# Activation of Local Aldosterone System in Podocytes under Diabetic Conditions

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Directed by Professor Shin-Wook Kang

Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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**Dec 2008**

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## ABSTRACT

**Activation of Local Aldosterone System**
**in Podocytes under Diabetic Conditions**

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(Directed by Professor Shin-Wook Kang)

**Background:** Accumulating evidence has suggested that aldosterone plays an important role in the pathogenesis and progression of diabetic nephropathy. Besides adrenal gland, previous studies have shown that endothelial cells, vascular smooth muscle cells and the heart can also produce aldosterone, which may play a more important role in the development of vascular and myocardial fibrosis. In the kidney, glomerular mesangial cells and tubular epithelial cells were found to produce aldosterone based on the presence of aldosterone synthase (CYP11B2). However, the expression of CYP11B2 and

aldosterone biosynthesis was not fully explored in podocytes. In addition, although CYP11B2 expression was found to be increased in diabetic kidney, it was not elucidated whether the glomerular cells are responsible for this increase. This study was undertaken to investigate whether a local aldosterone system is present in podocytes and whether aldosterone production and the expression of CYP11B2 and mineralocorticoid receptor (MCR) are changed in podocytes cultured under high glucose conditions. Further, the changes in CYP11B2 and MCR expression were verified in diabetic glomeruli.

**Methods:** In vitro, immortalized mouse podocytes were exposed to medium containing 5.6 mM glucose (NG), NG + 24.4 mM mannitol (NG+M), NG + $10^{-7}$ M Angiotensin II (NG+AII), or 30 mM glucose (HG) with or without pretreatment of angiotensin II type I receptor blocker (ARB) for 6-hours. In vivo, 16 Sprague-Dawley rats were injected either with diluent (n=8, C) or with streptozotocin (65 mg/kg) intraperitoneally (IP) (n=8, DM). CYP11B2 and MCR mRNA expression were determined by real-time PCR and their protein expression by Western blot. AII and aldosterone levels were determined by ELISA and radioimmunoassay, respectively. Double immunofluorescence staining using renal tissue were also performed to identify which glomerular cells were responsible for the changes in CYP11B2 and MCR protein expression under diabetic conditions.

**Results:** CYP11B2 and MCR mRNA and protein expression were significantly increased in HG-stimulated podocytes compared to NG cells ($P<0.05$), and these increases in HG cells were attenuated with ARB treatment. AII also induced CYP11B2 ($P<0.05$) and MCR mRNA and protein expression ($P<0.01$) in cultured podocytes. AII levels were significantly higher in HG-conditioned media (2.54±0.33 pg/μl) compared to NG-conditioned media (1.13±0.12 pg/μl) ($P<0.01$). Aldosterone levels were also significantly higher in NG+AII-conditioned media (377.2±53.3 pg/mL) and HG-conditioned media (318.5±41.6 pg/mL) compared to the NG medium (129.0±15.3 pg/mL) ($P<0.01$), and this increase of aldosterone concentrations in the HG-conditioned medium was abrogated by ARB ($P<0.05$). Compared to C rats (0.37±0.07 mg/day), 24-hour urinary albumin excretion was significantly higher in DM rats (1.69±0.30 mg/day, $P<0.05$). The changes in CYP11B2 and MCR mRNA and protein expression in DM glomeruli were similar to those in high glucose-stimulated podocytes. Double immunofluorescence staining for CYP11B2 and MCR with synaptopodin revealed that the increases in CYP11B2 and MCR protein expression in DM glomeruli were mainly attributed to their increases in podocytes.

**Conclusion:** CYP11B2 and MCR mRNA and protein expression were significantly increased in diabetic glomeruli and cultured podocytes under

high glucose conditions along with increased aldosterone levels. The increases of CYP11B2 and MCR expression in high glucose-stimulated podocytes were ameliorated by ARB. These findings suggest that a local aldosterone system is activated in podocytes under diabetic conditions via renin-angiotensin system pathway, and its activation may play an important role in the pathogenesis of diabetic nephropathy.

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**Key words**: diabetic nephropathy, podocytes, aldosterone, aldosterone synthase, mineralocorticoid receptor

# Activation of local aldosterone system in podocytes under diabetic conditions

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## I. INTRODUCTION

Podocytes are terminally differentiated and highly specialized cells[1]. They line the urinary side of the glomerular basement membrane and function as a fine filter contributing ultimate size-selectivity, permitting permeability to molecules smaller than albumin[2]. Recent studies have shown that podocyte injury plays a role in the pathogenesis of various glomerular diseases[3], including diabetic nephropathy, the leading cause of end-stage renal disease in many countries[4,5]. Among the characteristic findings of diabetic nephropathy, podocytes are involved in the development of glomerular hypertrophy, glomerulosclerosis, foot process effacement, and podocytopenia[6]. Even though the molecular and cellular mechanisms responsible for these changes remain incompletely resolved, previous studies have demonstrated that the

diabetic milieu *per se*, hemodynamic changes, and local growth factors such as angiotensin II (AII) and transforming growth factor-β1 (TGF-β1) mediate the process of podocyte injury in diabetic nephropathy. Recently, accumulating evidence has suggested that aldosterone also plays an important role in the pathogenesis of diabetic nephropathy[7,8].

Aldosterone has been known to be originally produced in the glomerulosa zone of the adrenal cortex by a series of enzymatic steps, which can largely be divided into two phases. Acutely, aldosterone biosynthesis is controlled by the movement of cholesterol into the mitochondria, while its production is chronically regulated by the expression level of aldosterone synthase (CYP11B2). Besides the adrenal glands, previous studies have shown that various cells or tissues, such as endothelial cells, vascular smooth muscle cells, and the heart[9], can also produce aldosterone and that locally produced aldosterone may play a more important role in the development of vascular and myocardial fibrosis[10-12].

In the kidney, mesangial cells are known to produce aldosterone in response to AII, resulting in extracellular matrix accumulation (ECM)[13]. CYP11B2 mRNA and protein were demonstrated in cultured tubular epithelial cells and renal tissues, and its expression was upregulated by AII[14]. In addition, CYP11B2 mRNA and protein expression were increased in diabetic kidney,

and these increases were inhibited by AII type 1 receptor blocker (ARB) treatment[15]. These findings suggest that AII, the most biologically active peptide of the renin-angiotensin system (RAS), is an important mediator of aldosterone production in renal cells as in adrenal glomerulosa cells. Since local RAS and AII type 1 receptor (AT1R) have been demonstrated in podocytes[16], there is also a possibility that a local aldosterone system exists in podocytes. However, aldosterone biosynthesis and the expression of CYP11B2 have not yet been investigated in podocytes.

The present study was undertaken to investigate whether a local aldosterone system is present in podocytes and whether aldosterone production and the expression of CYP11B2 and mineralocorticoid receptor (MCR) are changed in podocytes cultured under high glucose conditions. In addition, the changes in CYP11B2 and MCR expression were verified in diabetic glomeruli.

## II. MATERIALS AND METHODS

### 1. Culture of podocytes

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (Albert Einstein College of Medicine, Bronx, NY) and were cultured as previously described[17]. Briefly, frozen podocytes were first grown under permissive conditions at 33°C in collagen coated flasks with RPMI 1640 medium containing 10% fetal bovine serum, 50 U/ml γ-interferon, and 100 U/ml of penicillin/streptomycin. The γ-interferon concentration was gradually reduced to 10 U/ml in successive passages. Cells were then trypsinized and subcultured without γ-interferon (non-permissive conditions) and allowed to differentiate at 37°C with medium changed on alternate days. The differentiation of podocytes grown for 14 days at 37°C was confirmed by the identification of synaptopodin, a podocyte differentiation marker, by reverse transcription-PCR and Western blotting.

After confirming differentiation of podocytes and serum restriction for 24 hours, the medium was changed to serum-free RPMI medium containing normal glucose (5.6 mM, NG), NG + mannitol (24.4 mM, NG+M), NG + $10^{-7}$ M AII (NG+AII), or high glucose (30 mM, HG) with or without 6-hours pretreatment of a ARB ($10^{-7}$ M L-158,809, a generous gift from Merck Sharp

and Dohme, Korea). The concentrations of L-158,809 used in this study were determined in preliminary experiments. At 24 hours after the medium change, cells were harvested for either RNA or protein, and the conditioned media were collected for the measurement of AII and aldosterone levels.

**2. Animals**

All animal studies were conducted using a protocol approved by the committee for the care and use of laboratory animals of Yonsei University College of Medicine. Sprague-Dawley rats weighing 250-280 g were injected either with diluent [n=8, Control (C)] or with 65 mg/kg streptozotocin (STZ) intraperitoneally [n=8, Diabetes (DM)]. Blood glucose levels were measured 3 days after the streptozotocin injection and those levels above 300 mg/dl were considered as diabetes. Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the 3-months study period.

Body weights were checked monthly, and kidney weights were measured at the time of sacrifice. Blood glucose and 24-hour urinary albumin were also measured at the time of sacrifice. Blood glucose was measured by glucometer and 24-hour urinary albumin excretion was determined by ELISA (Nephrat II, Exocell, Inc., Philadelphia, PA).

## 3. Isolation of glomeruli

Glomeruli were isolated by sieving. Purity of the glomerular preparation was greater than 98% as determined by light microscopy.

## 4. Isolation of total RNA

Podocytes RNA from each plate was extracted as previously described[18]. Briefly, after cell scraping with 800 μl of RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA), the mixture was vortexed and stored for 5 minutes at room temperature. Next, 160 μl of chloroform was added, and the mixture was shaken vigorously for 30 seconds. After 3 minutes, the mixture was centrifuged at 12,000 g for 15 minutes at 4°C, and the upper aqueous phase containing the extracted RNA was transferred to a new tube. The RNA was precipitated from the aqueous phase by adding 400 μl of isopropanol and then pelleted by centrifugation at 12,000 g for 30 minutes at 4°C. The RNA precipitate was washed with 70% ice-cold ethanol, dried using a Speed Vac, and dissolved in DEPC-treated distilled water. The RNA yield and quality were assessed by spectrophotometrically at the wavelengths of 260 and 280 nm. Total RNA from sieved glomeruli was extracted similarly.

**5. Reverse transcription**

First strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Two μg of total RNA extracted from cultured podocytes and sieved glomeruli were reversely transcribed using 10 μM random hexanucleotide primer, 1 mM dNTP, 8 mM $MgCl_2$, 30 mM KCl, 50 mM Tris-HCl, pH 8.5, 0.2 mM dithiothreithol, 25 U RNAse inhibitor, and 40 U AMV reverse transcriptase. The mixture was incubated at 30°C for 10 minutes and 42°C for 1 hour followed by inactivation of the enzyme at 99°C for 5 minutes.

**6. Real-time PCR**

The primers used in the experiments are summarized in Table 1. Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), the PCR was performed with a total volume of 20 μl in each well, containing 10 μl of SYBR Green® PCR Master Mix (Applied Biosystems), 5 μl of cDNA corresponding to 25 ng of RNA, and 5 pmol sense and antisense primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. The PCR conditions were as follows: 40 cycles, denaturation at 95°C for 45 seconds, annealing at the corresponding temperature for 45 seconds, and

extension at 72°C for 1 minute. Initial heating at 95°C for 9 minutes and a final extension at 72°C for 7 minutes were performed for all PCRs. Each sample was run in triplicate in separate tubes to permit quantification of the CYP11B2, MCR, and 11β-hydroxysteroid dehydrogenase type 2 (11ß-HSD2) genes normalized to the GAPDH gene. After real-time PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min to construct a melting curve. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using a comparative $C_T$ method with $2^{-\Delta\Delta CT}$. The results are given as relative expression normalized to the GAPDH gene and expressed in arbitrary units. Signals from NG podocytes and C glomeruli were assigned a relative value of 1.0. In pilot experiments, PCR products revealed a single band on agarose gels.

**Table 1. Sequence of primers**

| | | Sequence (5'→ 3') |
|---|---|---|
| CYP11B2 | Sense | CCTGCACCACCAACTGCTTAGC |
| | Antisense | CCAGTGAGCTTCCCGTTCAGC |
| MCR | Sense | CTACCACAGTCTCCCTGAAG |
| | Antisense | CGTTGACAATCTCCATGTAG |
| 11ß-HSD2 | Sense | GCTGCTCCAGGCCTATGTT |
| | Antisense | AGGTCAGGCAATGCCATTCT |
| GAPDH | Sense | CGTGAGAGTGTCTAACGGG |
| | Antisense | CGAGTCAGGCATTTGGTCC |

**7. Western blot analysis**

Podocytes harvested from plates and sieved glomeruli were lysed in sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10 mM Tris-HCl, pH 6.8, 10% [vol/vol] glycerol). Aliquots of 50 μg of protein were treated with Laemmli sample buffer, heated at 100°C for 5 minutes, and electrophoresed with 50 μg/lane in an 8-12% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane (Amersham Life Science, Inc., Arlington Heights, IL, USA) using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA), and the membrane was then incubated in blocking buffer A (1 X PBS, 0.1% Tween-20, and 8% nonfat milk) for 1 hour at room temperature, followed by an overnight incubation at

4°C with a 1:1000 dilution of polyclonal antibodies to CYP11B2, MCR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or a 1:2000 dilution of polyclonal β-actin antibody (Santa Cruz Biotechnology, Inc.). The membrane was then washed once for 15 minutes and twice for 5 minutes in 1 X PBS with 0.1% Tween-20. Next, the membrane was incubated in buffer A containing a 1:1000 dilution of horseradish peroxidase-linked goat anti-rabbit IgG (Amersham Life Science, Inc.). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc.). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany) and were used for analysis.

**8. Measurement of angiotensin II and aldosterone levels**

Angiotensin II (AII) levels were determined in conditioned culture media using a commercial ELISA kit (Peninsula Laboratories, Belmont, CA, USA) by the avidin-streptavidin method, as previously described[19]. Briefly, samples or standards were incubated with anti-AII antibody and biotinylated AII (B-AII) in 96-well plates coated with *Staphylococcus aureus* Protein A. After incubation, the unbound B-AII was removed by washing, and the bound B-AII was determined by reaction of streptavidin-HRP in the wells using TMB (3,3',5,5'-tetramethylbenzidine dihydrochloride) and $H_2O_2$ as a substrate. The

reaction was terminated with 2 N HCl, and the color intensity in each well was read at 450 nm using an ELISA microtiter plate reader. The AII amount in each well was calculated from the standard curve and normalized with the total protein content, which had been previously determined by a modified Lowry method.

The concentrations of aldosterone in the conditioned media were measured with a specific radioimmunoasssay kit (SPAC RIA Kit, Daiichi Radio-isotope Co., Tokyo, Japan), as previously described[20]. The sensitivity of the radioimmunoassay was 6.9 fmol/tube, and the overall recovery in the radioimmunoassay was 90-95%.

**9. Immunofluorescence staining**

Slices of kidney for immunofluorescence staining were snap-frozen in optimal cutting temperature solution and 4 μm sections of tissues were utilized. Slides were fixed in acetone for 10 minutes at 4°C, air dried for 10 minutes at room temperature, and blocked with 10% donkey serum for 20 minutes at room temperature. For double immunofluorescence staining, primary polyclonal antibodies to CYP11B2 and MCR (Santa Cruz Biotechnology, Inc.) was diluted in 1:100 with antibody diluent (DAKO, Glostrup, Denmark) and was applied for 3 hours at room temperature. After

washing, Cy3 (red)-conjugated anti-rabbit IgG antibody (Research Diagnostics, Inc., Flanders, NJ, USA) was added for 60 minutes. A 1:200 dilution of polyclonal anti-synaptopodin antibody (Santa Cruz Biotechnology, Inc.) was then applied, followed by Cy2 (green)-conjugated anti-goat IgG antibody.

**10. Statistical analysis**

All values were expressed as the mean±standard error of the mean (SEM). Statistical analysis was performed using SPSS for Windows Ver. 11.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were confirmed by the Mann-Whitney U test. *P* values less than 0.05 were considered to be statistically significant.

## III. RESULTS

### 1. CYP11B2 mRNA and protein expression in cultured podocytes

High glucose significantly increased CYP11B2 mRNA and protein expression in cultured podocytes (*P*<0.05). However, there was no difference in GAPDH mRNA and β-actin protein expression among the groups (data not shown). The CYP11B2/GAPDH mRNA ratio and CYP11B2 protein expression were 1.9- and 1.7-fold higher, respectively, in HG-stimulated podocytes relative to NG cells (*P*<0.05), and these increases were inhibited by 78% and 73%, respectively, with ARB treatment (*P*<0.05) (Fig. 1, 2). The CYP11B2/GAPDH mRNA ratio and protein expression were also significantly increased by 118% and 97%, respectively, in AII-treated podocytes compared to NG cells (*P*<0.05) (Fig. 1, 2).

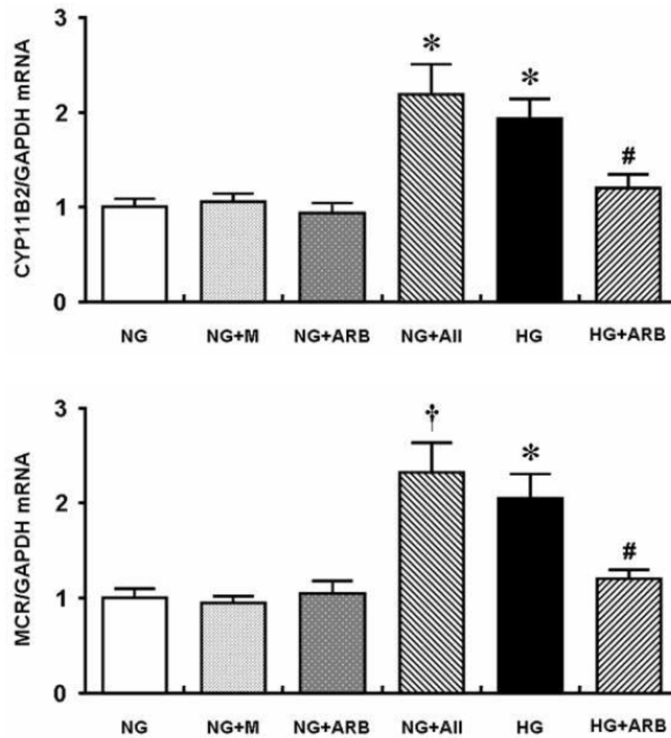
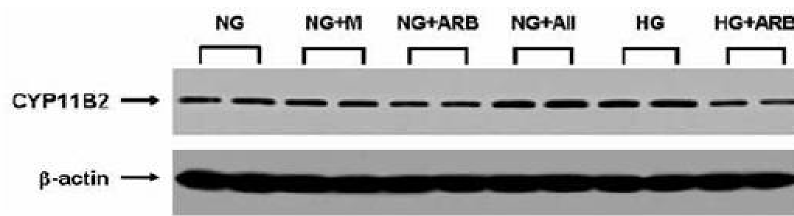



**Figure 1.** CYP11B2/GAPDH mRNA ratios in cultured podocytes. The podocytes were exposed to NG, NG+24.4 mM mannitol (NG+M), NG+$10^{-7}$ M L-158,809 (NG+ARB), NG+$10^{-7}$ M AII (NG+AII), HG, and HG+ARB (N=6). There was a 1.9-fold increase in CYP11B2/GAPDH mRNA ratio in HG-stimulated podocytes relative to NG cells, and this increase in HG cells was significantly inhibited with ARB treatment. The CYP11B2/GAPDH mRNA ratio was also significantly increased by 118% in AII-treated podocytes compared to NG cells. *; $P<0.05$ vs. NG, NG+M and NG+ARB groups, #; $P<0.05$ vs. HG group



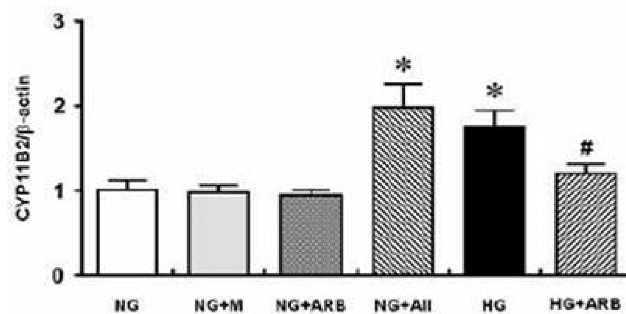




**Figure 2.** A representative Western blot of cellular CYP11B2 in cultured podocytes. The podocytes were exposed to NG, NG+24.4 mM mannitol (NG+M), NG+$10^{-7}$ M L-158,809 (NG+ARB), NG+$10^{-7}$ M AII (NG+AII), HG, and HG+ARB (representative of four blots). CYP11B2 protein expression was 1.7-fold higher in podocytes exposed to HG compared to NG cells, and ARB treatment abrogated this increase in HG cells. AII also significantly induced CYP11B2 protein expression in cultured podocytes. *; $P$<0.05 vs. NG, NG+M and NG+ARB groups, #; $P$<0.05 vs. HG group

**2. MCR mRNA and protein expression in cultured podocytes**

The MCR mRNA expression was significantly increased in podocytes exposed to NG+AII and HG versus NG cells ($P$<0.05). The MCR/GAPDH mRNA ratios were 2.3- ($P$<0.01) and 2.0-fold ($P$<0.05) higher in AII- and HG-, respectively, relative to NG-stimulated podocytes, and ARB treatment

significantly ameliorated the increase in MCR mRNA expression by 81% in HG cells ($P<0.05$) (Fig. 3). The expression of MCR protein showed a similar pattern to its mRNA expression (Fig. 4).

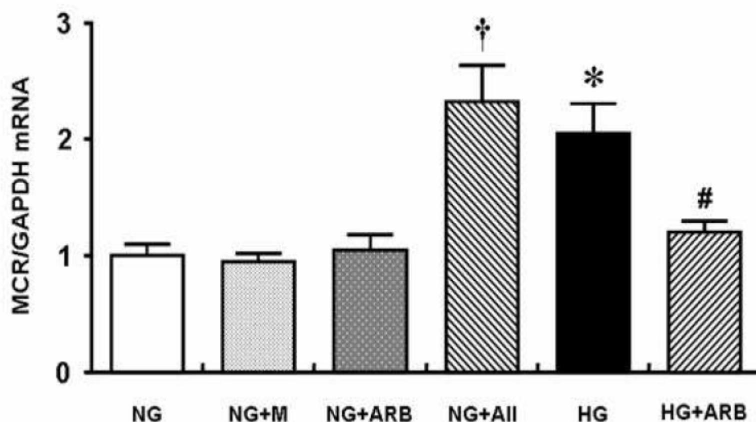



**Figure 3.** MCR/GAPDH mRNA ratios in cultured podocytes. The podocytes were exposed to NG, NG+24.4 mM mannitol (NG+M), NG+$10^{-7}$ M L-158,809 (NG+ARB), NG+$10^{-7}$ M AII (NG+AII), HG, and HG+ARB (N=6). There was a 2.0-fold increase in MCR/GAPDH mRNA ratio in HG-stimulated podocytes relative to NG cells, and this increase in HG cells was significantly ameliorated with ARB treatment. The CYP11B2/GAPDH mRNA ratio was also significantly increased by 133% in AII-treated podocytes compared to NG cells. †; $P<0.01$ vs. NG, NG+M and NG+ARB groups, *; $P<0.05$ vs. NG, NG+M and NG+ARB groups, #; $P<0.05$ vs. HG group

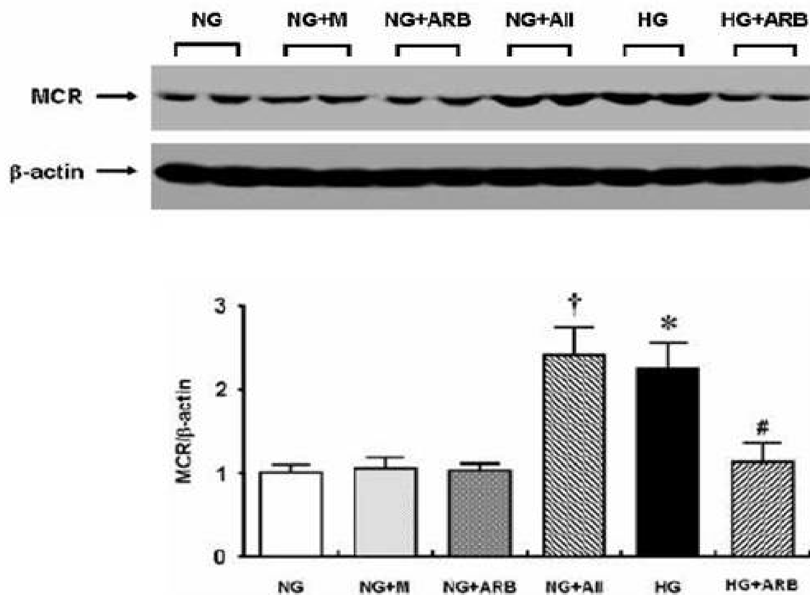



**Figure 4.** A representative Western blot of cellular MCR in cultured podocytes. The podocytes were exposed to NG, NG+24.4 mM mannitol (NG+M), NG+$10^{-7}$ M L-158,809 (NG+ARB), NG+$10^{-7}$ M AII (NG+AII), HG, and HG+ARB (representative of four blots). MCR protein expression was 1.9-fold higher in podocytes exposed to HG compared to NG cells, and ARB treatment attenuated this increase in HG cells. AII also significantly induced MCR protein expression in cultured podocytes. †; $P<0.01$ vs. NG, NG+M and NG+ARB groups, *; $P<0.05$ vs. NG, NG+M and NG+ARB groups, #; $P<0.05$ vs. HG group

**3. Angiotensin II levels in conditioned culture media**

AII levels were significantly higher in HG-conditioned medium (2.54±0.33 pg/μl) compared to NG-conditioned medium (1.13±0.12 pg/μl) ($P<0.01$) (Fig. 5). On the other hand, mannitol had no effect on AII concentrations in

conditioned media.

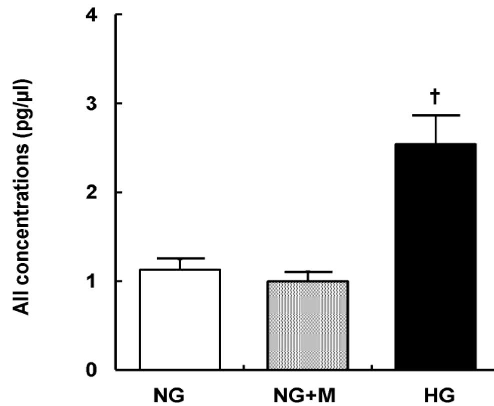



**Figure 5.** Angiotensin II levels in cultured podocytes. The podocytes were exposed to NG, NG+24.4 mM mannitol (NG+M), and HG medium. AII levels were significantly higher in HG- compared to NG-conditioned media. On the other hand, mannitol had no effect on AII concentrations in conditioned media. †; $P$<0.01 vs. NG and NG+M groups

### 4. Aldosterone levels in conditioned culture media

Aldosterone levels were significantly higher in NG+AII- (377.2±53.3 pg/mL) and HG-conditioned media (318.5±41.6 pg/mL) compared to the NG medium (129.0±15.3 pg/mL) ($P$<0.01), and this increase in aldosterone concentrations in the HG-conditioned medium was abrogated by ARB ($P$<0.05) (Fig. 6). On the other hand, mannitol had no effect on aldosterone production in cultured podocytes.

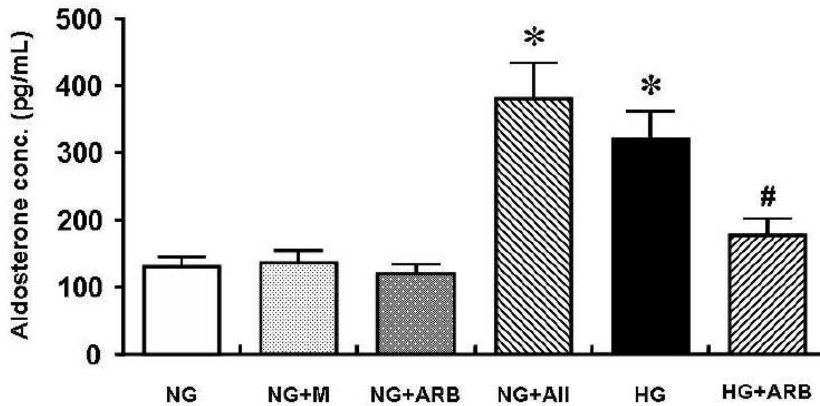



**Figure 6.** Aldosterone levels in podocytes. The podocytes were exposed to NG, NG+24.4 mM mannitol (NG+M), NG+$10^{-7}$ M L-158,809 (NG+ARB), NG+$10^{-7}$ M AII (NG+AII), HG, and HG+$10^{-7}$ M L-158,809 (HG+ARB) (N=6). Aldosterone levels were significantly higher in NG+AII- and HG-conditioned media compared to the NG medium, and this increase in aldosterone concentrations in HG-conditioned medium was abrogated by ARB. On the other hand, mannitol had no effect on aldosterone production in cultured podocytes. *; $P<0.01$ vs. NG, NG+M and NG+ARB groups, #; $P<0.05$ vs. HG group

**5. 11β-Hydroxysteroid dehydrogenase type 2 mRNA expression in culture podocytes**

To examine whether the non-selective MCR activation by cortisol also plays a role in the activation of local aldosterone system under high glucose conditions, the mRNA expression of 11β-HSD2, an enzyme that converts

biologically active 11-hydroxy-steroid (cortisol) to the inactive 11-ketosteroid form (cortisone), was determine by real-time PCR. There was no difference in 11β-HSD2/GAPDH mRNA ratio among NG, NG+M, and HG-stimulated podocytes (Fig. 7).

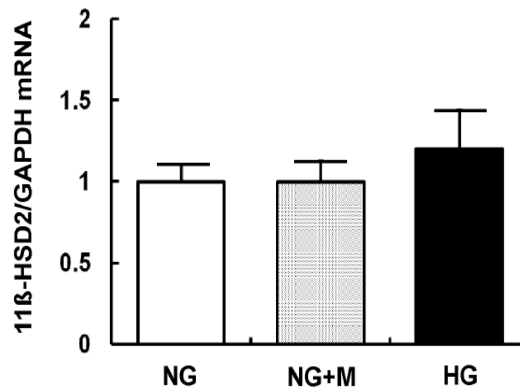



**Figure 7.** 11β-HSD2/GAPDH mRNA ratios in cultured podocytes. The podocytes were exposed to NG, NG+24.4 mM mannitol (NG+M), and HG. There was no difference in 11β-HSD2/GAPDH mRNA ratio among the three groups.

**6. Animal data**

All animals gained body weights over the 3-month experimental period, but weight gain was much lesser in DM rats ($P$<0.01). The ratio of kidney weight to body weight in DM rats (1.51±0.10%) was significantly higher than that in

C (0.60±0.06%) (*P*<0.01). Mean blood glucose levels of C and DM rats were 111.8±5.4 mg/dL and 468.5±11.5 mg/dL, respectively (*P*<0.01). Compared to the C group (0.37±0.07 mg/day), 24-hour urinary albumin excretion was significantly higher in the DM group (1.69±0.30 mg/day, *P*<0.05).

**Table 2.** Animal data

| | Control (N=8) | DM (N=8) |
|---|---|---|
| Body weight (Bwt, g) | 510±4 | 266±3* |
| Kwt/Bwt (%) | 0.60±0.06 | 1.51±0.10* |
| Blood glucose (mg/dl) | 111.8±5.4 | 468.5±11.5* |
| UAE (mg/day) | 0.37±0.07 | 1.69±0.30# |

Bwt, body weight; Kwt, kidney weight; UAE, 24-hour urinary albumin excretion, *; *P*<0.01 vs. control group, #; *P*<0.05 vs. control group

**7. Glomerular CYP11B2 and MCR mRNA and protein expression**

The changes in CYP11B2 and MCR mRNA and protein expression in diabetic glomeruli were similar to those in high glucose-stimulated podocytes. The CYP11B2/GAPDH mRNA and MCR/GAPDH mRNA ratios were significantly higher in diabetic glomeruli by 83% (*P*<0.05) and 139% (*P*<0.01), respectively, compared to C glomeruli (Fig. 8). Western blot analysis also revealed significant increases in CYP11B2 and MCR protein expression in diabetic glomeruli (Fig. 9).

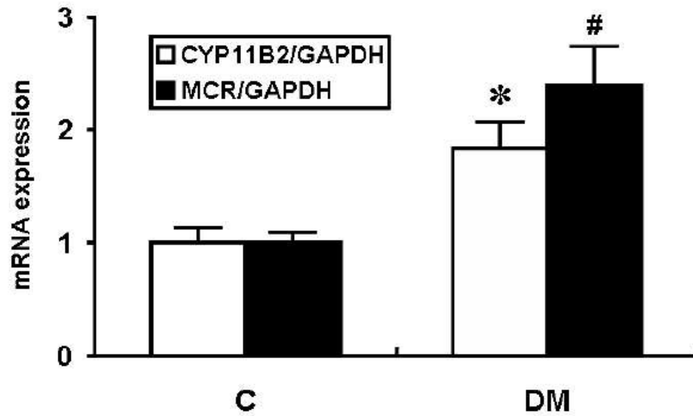



**Figure 8.** CYP11B2/GAPDH mRNA and MCR/GAPDH mRNA ratios in C and diabetic glomeruli. The CYP11B2/GAPDH mRNA and MCR/GAPDH mRNA ratios were significantly higher in diabetic glomeruli by 83% and 139%, respectively, compared to C glomeruli. *; $P<0.05$ vs. C group, #; $P<0.01$ vs. C group

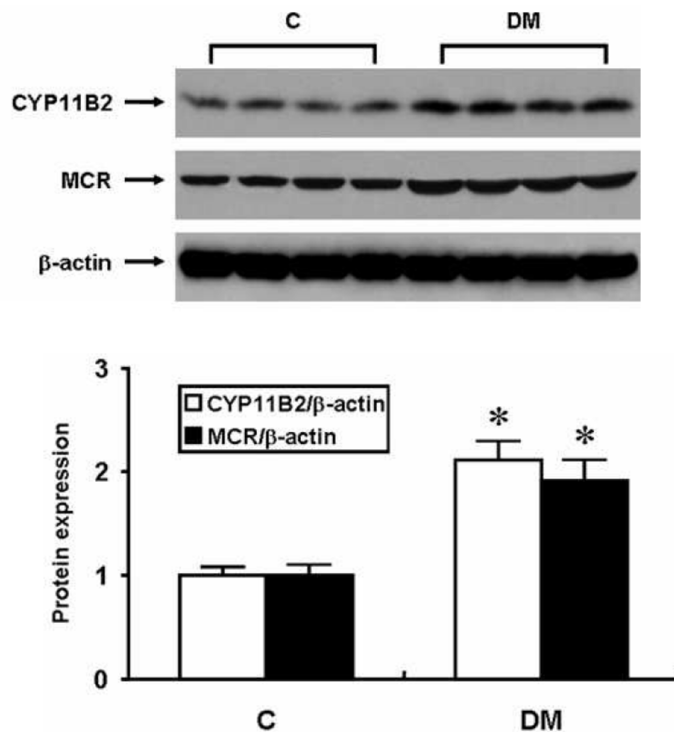



**Figure 9.** A representative Western blot of CYP11B2 and MCR in C and diabetic glomeruli (representative of four blots). There were 2.1- and 1.9-fold increases in CYP11B2 and MCR protein expression, respectively, in diabetic relative to C glomeruli. *; $P<0.05$ vs. C group

**8. Double immunofluorescence staining**

To identify which glomerular cells were responsible for the increase in CYP11B2 and MCR protein expression in diabetic glomeruli, double immunofluorescence staining with antibodies to CYP11B2 and MCR with synaptopodin was performed. Double immunofluorescence staining for CYP11B2 and MCR with synaptopodin revealed that the increases in

CYP11B2 and MCR protein expression were mainly attributed to their increases in podocytes (Fig. 10).

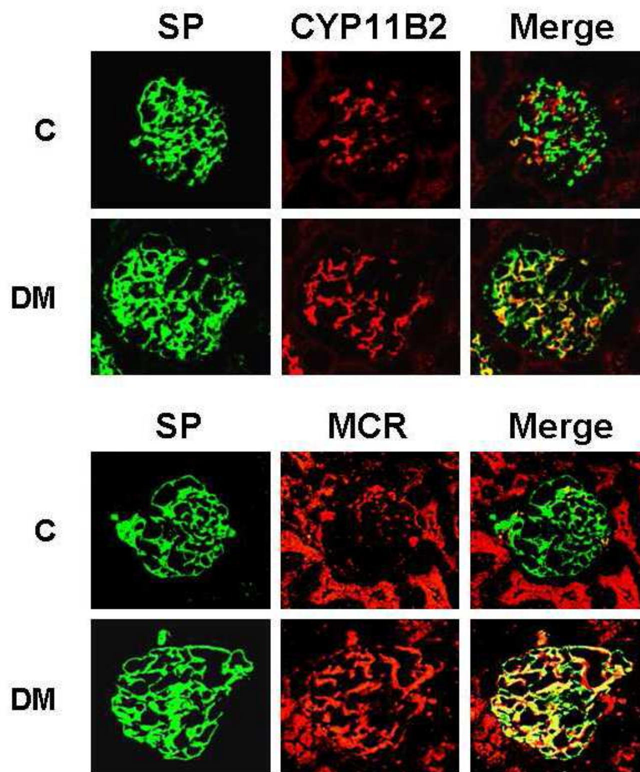



**Figure 10.** Double immunofluorescence staining for CYP11B2 and MCR with synaptopodin (SP). Compared to C, immunofluorescence staining for CYP11B2 and MCR were increased in diabetic glomeruli, and double immunofluorescence staining revealed that the increases in CYP11B2 and MCR protein expression were mainly attributed to their increases in podocytes (x 400).

## IV. DISCUSSION

Recent clinical and experimental studies have demonstrated that local aldosterone acts as a mediator in the development and progression of diabetic nephropathy, but the presence of local aldosterone system and its changes under diabetic conditions have not yet been investigated in podocytes. This study demonstrates for the first time that CYP11B2 is present in podocytes and that its expression is induced by high glucose via AT1R, resulting in increased aldosterone production.

Aldosterone is classically produced by zona glomerulosa cells in the adrenal cortex via conversion from cholesterol[21]. The conversion process is regulated at two critical enzymatic steps: (1) the early phase, the initial step converting cholesterol to pregnenolone; and (2) the late phase, the latter step mediated by CYP11B2, which converts 11-deoxycorticosterone to corticosterone and subsequently to aldosterone. The production of aldosterone in the adrenal gland is largely controlled by the expression levels of the CYP11B2 gene, which is regulated by various factors including adrenocorticotrophic hormone (ACTH), AII, and potassium[22,23]. On the other hand, accumulating evidence has suggested that aldosterone can be synthesized in non-adrenal cells, such as vascular endothelial and smooth

muscle cells, and cardiac myocytes[10-13]. Glomerular mesangial and tubular epithelial cells are also found to express the CYP11B2 gene[7]. Moreover, Xue and Siragy[15] demonstrated that CYP11B2 mRNA and protein expression were localized mainly in the renal cortex and were upregulated by AII and low salt intake and that even though aldosterone was absent in adrenalectomized rat, it was present in renal interstitium and tissue, suggesting the presence of local aldosterone system in the kidney. The present study shows for the first time that CYP11B2 gene is also expressed in podocytes. In addition, AII and high glucose increase CYP11B2 mRNA and protein expression, and these increments in CYP11B2 expression are abrogated by ARB. Since AII levels were increased in high glucose-conditioned culture media in this study in accordance with previous study[16], the results of the present study suggest that a local aldosterone system is present in nearly all types of cells in the kidney and that AII is a potent inducer of the CYP11B2 gene in podocytes as in adrenal zona glomerulosa cells, vascular smooth muscle cells, cardiac myocytes, and glomerular mesangial cells. Moreover, it is supposed that increased CYP11B2 expression in high glucose-stimulated podocytes is partly attributed to the increase in AII levels by high glucose. Several recent studies also showed that CYP11B2 expression was increased in diabetic glomeruli[15,24], with which the results of this study were consistent, but it was not elucidated

which glomerular cells are responsible for this increase. Using double immunofluorescence staining, it is demonstrated for the first time that the increase in glomerular CYP11B2 protein expression was mainly attributed to its increased expression in podocytes. Although the expression of CYP11B2 in mesangial cells was not focused in the present study, there is a possibility that the increase in CYP11B2 expression in mesangial cells may also contributes to the increase in glomerular CYP11B2 expression.

In addition to the effect of circulating aldosterone on regulating fluid and electrolyte balance, previous studies have revealed that aldosterone directly induces cellular hypertrophy and apoptosis, and regulates ECM metabolism in cardiac myocytes[25-27]. Since cellular hypertrophy, ECM accumulation, and apoptosis are characteristic findings of diabetic nephropathy[28-30], aldosterone is considered a potential mediator in the pathogenesis of diabetic nephropathy. In support of this theory, recent experimental studies have shown that aldosterone receptor blockade prevented the development and progression of diabetic nephropathy by ameliorating glomerular and tubulointerstitial ECM accumulation and by inhibiting macrophage infiltration[8,31,32]. In cultured mesangial and proximal tubular cells, aldosterone induced a significant increase in connective tissue growth factor expression associated with increased collagen synthesis, which was abolished by pretreatment with

spironolactone[7]. Lai et al[13] also demonstrated that aldosterone promoted fibronectin production in cultured mesangial cells and blocking the TGF-β1 signaling pathway by knocking down Smad2 significantly inhibited this increase in fibronectin synthesis, suggesting that aldosterone-induced fibronectin production was dependent on the TGF-β1/Smad2 pathway.

Recently, podocytes have been the focus of numerous studies as a target of injury in diabetic nephropathy. The number of podocytes, which reflects the balance between podocyte loss and proliferation, is decreased in diabetic glomeruli due to increased loss and/or lack of proliferation, and apoptosis is implicated as a potential mechanism of podocyte loss, while impaired DNA synthesis and hypertrophy contribute to lack of proliferation[3]. Apoptosis, usually known as programmed cell death, removes damaged or unwanted cells and has been implicated in the pathogenesis of numerous diseases such as malignancy, rheumatoid arthritis, and Alzheimer's disease[33]. Previous studies have demonstrated that apoptosis in cardiac myocytes and skeletal muscle cells is induced by aldosterone[34,35], suggesting that aldosterone is involved in the process of apoptosis. Moreover, one recent study by Nagase et al[36] revealed that podocyte injury in Dahl salt-hypertensive rats was prevented by an aldosterone blocker, suggesting the involvement of aldosterone in the process of podocyte damage. Even though the changes in TGF-β1 expression

were analyzed in that study, the number of apoptotic cells and the changes in apoptosis-related molecules were not determined. Another recent study demonstrated that MCR is consistently expressed in podocytes and that aldosterone could directly modulate podocyte function via MCR, possibly through the induction of oxidative stress and aldosterone effector kinase Sgk1[37]. Based on these findings, there is a possibility that locally activated aldosterone system under diabetic conditions may induce podocyte injury such as podocyte apoptosis, but it has never been explored. Further study is needed to clarify the exact role of local aldosterone system within podocytes and the functional significance of its increase under diabetic conditions.

In summary, CYP11B2 and MCR mRNA and protein expression were significantly increased in diabetic glomeruli and cultured podocytes under high glucose conditions along with increased aldosterone levels, and these changes in CYP11B2 and MCR were ameliorated with ARB. These findings suggest that a local aldosterone system is activated in podocytes under diabetic conditions via RAS pathway, and its activation may play an important role in the pathogenesis of diabetic nephropathy.

## V. CONCLUSION

This study examined whether a local aldosterone system was present in podocytes and whether aldosterone production and the expression of CYP11B2 and MCR were changed in high glucose-stimulated podocytes and experimental diabetic glomeruli. The results were as follows;

1. High glucose significantly increased CYP11B2 mRNA and protein expression in cultured podocytes, and these increases were significantly abrogated with ARB treatment. CYP11B2 mRNA and protein expression were also significantly increased in AII-treated podocytes compared to NG cells.
2. MCR mRNA and protein expression were significantly increased in podocytes exposed to NG+AII and HG versus NG cells, and ARB treatment significantly ameliorated the increase in MCR expression in HG podocytes.
3. AII levels were significantly higher in HG-conditioned medium compared to NG-conditioned medium.
4. Aldosterone levels were significantly higher in NG+AII- and HG-conditioned media relative to the NG medium, and this increase in aldosterone concentrations in the HG-conditioned medium was attenuated by ARB.

5. Compared to C rats, 24-hour urinary albumin excretion was significantly higher in DM rats.
6. Glomerular CYP11B2 and MCR mRNA and protein expression were significantly increased in DM compared to C rats.
7. Double immunofluorescence staining for CYP11B2 and MCR with synaptopodin revealed that the increases in CYP11B2 and MCR protein expression in DM glomeruli were mainly attributed to their increases in podocytes.

In summary, CYP11B2 and MCR mRNA and protein expression were significantly increased in diabetic glomeruli and cultured podocytes under high glucose conditions along with increased aldosterone levels, and these increases in CYP11B2 and MCR expression in high glucose-stimulated podocytes were inhibited by ARB. These findings suggest that a local aldosterone system is activated in podocytes under diabetic conditions via RAS pathway, and its activation may play an important role in the pathogenesis of diabetic nephropathy.

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## ABSTRACT (In Korean)


### 당뇨 조건 하에서 국소적 알도스테론계의 활성화

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**이 정 은**

**서론:** 최근의 연구들에 의하면 알도스테론이 당뇨병성 신병증의 발생 및 진행에 중요한 역할을 담당하는 것으로 보고되고 있다. 원래 알도스테론은 부신 피질의 사구층 세포에서 생성되는 것으로 알려져 있었으나, 생체 내외의 많은 연구를 통하여 부신 피질 이외에 혈관 내피세포, 혈관 평활근 세포, 그리고 심근세포에서도 생성이 일어나며, 이러한 국소적 알도스테론 생성이 각종 심혈관계 질환의 발생과 관련이 있는 것으로 보고되고 있다. 신장에서는 사구체 혈관사이 세포와 세뇨관 세포에 알도스테론 합성에 가장 중요한 효소인 aldosterone synthase (CYP11B2)가 존재하는 것으로 미루어 보아, 이들 세포에서도 국소적 알도스테론 합성이 이루어질 것으로 생각되나, 족세포에서의 국소적 알도스테론 생성에 대한

연구는 아직까지 극히 미미한 실정이다. 또한, 최근의 연구에 의하면 당뇨 흰쥐에서 추출한 신장 내 CYP11B2의 발현이 증가되는 것으로 알려져 있으나, 당뇨 조건 하에서 어떠한 신장 세포에서 CYP11B2의 발현이 증가되는 지에 대한 연구는 전무한 상태이다. 이에 본 연구자는 고포도당 조건 하에서 배양한 족세포 및 당뇨 흰쥐의 사구체에서 레닌-안지오텐신계가 활성화되면서 국소적 알도스테론계의 활성화가 동반될 것이라는 가정하에 족세포 내 CYP11B2와 mineralocorticoid receptor (MCR)의 존재를 확인함과 동시에 생체 내외의 실험을 통하여 당뇨 조건 하에서 이들의 발현 변화를 관찰하고자 하였다.

**방법:** 생체 외 실험으로는 불멸 생쥐 족세포 (immortalized mouse podocytes)를 정상 포도당군 (5.6 mM), 정상 포도당 + 만니톨군 (24.4 mM 만니톨), 정상 포도당 + 안지오텐신 ($10^{-7}$ M), 그리고 고포도당군 (30 mM)으로 나누어 배양하였으며, 각 군에 안지오텐신 제 1형 수용체 차단제 ($10^{-7}$ M L-158,809)를 6시간 전처치한 족세포도 배양하였다. 생체 내 실험으로는 16마리의 Sprague-Dawley 백서를 대조군 (8마리)과 복강 내로 streptozotocin을 투여하여 당뇨를 유발시킨 당뇨군 (8마리)으로 나누어 실험하였다. 배양 족세포와 백서 신장으로부터 분리한 사구체에서 CYP11B2와 MCR의 mRNA 및 단백 발현은 각각 real-time PCR과 Western blot을 이용하여

분석하였다. 세포 배양액 내 안지오텐신 II 농도는 ELISA를, 그리고 알도스테론 농도는 radioimmunoassay를 이용하여 측정하였다. 신장 조직을 이용한 이중 면역형광염색을 시행하여 당뇨 조건 하에서 족세포 내 CYP11B2와 MCR의 발현 변화를 관찰하였다.

**결과:** CYP11B와 MCR의 mRNA 및 단백 발현은 정상 포도당군에 비하여 고포도당으로 자극한 족세포군에서 유의하게 증가되었으며 (*P*<0.05), 안지오텐신 제 1형 수용체 차단제로 전처치한 경우 고포도당군에서의 발현 증가가 의미있게 억제되었다. 안지오텐신 II도 배양 족세포에서 CYP11B2 (*P*<0.05)와 MCR (*P*<0.01)의 mRNA 및 단백 발현을 의의있게 증가시켰다. 세포 배양액 내 안지오텐신 II 농도는 고포도당군 (2.54±0.33 pg/μl)에서 정상 포도당군(1.13±0.12 pg/μl)에 비하여 유의하게 높았다 (*P*<0.01). 알도스테론 농도도 고포도당군 (318.5±41.6 pg/mL)과 안지오텐신 II으로 자극한 군 (377.2±53.3 pg/mL)에서 정상 포도당군 (129.0±15.3 pg/mL)에 비하여 의미있게 높았으며 (*P*<0.01), 고포도당군에서 증가된 알도스테론 농도는 안지오텐신 제 1형 수용체 차단제에 의하여 의의있게 감소되었다 (*P*<0.05). 24시간 뇨알부민 배설량은 대조군 (0.37±0.07 mg/day)에 비하여 당뇨군 (1.69±0.30 mg/day)에서 유의하게 많았다 (*P*<0.05). 사구체 내 CYP11B2와 MCR의 mRNA 및 단백 발현은 당뇨군에서 대조군에 비하여 의미있게 증가되었으며, CYP11B2 및

MCR과 synaptopodin을 이용한 이중 면역형광염색을 시행한 결과, 당뇨 조건 하에서 사구체 내 CYP11B2 및 MCR 단백의 발현 증가는 주로 족세포 내에서의 증가에 기인함을 알 수 있었다.

**결론:** 이상의 실험 결과로 미루어 보아, 당뇨 조건 하에서 족세포 내 국소적 알도스테론계가 활성화되며, 이러한 활성화는 국소적 레닌-안지오텐신계의 활성화와 관련이 있을 것으로 생각된다. 또한, 이러한 족세포 내 국소적 알도스테론계의 활성화가 당뇨병성 신병증의 발생 및 진행과 일부 연관되어 있을 것으로 사료된다.

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**핵심 되는 말**: 당뇨병성 신병증, 족세포, 알도스테론, aldosterone synthase, mineralocorticoid receptor