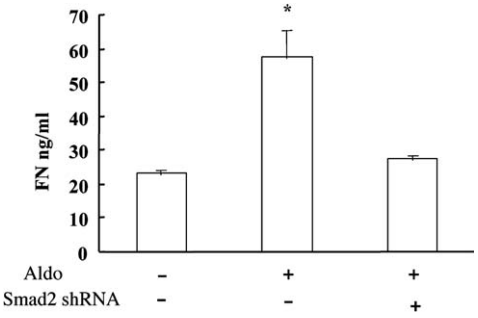

Fig. 8. Effect of Smad2 shRNA on aldosterone induced fibronedtin (FN) synthesis. Cultured renal mesangial cells were transfected with vector encoding Smad2 shRNA or empty vector (neg). Twenty-four hours later, the cells were treated with aldosterone (10^{-7} M) or vehicle for 24 h. Culture medium fibronectin was determined as described. $^{*}P < 0.05$, versus control and Smad2 knock-down.

Discussion

As early as four decades ago, it was shown that overproduction of aldosterone was associated with proteinuria [19]. Later studies demonstrate that in patients with chronic kidney disease, plasma aldosterone concentration is increased and renal dysfunction is comparable with plasma aldosterone level [20]. Recent studies show that aldosterone blockade is associated with reduced proteinuria and glomerulosclerosis in chronic kidney diseases in human as well as in animal models [6–9]. The present study, at cellular level, demonstrated that (1) renal mesangial cells were able to produce all the components that were required for local aldosterone action, including aldosterone synthase, aldosterone, mineralocorticoid receptor, and 11β-HSD2. (2) Aldosterone directly stimulated extra-cellular matrix protein fibronectin production in cultured renal mesangial cells. (3) TGF-β1/Samd2 signaling mediated aldosterone induced fibronectin generation. The present studies provided important evidence which suggests that renal mesangial cells are target of local aldosterone action, which may play an important role in glomerular damage in chronic kidney disease.

According to classic mechanism, aldosterone is a circulating hormone, primarily produced in zona glomerulosa of adrenal gland. Aldosterone synthase (CYP11B2) is a key enzyme for aldosterone synthesis. Production of aldosterone is stimulated by angiotensin II, potassium, and ACTH [21]. The circulating aldosterone binds to mineralocorticoid receptors (MR) in target cells, mainly epithelial cells in distal nephron and salivary gland, promoting sodium reabsorption and potassium excretion [22]. Since MR and glucocorticoid receptor (GR), a receptor for glucocorticoid, have extensive sequence homology in their hormone-binding domains (57% identity), the MR can also be activated by glucocorticoid, whose blood concentration is at least 100-fold higher than that of aldosterone [23]. However, because aldosterone targeting cells express an NAD-dependent enzyme 11β-HSD2, which converts cortisol and corticosterone into inactive cortisone and 11-dehydrocorticosterone, respectively, the MR in these cells can only be activated by aldosterone [24]. Accumulating evidence indicates that, beyond regulating sodium homeostasis, aldosterone has been shown as an independent factor inducing inflammation, fibrosis in the heart, vasculature, and kidney [4,25,26]. Aldosterone has been reported to be synthesized in the heart, endothelial cells, etc. [15,21]. Mineralocorticoid receptor can also be detected in these nonepithelial cells [27–29]. These studies suggest a local pro-inflammatory and pro-fibrotic effect of aldosterone in these tissues. In the present study, aldosterone was detected in renal mesangial cells, and aldosterone synthase mRNA expression in the mesangial cells was stimulated by angiotensin II and potassium as in adrenal gland. Furthermore, aldosterone receptor MR and 11β-HSD2 were also detected in these mesangial cells, suggesting that aldosterone may function locally on mesangial cells in an autocrine–paracrine fashion. These studies also demonstrated that aldosterone significantly increased expression of fibronectin, an important extra-cellular matrix protein in the development of sclerosis and fibrosis, in cultured renal mesangial cells. These studies suggest that local action of aldosterone on mesangial cells may be involved in sclerotic process. The effect of aldosterone on fibronectin expression was abolished by MR antagonist spironolactone, suggesting that the effect of aldosterone on fibronectin is MR-dependent.

It is well documented that TGF-β is a signaling pathway mediating extra-cellular matrix protein, including fibronectin expression in renal mesangial cells [30–34]. Studies show that activation of MR by aldosterone is associated with activation of TGF-β1 signaling [35–38]. In the present study, we examined the role of TGF-β1 signaling in aldosterone induced fibronectin expression in renal mesangial cells. Exogenous aldosterone significantly stimulated endogenous TGF-β1 activity determined using a TGF-β1 reporter system. The increase in TGF-β1 activity was, at least in part, associated with increased TGF-β1 protein level following aldosterone treatment. The TGF-β1 signaling pathway is propagated by Smad2, a transcription factor that is phosphorylated and translocated to the nucleus in response to TGF-β1 signaling [39]. To further determine whether the TGF-β1/Smad pathway mediates aldosterone induced fibronectin expression, Smad2 was down regulated by a siRNA. As shown in Fig. 8, Smad2 knock-down significantly blunted aldosterone stimulated fibronectin expression, supporting the role of TGF-β1/Smad2 signaling in aldosterone associated fibronectin expression.

In summary, we have shown that the aldosterone producing and response system was detected in cultured renal mesangial cells. Aldosterone increased extra-cellular matrix protein fibronectin expression, which was blocked by mineralocorticoid receptor antagonist spironolactone. TGF-β1/Smad2 signaling mechanism mediated aldosterone stimulated fibronectin production.

Acknowledgments

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