Activation of local aldosterone system within podocytes is involved in apoptosis under diabetic conditions

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[1]*Department of Internal Medicine, College of Medicine, Brain Korea 21 for Medical Science, Yonsei University, and* [3]*Department of Internal Medicine, College of Medicine, Ewha Womans University, Seoul, Korea; and* [2]*Nephrology and Dialysis Unit, Department of Internal Medicine, The Affiliated Hospital, YanBian University Medical College, JiLin, China*

Lee SH, Yoo T, Nam B, Kim DK, Li JJ, Jung D, Kwak S, Ryu D, Han SH, Lee JE, Moon SJ, Han DS, Kang S. Activation of local aldosterone system within podocytes is involved in apoptosis under diabetic conditions. *Am J Physiol Renal Physiol* 297: F1381–F1390, 2009. First published August 26, 2009; doi:10.1152/ajprenal.00101.2009.— Previous studies have shown that mineralocorticoid receptor (MCR) blocker reduces proteinuria in diabetic nephropathy (DN), but the role of aldosterone in podocyte injury has never been explored in DN. This study was undertaken to elucidate whether a local aldosterone system existed in podocytes and to examine its role in podocyte apoptosis under diabetic conditions. In vitro, immortalized podocytes were exposed to 5.6 mM glucose (NG), NG + 24.4 mM mannitol, and 30 mM glucose (HG) with or without 10^{-7} M spironolactone (SPR). In vivo, 32 Sprague-Dawley rats were injected with diluent (C, $n = 16$) or streptozotocin intraperitoneally [diabetes mellitus (DM), $n = 16$], and 8 rats from each group were treated with SPR for 3 mo. Aldosterone synthase (CYP11B2) and MCR mRNA and protein expression were determined by real-time PCR and Western blot, respectively, and aldosterone levels by radioimmunoassay. Western blot for apoptosis-related molecules, Hoechst 33342 staining, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay were performed to determine apoptosis. CYP11B2 and MCR expression were significantly higher in HG-stimulated podocytes and DM glomeruli compared with NG cells and C glomeruli, respectively, along with increased aldosterone levels. Western blot analysis revealed that cleaved caspase-3 and Bax expression was significantly increased, whereas Bcl-2 expression was significantly decreased in HG-stimulated podocytes and in DM glomeruli. Apoptosis determined by Hoechst 33342 staining and TUNEL assay were also significantly increased in podocytes under diabetic conditions. These changes in the expression of apoptosis-related proteins and the increase in apoptotic cells were inhibited by SPR treatment. These findings suggest that a local aldosterone system is activated and is involved in podocyte apoptosis under diabetic conditions.

diabetic nephropathy; podocytes; aldosterone; apoptosis; high glucose

PODOCYTES ARE TERMINALLY DIFFERENTIATED and highly specialized cells (24). They line the urinary side of the glomerular basement membrane and function as a fine filter contributing to ultimate size-selectivity and permitting permeability to molecules smaller than albumin (33). Recent studies have shown that podocyte injury plays a role in the pathogenesis of various glomerular diseases (26), including diabetic nephropathy, the leading cause of end-stage renal disease in many countries (4,

34). Among the characteristic findings of diabetic nephropathy, podocytes are involved in the development of glomerular hypertrophy, glomerulosclerosis, foot process effacement, and podocytopenia (40). Even though the molecular and cellular mechanisms responsible for these changes remain incompletely resolved, previous studies have demonstrated that diabetic milieu per se, hemodynamic changes, and local growth factors such as angiotensin II (ANG II) and transforming growth factor-β1 (TGF-β1) mediate the process of podocyte injury in diabetic nephropathy (39, 40). Recently, accumulating evidence has suggested that aldosterone also plays an important role in the pathogenesis of diabetic nephropathy (6, 7, 9, 29, 31, 45). Moreover, most previous studies have shown mineralocorticoid receptor (MCR) blocker reduces proteinuria in diabetic nephropathy (7, 9, 31, 45). Although a recent study demonstrated that aldosterone stimulated oxidative stress generation in cultured podocytes (27), the role of aldosterone in podocyte injury has never been explored in diabetic nephropathy.

Aldosterone is 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 in the mitochondria, whereas its production is chronically regulated by the expression level of aldosterone synthase (CYP11B2) (19, 38). Besides the adrenal glands, recent studies have shown that various cells or tissues, such as endothelial cells, vascular smooth muscle cells, and the heart (11, 28, 32), can also produce aldosterone and that locally produced aldosterone may play a more important role in the development of vascular and myocardial fibrosis (30).

In the kidney, mesangial cells are known to produce aldosterone in response to ANG II, resulting in extracellular matrix (ECM) accumulation (15). CYP11B2 mRNA and protein were demonstrated in cultured tubular epithelial cells and renal tissues, and its expression was upregulated by ANG II (41, 43). In addition, CYP11B2 expression was increased in diabetic kidney (29, 31, 43), and this increase was inhibited by ANG II type 1 receptor (AT1R) blocker (ARB) treatment (43). These findings suggest that ANG II, 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 (38). Because local RAS and AT1R have been demonstrated in podocytes (44), 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.

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In this study, we examined whether a local aldosterone system was present in podocytes and whether aldosterone production and the expression of CYP11B2 and MCR were changed in podocytes cultured under high glucose conditions. In addition, the changes in CYP11B2 and MCR expression were verified in diabetic glomeruli. Because aldosterone is known to induce apoptosis in cardiac myocytes and skeletal muscle cells (2, 17), and apoptosis is implicated as a potential mechanism of podocyte loss characterized in diabetic nephropathy (40), we also observed whether a local aldosterone system was involved in podocyte apoptosis under diabetic conditions.

METHODS

Podocyte Culture

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (Albert Einstein College of Medicine, Bronx, NY) and were cultured as previously described (20). Differentiated podocytes were serum restricted for 24 h, and after then 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^{-6} M ANG II (NG + ANG II), NG + 10^{-7} M aldosterone (NG + ALDO), or high glucose (30 mM, HG) with or without 6-h pretreatment of MCR blocker [10^{-7} M spironolactone (SPR); Sigma Chemical, St. Louis, MO] or a selective ARB (10^{-7} M L-158,809, a generous gift from Merck Sharp and Dohme). The concentrations of SPR and L-158,809 used in this study were determined in preliminary experiments. After the medium was changed (24 h), cells were harvested for either RNA or protein, and the conditioned culture medium was collected for the measurement of aldosterone levels.

Animal Study

All animal studies were approved by the committee for the care and use of laboratory animals of Yonsei University College of Medicine. Thirty-two male Sprague-Dawley rats weighing 250–280 g were injected either with diluent [$n = 16$, control (C)] or with 65 mg/kg streptozotocin (STZ) intraperitoneally [$n = 16$, diabetes mellitus
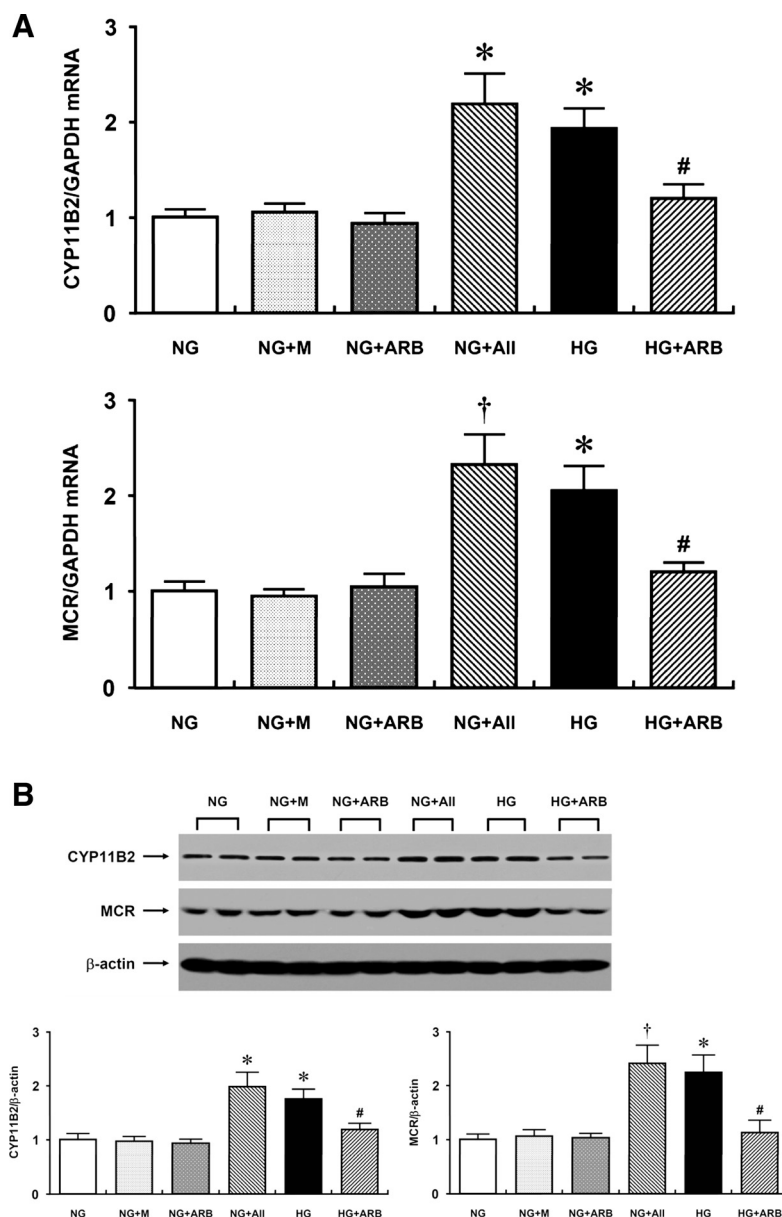

Fig. 1. *A*: CYP11B2-to-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and mineralocorticoid receptor (MCR)-to-GAPDH mRNA ratios in cultured podocytes exposed to normal glucose (NG), NG + 24.4 mM mannitol (NG + M), NG + 10^{-7} M L-158,809 [NG + ANG II type 1 receptor blocker (ARB)], NG + 10^{-6} M ANG II (NG + ANG II), high glucose (HG), and HG + ARB ($n = 6$). There were 1.9- and 2.0-fold increases in CYP11B2-to-GAPDH mRNA and MCR-to-GAPDH mRNA ratios, respectively, in HG-stimulated podocytes relative to NG cells, and these increases in HG cells were significantly inhibited with ARB treatment. The CYP11B2-to-GAPDH mRNA and MCR-to-GAPDH mRNA ratios were also significantly increased by 118 and 133%, respectively, in ANG II-treated podocytes compared with NG cells. *B*: representative Western blot of cellular CYP11B2 and MCR in cultured podocytes (representative of 4 blots). CYP11B2 and MCR protein expression were 1.7- and 1.9-fold higher, respectively, in podocytes exposed to HG compared with NG cells, and ARB treatment abrogated these increases in HG cells. ANG II also significantly induced CYP11B2 and MCR protein expression in cultured podocytes. $P < 0.05$ vs. NG, NG + M, and NG + ARB groups (*) and vs. HG group (#). †$P < 0.01$ vs. NG, NG + M, and NG + ARB groups.
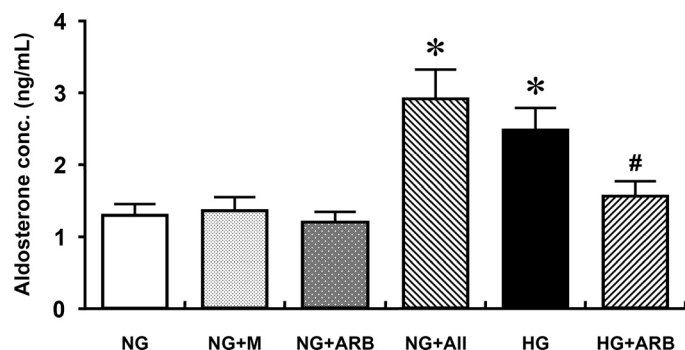
Fig. 2. Aldosterone levels in podocytes exposed to NG, NG + M, NG + ARB, NG + ANG II, HG, and HG + 10^{-7} M L-158,809 (HG + ARB) ($n = 6$). Aldosterone levels were significantly higher in NG + ANG II- and HG-conditioned media compared with the NG medium, and this increase in aldosterone concentrations in HG-conditioned medium was ameliorated 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.

(DM)]. Diabetes was confirmed by tail vein blood glucose levels on the 3rd day postinjection. After diabetes was confirmed, eight rats from each group were treated with 20 mg·kg^{-1}·day^{-1} of SPR (Sigma Chemical) by gavage (C + SPR, DM + SPR) for 3 mo. Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the 3-mo study period.

Body weights were checked monthly, and kidney weights were measured at the time of death. Systolic blood pressure was measured by tail-cuff phlethysmography at 3 mo. Serum glucose and 24-h urinary albumin were also measured at the time of death. Blood glucose was measured by glucometer, and 24-h urinary albumin excretion was determined by enzyme-linked immunosorbent assay (Nephrat II; Exocell, Philadelphia, PA).

Glomerular Isolation

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

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from cultured podocytes and isolated glomeruli as previously described (42), and first-strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). Total RNA (2 μg) extracted from podocytes and glomeruli was reverse transcribed as previously described (42).

Real-Time PCR

The primers used for CYP11B2, MCR, 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: CYP11B2 sense 5′-CCATGCCCTGCAT-TCTATGTT-3′, antisense 5′-GTGCTTGTCCAGCGAGTCAA-3′; MCR sense 5′-CTACCACAGTCTCCCTGAAG-3′, antisense 5′-CGTTGACAATCTCCATGTAG-3′; 11β-HSD2 sense 5′-GCTGCTC-CAGGCCTATGTT-3′, antisense 5′-AGGTCAGGCAATGCCATTCT-3′; and GAPDH sense 5′-CGTGAGAGTGTCTAACGGG-3′, antisense 5′-CGAGTCAGGCATTTGGTCC-3′. With the use of the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), PCR was performed as previously described (44). 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 cycle threshold (C_T) method with $2^{-\Delta\Delta C_T}$. The results are given as relative expression normalized to the GAPDH gene and expressed in arbitrary units. Signals from NG cells and C glomeruli were assigned a relative value of 1.0. In pilot experiments, PCR products revealed a single band on agarose gels.

Western Blot Analysis

Podocytes harvested from plates and sieved glomeruli were lysed in SDS sample buffer [2% SDS, 10 mM Tris·HCl, pH 6.8, and 10% (vol/vol) glycerol]. Aliquots of 50 μg of protein were treated with Laemmli sample buffer, heated at 100°C for 5 min, 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, Arlington Heights, IL) using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA), and the membrane was then incubated in *blocking buffer A* (1× PBS, 0.1% Tween 20, and 8% nonfat milk) for 1 h at room temperature, followed by overnight incubation at 4°C with a 1:1,000 dilution of polyclonal antibodies to CYP11B2 (Chemicon International, Temecula, CA), MCR (Affinity BioReagents, Golden, CO), Bax, Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), active fragments of caspase-3 (Cell Signaling, Beverly, MA), or β-actin (Santa Cruz Biotechnology). The membrane was then washed one time for 15 min and two times for 5 min in 1× PBS with 0.1% Tween 20. Next, the membrane was incubated in *buffer A* containing a 1:1,000 dilution of horseradish peroxidase-linked goat anti-rabbit IgG (Amersham Life Science). Washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science). Band densities were measured using TINA image software (Raytest, Straubenhardt, Germany) and were used for analysis.
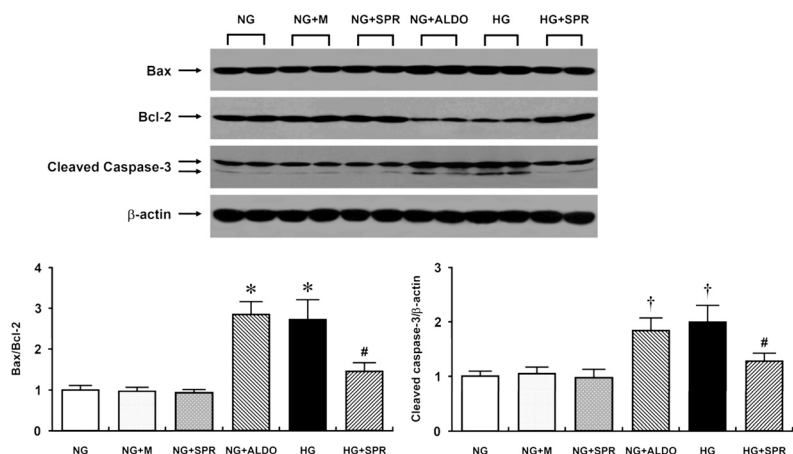

Fig. 3. A representative Western blot of Bax, Bcl-2, and active fragments of caspase-3 protein expression in cultured podocytes exposed to NG, NG + M, NG + 10^{-7} M spironolactone (NG + SPR), NG + 10^{-7} M aldosterone (NG + ALDO), HG, and HG + SPR (representative of 4 blots). Aldosterone significantly induced Bax and active fragments of caspase-3 protein expression and significantly reduced Bcl-2 protein expression in cultured podocytes. Bax and active fragments of caspase-3 protein expression were also significantly increased, whereas Bcl-2 protein expression was significantly decreased, in podocytes cultured under HG medium compared with NG cells, and these changes were attenuated by SPR. *$P < 0.01$ vs. NG, NG + M, and NG + SPR groups. $P < 0.05$ vs. HG group (#) and vs. NG, NG + M, and NG + ARB groups (†).

Hoechst 33342 Staining and TUNEL Assay

In addition to the changes in the expression of apoptosis-related molecules, apoptosis was also identified in cultured podocytes seeded on cover slips by Hoechst 33342 (Molecular Probes, Eugene, OR) staining and within glomeruli by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using a commercially available kit (Chemicon International). Apoptosis was defined as the presence of nuclear condensation on Hoechst staining and TUNEL-positive cells within glomeruli. The percentage of podocytes with nuclear condensation and TUNEL-positive glomerular cells in formalin-fixed renal tissue were determined by examining at least 300 cells/condition and 30 glomeruli, respectively, at ×400 magnification.

Measurement of Aldosterone Levels

The concentrations of aldosterone in the conditioned media, plasma, and glomerular lysates were measured with a specific radio-immunoasssay kit (SPAC RIA Kit; Dangiichi Radio-isotope, Tokyo, Japan), as previously described (23), and aldosterone levels in the conditioned media and glomerular lysates were normalized to the total protein amount. The sensitivity of the radioimmunoassay was 6.9 fmol/tube, and the overall recovery in the radioimmunoassay was 90–95%.

Immunofluorescence

Slices of kidney for immunofluorescence staining were snap-frozen in optimal cutting temperature solution, and 4-μm sections of tissues were used. Slides were fixed in acetone for 10 min at 4°C, air-dried for 10 min at room temperature, and blocked with 10% donkey serum for 20 min at room temperature. For double-immunofluorescence staining, primary polyclonal antibodies to CYP11B2 or MCR (Santa Cruz Biotechnology.) were diluted 1:100 with antibody diluent (DAKO, Glostrup, Denmark) and were applied for 3 h at room temperature. After washing, Cy3 (red)-conjugated anti-rabbit IgG antibody (Research Diagnostics, Flanders, NJ) was added for 60 min. A 1:200 dilution of polyclonal anti-synaptopodin antibody (Santa Cruz Biotechnology) was then applied, followed by Cy2 (green)-conjugated anti-goat IgG antibody.

Determination of Podocyte Numbers

Immunohistochemical staining for WT-1 was also performed to determine the number of podocytes as previously described (25).

Statistical Analysis

All values are expressed as the means ± SE. Statistical analysis was performed using the statistical package SPSS for Windows version 11.0 (SPSS, Chicago, IL). Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences determined by the Kruskal-Wallis test were further confirmed by the Mann-Whitney U-test. P values <0.05 were considered statistically significant.

RESULTS

Cultured Podocyte Studies

CYP11B2 and MCR mRNA and protein expression. HG 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-to-GAPDH mRNA ratio and CYP11B2 protein expression were 1.9- and 1.7-fold higher, respectively, in HG relative to NG podocytes ($P < 0.05$), and these increments were inhibited by 78 and 73%, respectively, with ARB treatment ($P < 0.05$) (Fig. 1, *A* and *B*). The CYP11B2-

to-GAPDH mRNA ratio and protein expression were also significantly increased by 118 and 97%, respectively, in ANG II-treated podocytes compared with NG cells ($P < 0.05$) (Fig. 1, *A* and *B*).

The MCR mRNA expression was also significantly increased in podocytes exposed to NG + ANG II and HG vs. NG cells ($P < 0.05$). The MCR-to-GAPDH mRNA ratio was 2.3 ($P < 0.01$)- and 2.0 ($P < 0.05$)-fold higher in ANG II- and HG-, respectively, relative to NG-stimulated podocytes, and ARB treatment significantly inhibited the increase in MCR mRNA expression by 81% in HG cells ($P < 0.05$) (Fig. 1A). The expression of MCR protein showed a similar pattern to its mRNA expression (Fig. 1B).

Aldosterone levels. Aldosterone levels were significantly higher in NG + ANG II (3.77 ± 0.53 pg/μg)- and HG (3.19 ± 0.42 pg/μg)-conditioned media compared with the NG medium (1.29 ± 0.15 pg/μg) ($P < 0.01$), and this increase in
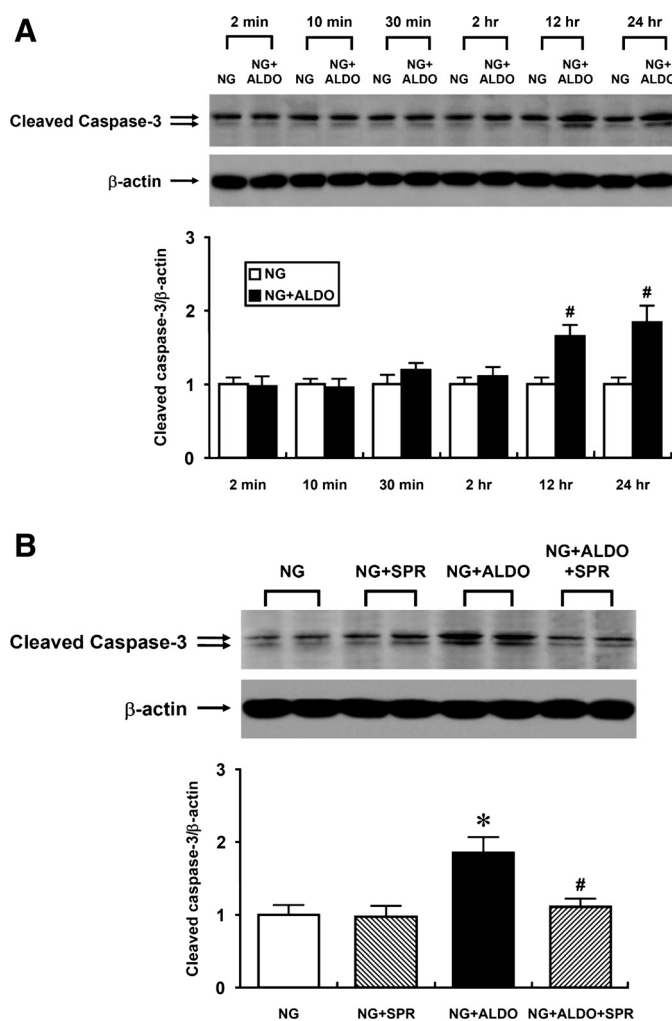
Fig. 4. *A*: representative Western blot of time-course changes in active fragments of caspase-3 protein expression in cultured podocytes exposed to NG and NG + ALDO (representative of 4 blots). Active fragment of caspase-3 protein expression was not significantly increased until 12 h after aldosterone treatment. *B*: representative Western blot of active fragments of caspase-3 protein expression in cultured podocytes exposed to NG, NG + SPR, NG + ALDO, and NG + 10^{-7} M aldosterone + 10^{-7} M spironolactone (NG + ALDO + SPR) (representative of 4 blots). Aldosterone-induced podocyte apoptosis was significantly abrogated by SPR. $P < 0.05$ vs. NG group (*) and vs. NG + ALDO group (#).

aldosterone concentrations in the HG-conditioned medium was attenuated by ARB ($P < 0.05$) (Fig. 2). Mannitol had no effect on aldosterone production in cultured podocytes.

Bax, Bcl-2, and active fragments of caspase-3 protein expression. The effects of aldosterone on apoptosis-related molecules and SPR on HG-induced changes in these molecules in cultured podocytes are shown in Fig. 3. Aldosterone significantly induced Bax and active fragments of caspase-3 protein expression and significantly reduced Bcl-2 protein expression in cultured podocytes ($P < 0.05$). Bax and active fragments of caspase-3 protein expression were also significantly increased, whereas Bcl-2 protein expression was significantly decreased in podocytes cultured under HG medium compared with NG cells ($P < 0.05$), and these changes were inhibited by SPR treatment ($P < 0.05$).

Time-course effect of aldosterone on caspase-3 protein expression and the effect of SPR on aldosterone-induced apoptosis. To determine whether aldosterone-induced podocyte apop-

tosis is mediated through the genomic or nongenomic pathway, we examined not only the time-course effect of aldosterone on caspase-3 protein expression but also the effect of SPR on aldosterone-induced apoptosis in cultured podocytes. As shown in Fig. 4A, active fragment of caspase-3 protein expression was not significantly increased until 12 h after aldosterone treatment. In addition, aldosterone-induced podocyte apoptosis was significantly abrogated by SPR (Fig. 4B).

Hoechst 33342 staining. Apoptotic cells assessed by Hoechst 33342 staining were significantly increased in HG- vs. NG-stimulated cells ($P < 0.01$), and this increment in apoptotic cells was ameliorated by SPR ($P < 0.05$). Aldosterone also significantly increased the number of apoptotic cells in cultured podocytes ($P < 0.01$) (Fig. 5).

11β-HSD2 mRNA expression in culture podocytes. Because nonselective MCR activation by glucocorticoids may also play a role in podocyte apoptosis under HG conditions, we examined the mRNA expression of 11β-HSD2, an enzyme that protects against glucocorticoid-induced MCR activation by converting biologically active 11-hydroxysteroid (cortisol) to the inactive 11-ketosteroid form (cortisone). 11β-HSD2 enzyme was present in cultured podocytes, but there was no difference in the 11β-HSD2-to-GAPDH mRNA ratio among NG, NG + M, and HG-stimulated podocytes (Fig. 6).

Animal Studies

Animal data. All animals gained weight over the 3-mo experimental period, but weight gain was highest in C rats ($P < 0.01$). The ratio of kidney weight to body weight in DM rats ($1.51 \pm 0.10\%$) was significantly higher than those in C ($0.60 \pm 0.06\%$), C + SPR (SPR) (0.64 ± 0.06) ($P < 0.01$), and DM + SPR rats ($0.97 \pm 0.08\%$) ($P < 0.05$). Mean blood glucose levels of C, C + SPR, DM, and DM + SPR rats were 111.8 ± 5.4, 113.7 ± 6.7, 468.5 ± 11.5, and 475.4 ± 10.9 mg/dl, respectively ($P < 0.01$). Compared with the C group (0.37 ± 0.07 mg/day), 24-h urinary albumin excretion was significantly higher in the DM group (1.69 ± 0.30 mg/day,
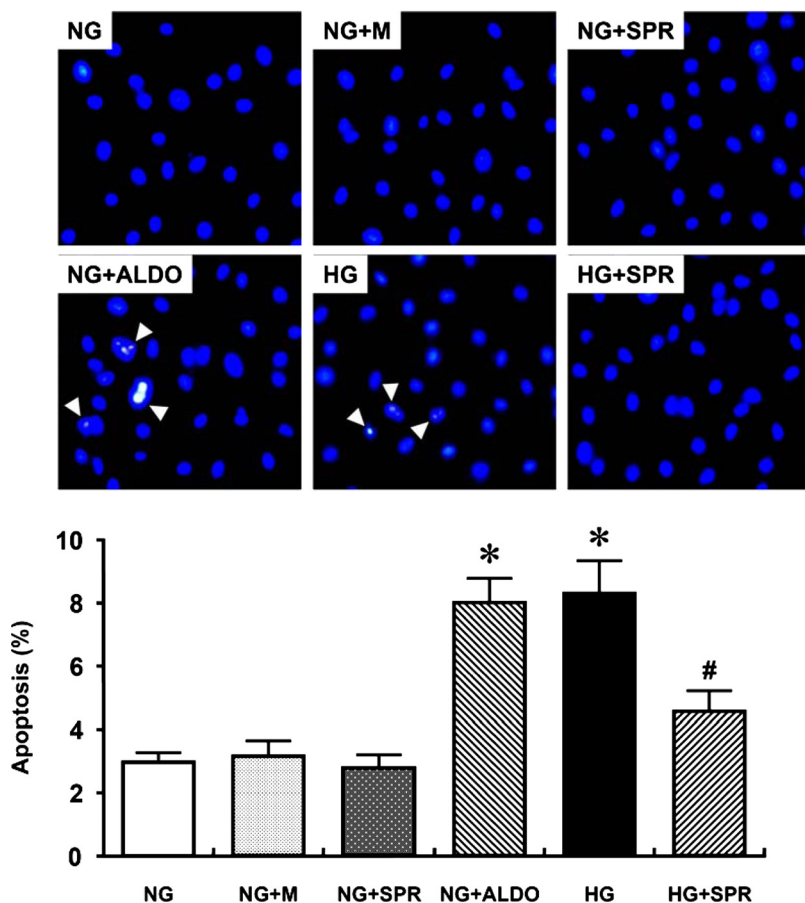

Fig. 5. Apoptosis assessed by Hoechst 33342 staining. There was a significant increase in apoptotic cells (arrowheads) in HG- vs. NG-stimulated cells, and this increase in apoptotic cells was ameliorated with SPR treatment. ALDO also significantly increased the no. of apoptotic cells in cultured podocytes. *$P < 0.01$ vs. NG, NG + M, and NG + SPR groups. #$P < 0.05$ vs. HG group.
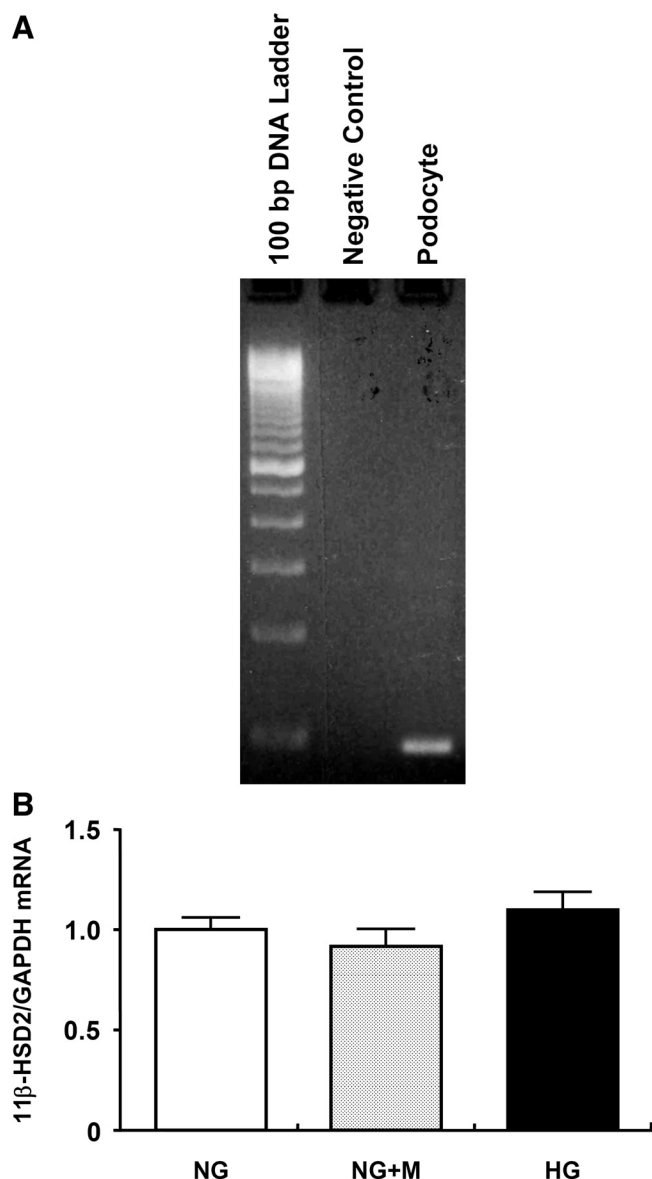
A

B

Fig. 6. *A*: representative RT-PCR for 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) with cDNA corresponding to 20 ng RNA extracted from cultured podocytes. RT-PCR without cDNA was performed as a negative control. *B*: 11β-HSD2-to-GAPDH mRNA ratio in cultured podocytes exposed to NG, NG + M, and HG. There was no difference in the 11β-HSD2-to-GAPDH mRNA ratio among NG-, NG + M-, and HG-stimulated podocytes.

$P < 0.05$), and SPR treatment significantly reduced albuminuria in DM rats (0.65 ± 0.13 mg/day, $P < 0.05$).

In the preliminary experiments using diabetic rats treated with 2 U/day of insulin (Ultralente; Eli Lilly, Indianapolis, IN), the increases in CYP11B2, MCR, and cleaved caspase-3 expression in 3-mo diabetic kidney were significantly ameliorated by insulin treatment, suggesting that these changes in STZ-induced diabetic rats were not the result of STZ per se.

Glomerular CYP11B2 and MCR mRNA and protein expression. The changes in CYP11B2 and MCR mRNA and protein expression in DM glomeruli were similar to those in HG-stimulated podocytes. The CYP11B2-to-GAPDH mRNA and MCR-to-GAPDH mRNA ratios were significantly higher in DM glomeruli by 83% ($P < 0.05$) and 139% ($P < 0.01$),

respectively, compared with C glomeruli (Fig. 7*A*). Western blot analysis also revealed significant increases in CYP11B2 and MCR protein expression in DM glomeruli (Fig. 7*B*). On the other hand, SPR had no effect on the increases in glomerular CYP11B2 and MCR mRNA and protein expression in DM rats (Fig. 7, *A* and *B*). Double immunofluorescence staining for CYP11B2 or MCR with synaptopodin revealed that the increases in CYP11B2 and MCR protein expression were mainly attributed to their increases in podocytes (Fig. 7*C*).

Aldosterone levels. Plasma aldosterone levels were comparable between C and DM rats (333.9 ± 49.3 vs. 297.5 ± 30.2 pg/ml). In contrast, compared with C glomeruli (87.8 ± 11.3 pg/mg), there was a significant increase in the concentrations of aldosterone in DM glomeruli (139.5 ± 19.7 pg/mg) ($P < 0.05$), which was not affected by SPR (133.3 ± 20.5 pg/mg).

Bax, Bcl-2, and active fragments of caspase-3 protein expression. Bax and active fragments of caspase-3 protein expression were significantly increased, whereas Bcl-2 protein expression was significantly decreased in DM compared with C glomeruli ($P < 0.05$). The administration of SPR significantly abrogated the increases in the ratio of Bax to Bcl-2 ($P < 0.05$) and active fragments of caspase-3 protein expression in DM glomeruli ($P < 0.05$) (Fig. 8).

TUNEL assay. In addition to Bax, Bcl-2, and active fragments of caspase-3 protein expression, apoptosis in glomeruli was assessed by TUNEL assay. The number of glomerular apoptotic cells was significantly increased in DM compared with C and C + SPR rats ($P < 0.01$), and the increase in apoptotic cells in DM glomeruli was attenuated by SPR treatment ($P < 0.05$) (Fig. 9).

Podocyte numbers. Compared with C (171.2 ± 5.3) and C + SPR rats (173.5 ± 5.5), the number of podocyte was significantly decreased in DM rats (145.1 ± 4.7), and the reduction in podocyte numbers was inhibited in DM rats treated with SPR (167.3 ± 6.9) ($P < 0.05$).

DISCUSSION

Recent clinical and experimental studies have demonstrated that aldosterone acts as a mediator in the development and progression of diabetic nephropathy, but the direct effect of aldosterone on podocyte injury has not been well explored. In this study, we demonstrate for the first time that CYP11B2 is present in podocytes and that its expression is increased under diabetic conditions. In addition, the results of the present study reveal that aldosterone levels are significantly higher in diabetic compared with control glomeruli despite comparable plasma aldosterone concentrations between the two groups, suggesting intrarenal activation of the aldosterone system under diabetic conditions. Moreover, we show that MCR blocker inhibits podocyte apoptosis both in vivo and in vitro, indicating that a local aldosterone system may be involved in the process of podocyte injury under diabetic conditions.

Aldosterone is classically produced by zona glomerulosa cells in the adrenal cortex, and its production in the adrenal gland is largely controlled by the expression levels of the CYP11B2 gene, which is regulated by various factors, including adrenocorticotrophic hormone, ANG II, and potassium (38). However, accumulating evidence has suggested that aldosterone can be synthesized in nonadrenal cells, such as vascular endothelial and smooth muscle cells, and cardiac
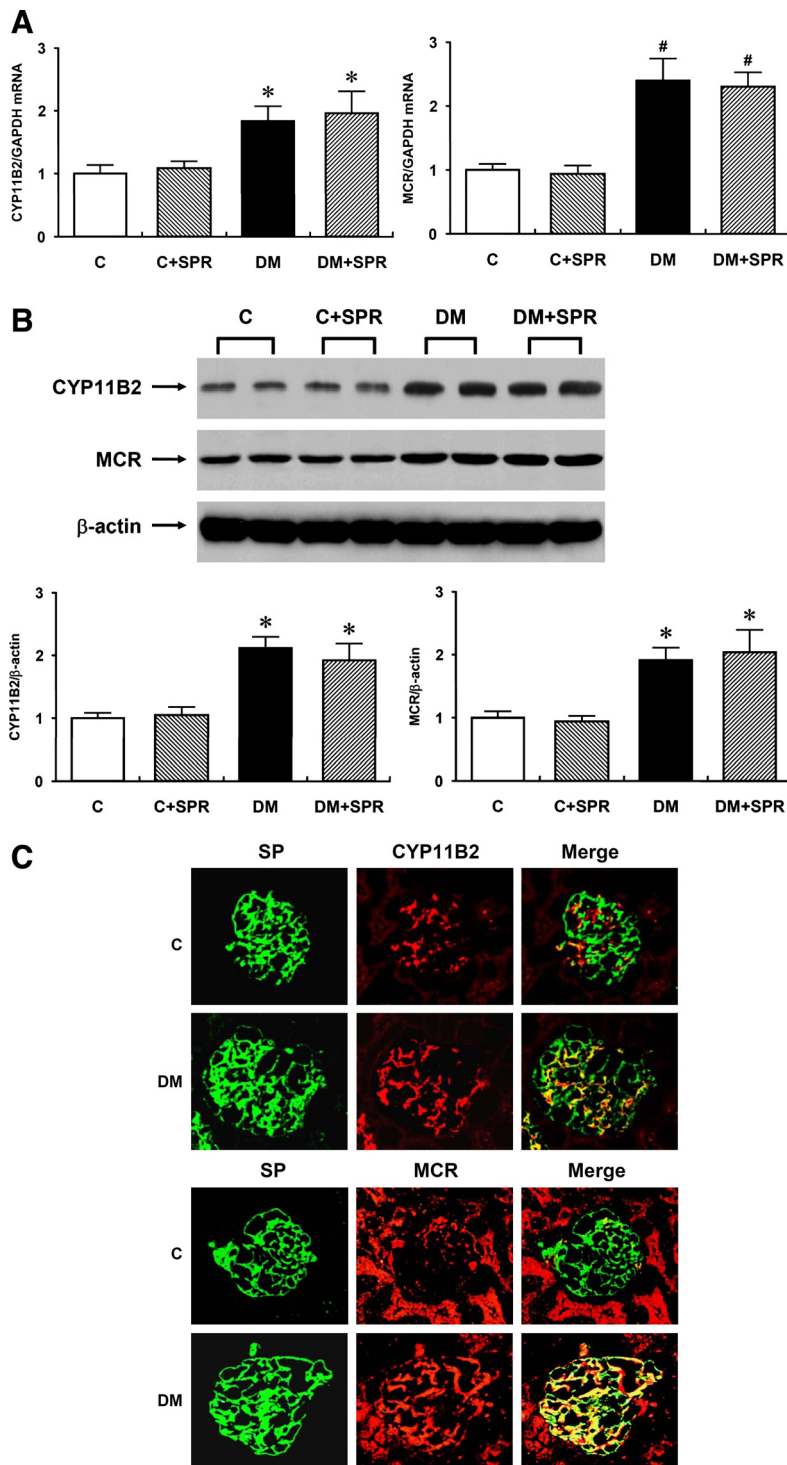
Fig. 7. *A*: CYP11B2-to-GAPDH mRNA and MCR-to-GAPDH mRNA ratios in control (C) and diabetes mellitus (DM) glomeruli. The CYP11B2-to-GAPDH mRNA and MCR-to-GAPDH mRNA ratios were significantly higher in DM glomeruli by 83 and 139%, respectively, compared with C glomeruli. On the other hand, SPR had no effect on the increases in glomerular CYP11B2 and MCR mRNA expression in DM rats. *B*: representative Western blot of CYP11B2 and MCR in C and DM glomeruli. There were 2.1- and 1.9-fold increases in CYP11B2 and MCR protein expression, respectively, in DM relative to C glomeruli. On the other hand, SPR had no effect on the increases in glomerular CYP11B2 and MCR protein expression in DM rats. *C*: double-immunofluorescence staining for CYP11B2 or MCR with synaptopodin. Compared with C, immunofluorescence staining for CYP11B2 and MCR was increased in DM glomeruli, and double-immunofluorescence staining revealed that the increases in CYP11B2 and MCR protein expression were mainly attributed to their increases in podocytes. Magnification ×400. *$P < 0.05$ vs. C group. #$P < 0.01$ vs. C group.

myocytes (11, 28, 32). Glomerular mesangial and tubular epithelial cells are also found to express the CYP11B2 gene (15, 41). Moreover, Xue and Siragy (43) demonstrated that CYP11B2 mRNA and protein expression was localized mainly in the renal cortex and was upregulated by ANG II and low salt intake and that, even though aldosterone was absent in adrenalectomized rats, it was present in renal interstitium and tissue. In this study, we show for the first time that podocytes also express the CYP11B2 gene. In addition, ANG II and HG

increase CYP11B2 mRNA and protein expression in cultured podocytes. 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 ANG II 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 (11, 12, 15, 38). Moreover, based on the previous results that HG increases ANG II levels in cultured podocytes (44) and the present findings that ARB treatment

Fig. 8. A representative Western blot of glomerular Bax, Bcl-2, and active fragments of caspase-3 in C, C + SPR, DM, and DM + SPR groups. Bax and active fragments of caspase-3 protein expression were significantly increased, whereas Bcl-2 protein expression was significantly decreased in DM compared with C glomeruli. Administration of SPR significantly ameliorated the increases in the ratio of Bax/Bcl-2 and active fragments of caspase-3 protein expression in DM glomeruli. *$P < 0.01$ vs. C and C + SPR groups. $P < 0.05$ vs. DM group (#) and vs. C and C + SPR groups (†).
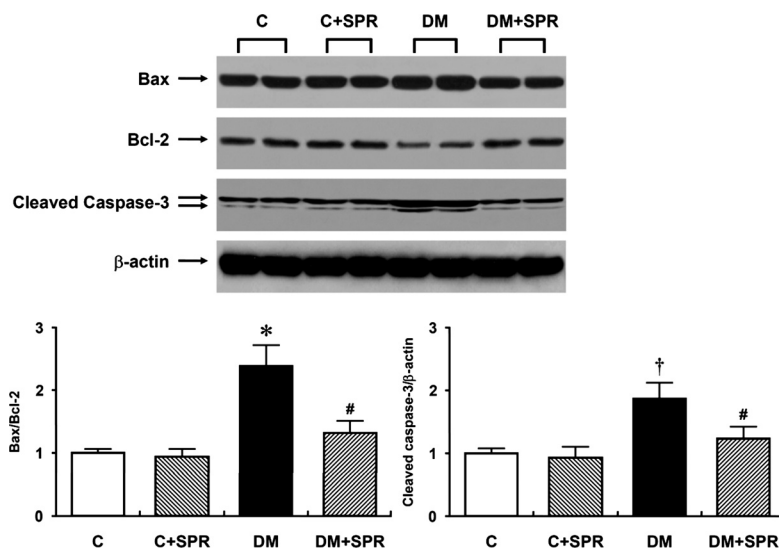
significantly abrogated the increment in CYP11B2 expression in podocytes exposed to HG, we speculate that increased CYP11B2 expression in HG-stimulated podocytes is partly attributed to the increase in ANG II levels by HG.

Several recent studies also showed that CYP11B2 expression was increased in diabetic glomeruli (31, 43), with which the results of our study were in agreement, but it was not elucidated which glomerular cells are responsible for this increase. Using double-immunofluorescence staining, we demonstrate for the first time that the increase in glomerular CYP11B2 protein expression was mainly attributed to its increased expression in podocytes. Although the exact role of these extra-adrenal aldosterone systems has not been completely elucidated, it is possible that locally produced aldosterone may exert autocrine or paracrine effects. On the other hand, the results of this study show that SPR treatment has no effect on the increases in CYP11B2 and MCR expression under diabetic conditions, which is inconsistent with a recent study by Taira et al. (31). They found that SPR prevented the increase in renal CYP11B2 and MCR expression in uninephrectomized STZ-induced diabetic rats. Although the reasons for these discrepant effects of SPR on CYP11B2 and MCR expression remain unclear, differences between animal models, duration of the animal experiments, administered dose of SPR, accompanying hypertension, or tissue used for the experiments may contribute to these disparities.

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 (2, 5, 13, 17). Because cellular hypertrophy, ECM accumulation, and apoptosis are characteristic findings of diabetic nephropathy (1, 40), aldosterone is considered a potential mediator in the pathogenesis of diabetic nephropathy. In support of this theory, recent experimental studies showed that MCR blocker prevented the development and progression of diabetic nephropathy by ameliorating glomerular and tubulointerstitial ECM accumulation and by inhibiting macrophage infiltration (6, 7, 9, 31, 45). 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 SPR (8). Lai et al. (14) 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.
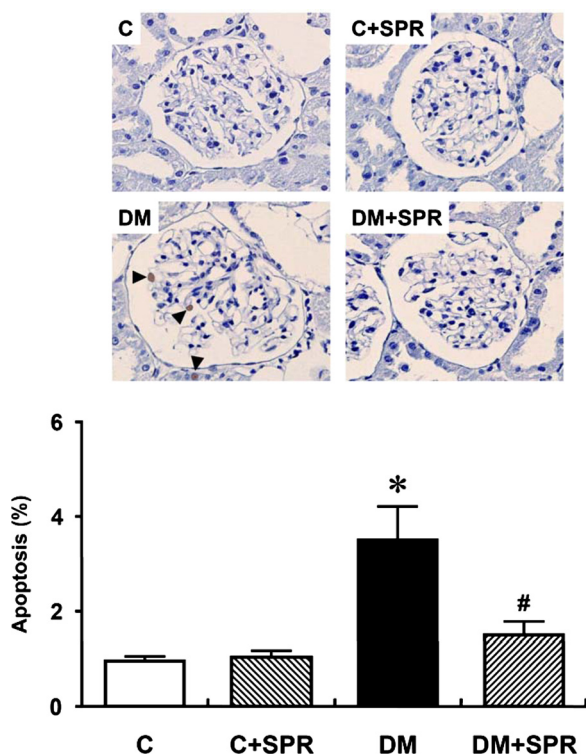

Fig. 9. Apoptosis assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay in C, C + SPR, DM, and DM + SPR rats. The no. of glomerular apoptotic cells (arrowheads) was significantly increased in DM compared with C and C + SPR rats, and the increase in apoptotic cells in DM glomeruli was attenuated by SPR treatment. *$P < 0.01$ vs. C and C + SPR groups. #$P < 0.05$ vs. DM group.

Aldosterone is also known to induce apoptosis in cardiac myocytes and skeletal muscle cells (2, 17), but the effect of aldosterone on podocyte apoptosis has never been explored. One recent study by Nagase et al. (21) revealed that podocyte injury in Dahl salt-hypertensive rats was prevented by a MCR 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 (27). In this study, we demonstrate for the first time that aldosterone induces apoptosis in cultured podocytes. Moreover, HG-induced CYP11B2 expression leads to increases in aldosterone levels, and MCR blocker inhibits apoptosis in podocytes exposed to HG medium and in diabetic glomeruli, suggesting that the activation of a local aldosterone system (i.e., increases in both CYP11B2 and MCR expression) contributes to podocyte apoptosis under diabetic conditions.

Besides the traditional (genomic) effects of aldosterone by binding to the cytoplasmic MCR and interacting with target genes, a number of rapid (nongenomic) effects have been described for aldosterone, which result from an interaction with a membrane receptor distinct from the classical steroid receptor and are insensitive to translation or transcription inhibitors (22). Rapid action of aldosterone, especially on electrolyte transport, and subsequent changes in intracellular electrolytes have been demonstrated in human mononuclear leukocytes (35) and vascular muscle cells (3, 36, 37), and previous studies have suggested that protein kinase C, Ca^{2+}, and inositol 1,4,5-triphosphate are involved in its nongenomic action (16, 18, 36, 37). However, direct evidence of the nongenomic action of aldosterone has been drawn from a study by Haseroth et al. (10). They showed that a rapid increase in intracellular Ca^{2+} induced by aldosterone was still present in cultured skin cells derived from MCR knockout mice. In the present study, we found that podocyte apoptosis was not apparent until 12 h after aldosterone treatment. Moreover, aldosterone-induced podocyte apoptosis was significantly abrogated by MCR blocker. Based on these findings, it seems that podocyte apoptosis induced by aldosterone is mainly mediated through the classical aldosterone-MCR pathway.

In summary, CYP11B2 and MCR expression are increased in HG-stimulated podocytes and in diabetic glomeruli along with increased podocyte apoptosis, and MCR blocker inhibited the increase in apoptosis in podocytes under diabetic conditions. These findings suggest that a local aldosterone system is activated and is involved in podocyte apoptosis under diabetic conditions.

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