The Role of Locally Activated Renin-Angiotensin System in Albumin Permeability in Glomerular Endothelial Cells under High Glucose Condition

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The Role of Locally Activated Renin-Angiotensin System in Albumin Permeability in Glomerular Endothelial Cells under High Glucose Condition

Directed by Professor Shin-Wook Kang

The Master's Thesis
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Ji Sun Paeng

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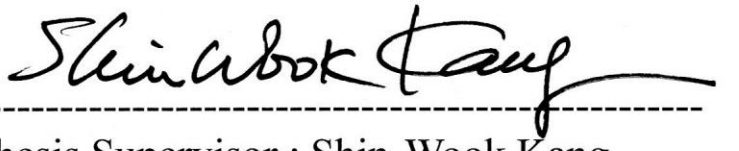
This certifies that the Master's Thesis
of Ji Sun Paeng is approved.



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ABSTRACT

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Background: Previous studies have demonstrated that local renin-angiotensin system (RAS) is activated in proximal tubular cells, mesangial cells, and podocytes under diabetic conditions. However, the role of RAS within glomerular endothelial cells (GECs) in the pathogenesis of diabetic nephropathy has not been fully explored. In this study, I investigated the existence and changes of RAS components in high glucose (HG)-stimulated GECs and the role of local RAS in morphological and functional changes of GECs under diabetic conditions.

Methods: In vitro, GECs were exposed to 5.6 mM glucose (NG), NG+24.4 mM mannitol, or 30 mM glucose (HG) with or without 10^{-7} M L-158,809, an angiotensin II type I receptor (AT1R) blocker, for 24 hours. In vivo, 32

Sprague-Dawley rats were injected with diluents (n=16, C) or streptozotocin intraperitoneally (n=16, DM), and 8 from each group were treated with 1 mg/kg/day of L-158,809 by oral gavage for 3 months. Real-time PCR, Western blot analysis, and enzyme-linked immunosorbent assay (ELISA) using cultured GECs were carried out to examine the activation of local RAS. Immunofluorescent (IF) staining for VE-cadherin and heparin sulfate glycosaminoglycans (HS-GAG) was also performed. In addition, the permeability of GECs to macromolecules was assessed by measuring the passage of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) across the monolayer of GECs. Moreover, the morphological changes were evaluated by scanning electron microscopy (SEM).

Results: There were 4.9- and 3.4-folds increases in angiotensinogen mRNA and protein expression in HG-stimulated GECs compared to NG cells, respectively ($P<0.01$). The concentrations of angiotensin I (AI) and AII in HG-conditioned cultured media were also significantly higher than those in NG media ($P < 0.05$). In contrast, there were no differences in the mRNA and protein expression of angiotensin-converting enzyme, renin, AT1R, and AT2R among the groups. IF staining showed that HS-GAG protein expression was significantly decreased ($P < 0.01$), while there was no change in VE-cadherin protein expression in GECs exposed to HG medium. The permeability to albumin assessed by FITC-BSA permeability assay was significantly higher in GECs cultured under HG conditions ($P < 0.001$), and L-158,809 treatment significantly abrogated the

increase in albumin permeability in HG cells (P < 0.01). On SEM examination, the mean size of fenestrae was significantly greater in HG-stimulated GECs (P < 0.01), and the enlarged fenestrae in HGcells were significantly ameliorated by AT1R blocker (P <0.05). In vivo, urinary albumin excretion and the size of GECs fenestrae were also significantly greater in DM rats compared to C rats (P <0.01), and these increases were significantly attenuated by L-158,809 (P <0.05).

Conclusion: Local RAS within GECs was activated under HG conditions, and this locally activated RAS seemed to be associated with the alteration in GEC fenestration along with a decrease in HS-GAG, resulting in the development of albuminuria in diabetic nephropathy.

Key words : GECs, fenestrae, RAS, diabetic nephropathy

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I. INTRODUCTION

The glomerular filtration barrier is comprised of three layers: a fenestrated endothelial layer, a glomerular basement membrane (GBM), and podocyte foot processes connected by a slit diaphragm[1]. Traditionally, the GBM and the slit diaphragm have been thought to function as a coarse and fine filter, respectively, that contributes to ultimate size-selectivity, while glomerular endothelium to serve as a charge-selective barrier, all together prohibiting permeability to large-sized and anionic-charged albumin[2].

Diabetic nephropathy, the leading cause of end-stage renal disease in many countries, is clinically characterized by proteinuria[3]. Since the underlying

pathologic change responsible for proteinuria is the loss of size-selective and/or charge-selective properties of the glomerular filtration barrier, a large number of previous studies have tried to elucidate the structural and functional changes in the glomerular filtration barrier, particularly in podocytes, in diabetic nephropathy[4]. In contrast to podocytes, glomerular endothelial cells (GECs) have not attracted a lot of attention in terms of the pathophysiology of proteinuria in diabetic nephropathy. Recently, however, accumulating evidence has indicated that the changes in GECs are also associated with the development of proteinuria in diabetic nephropathy[5]. While GECs are covered by 200-400 nm thickness of a protein-rich surface layer, glycocalyx, and possess numerous circular transcellular fenestrations 60-100 nm in diameter in normal state, a decrease in glycocalyx and changes in the size and number of fenestrations have been demonstrated in GECs under diabetic conditions[6]. Even though the diabetic milieu per se, hemodynamic changes, oxidative stress, and local growth factors are considered to be mediators in the pathogenesis of diabetic nephropathy[7], the underlying pathways mediating the changes of GECs are not well understood until recently.

Previous clinical and experimental studies in diabetic nephropathy have shown that blockade of the rennin-angiotensin system (RAS) by angiotensin-converting enzyme (ACE) inhibitors or angiotensin II (AII) receptor blockers reduced proteinuria that cannot be explained merely by their blood pressure-lowering effect[8]. These findings suggest that RAS blockade may

have direct effects on various renal cells. AII is the major effector molecule of the RAS[9]. It is a powerful direct vasoconstrictor, promotes vascular smooth muscle contraction and induces systemic hypertension[10]. Besides its hemodynamic effects, AII plays an important role in the pathogenesis of various renal diseases, including diabetic nephropathy. Especially, intrarenal RAS is known to be activated in spite of low plasma renin activity in diabetes[11,12]. In addition, previous studies have found that local RAS is activated in proximal tubular cells[13], mesangial cells[14,15], and podocytes[16] under diabetic conditions. However, the existence of local RAS and the changes in local RAS activity under diabetic conditions have never been explored in GECs.

In this study, we investigated the changes in RAS components, including angiotensinogen (AGT), renin, AI, ACE, AII, AII type 1 receptor (AT1R) and AT2R in GECs exposed to high glucose medium. Furthermore, the morphological change of GECs was clarified in GECs under diabetic conditions both in vitro and in vivo, and the effect of RAS blockade on diabetes-induced changes of GECs was elucidated in high glucose-stimulated GECs and diabetic glomeruli.

II. MATERIALS AND METHODS

1. Human GECs culture

Normal human GECs were purchased from Angio-Proteomie Inc. (Boston, MA, USA). The cells were cultured in ENDO-Growth medium (Angio-Proteomie Inc.) containing 5% fetal bovine serum (FBS), recombinant growth factors, 100 U/ml penicillin, and 100 μg/ml streptomycin on culture dishes coated with the coating solution (Quick Coating Solution, Angio-Proteomie Inc.) at 37℃ in humidified 5% CO2 in air. The medium was changed every 2 days. All experiments were performed using cells between the third and fifth passages.

For the experiments, subconfluent human GECs were incubated with medium containing 0.5% FBS for 24 hr to arrest and synchronize cell growth. After this time period, the cells were treated media containing 0.5% ENDO-Growth supplement (Angio-Proteomie Inc.) and 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), or 30 mM glucose (HG) with or without 10^{-7}M L-158,809, an AT1R blocker (ARB).

At 24 hr after the media change, cells were harvested for either RNA or protein, and the conditioned culture media were collected for the measurement of renin activity and the levels of AI and AII.

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. Twenty-four male Sprague-Dawley rats, weighing 250-280 g, were injected with either diluent [n=16, Control (C)] or 65 mg/kg STZ intraperitoneally [n=16, Diabetes (DM)]. Diabetes was confirmed by tail vein blood glucose levels above 300 mg/dl on the third day post-injection. After confirming diabetes, 8 rats from each group were treated daily with 1 mg/kg/day of L-158,809 by oral gavage (C+ARB and DM+ARB) for 3 months. The rats were given free access to water and standard laboratory chow during the 3 months study period. Body weights were checked monthly, and the kidney weights were measured at the time of sacrifice. Serum glucose and 24-hr urinary albumin were also measured at the time of sacrifice. Blood glucose was measured by a glucometer and 24-hr urinary albumin excretion was determined by enzyme-linked immunosorbent assay (ELISA) (Nephrat II, Exocell, Inc., Philadelphia, PA, USA).

3. Glomerular isolation

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

4. Total RNA extraction

Total ribonucleic acid (RNA) was extracted from cultured human GECs as previously described. Briefly, after cell scraping with 800 μl of RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA), the mixture vortexed, and stored for 5 min at room temperature, 160 μl of chloroform added, and the mixture shaken vigorously for 30 sec. After 3 min, the mixture was centrifuged at 12,000 g for 15 min at 4℃ and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by 400 μL isopropanol and pelleted with centrifugation at 12,000 g for 30 min at 4℃. The RNA precipitate was washed with 70% ice-cold ethanol, dried using Speed Vac, and dissolved in diethyl pyrocarbonate (DEPC)-treated distilled water.

The RNA yield and quality were assessed based on spectrophotometric measures at the wavelength of 260 and 280 nm.

5. Reverse transcription

First-strand complementary deoxyribonucleic acid (cDNA) was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Two μg of total RNA extracted from GECs was reverse transcribed using 10 μM random hexanucleotide primer, 1 mM, deoxynucleoside triphosphate (dNTP), 8 mM $MgCl_2$, 30 mM KCl, 50 mM Tris-HCl, pH 8.5, 0.2 mM dithiothreithol (DTT), 25 U RNAse inhibitor, and 40

U avian myeloblastoma virus (AMV) reverse transcriptase. The mixture was incubated at 30℃ for 10 min and 42℃ for 1 hr, followed by inactivation of enzyme at 99℃ for 5 min.

6. Real-time polymerase chain reaction (Real-time PCR)

The primers used for AGT, ACE, renin, ATIR, AT2R, and 18s amplification were as follows: AGT, sense 5'-AGC TGG TGC TAG TCG CTG CA-3', antisense 5'-AGG CCA GGG TGC CAA AGA CA-3'; ACE, sense 5'-ATG GAC CAG CTC TCC ACA GTG CA-3', antisense 5'-ACG TCC CCA ATG GCC TCA TG-3'; renin, sense 5'-AAG CCT GAA GGA ACG AGG TG-3', antisense 5'-TTC ATG GGT TGG CTC CAC TC-3'; AT1R, sense 5' GGG CTG TCT ACA CAG CTA TGG AA-3', antisense 5'-ATG ATG CAG GTG ACT TTG GCT A-3'; AT2R, sense 5'-TCT GGC AGA ACT TCA CGG AC-3', antisense 5'-TTG GCG GTG GAG TAG ATC CT-3'; and 18s, sense 5'-ACC GCG GTT CTA TTT TGT T-3', antisense 5'-CGG TCC AAG AAT TTC ACC TC-3'.

Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), 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, and 5 pM sense and antisense primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. Each sample was run in triplicate in

separate tubes to permit quantification of the gene normalized to the 18s. The PCR conditions were as follows: 35 cycles of denaturation at 94.5°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 minute. Initial heating at 95°C for 9 minutes and final extension at 72°C for 7 minutes were performed for all PCRs.

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 18s gene and expressed in arbitrary units. The results are given as relative expression of AGT, ACE, renin, AT1R, and AT2R normalized to the expression of the 18s housekeeping gene.

7. Western blot analysis

GECs harvested from plates 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 protein were treated with Laemmli sample buffer, heated at 100°C for 5 min, and electrophoresed at 50 μg/lane in a 8%-12% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA), and the membrane was then incubated in blocking buffer A [1 x phosphate-buffered saline (PBS), 0.1% Tween-20, and

8% nonfat milk] for 1 hr at room temperature, followed by an overnight incubation at 4°C in a 1:1000 dilution of polyclonal antibodies to AGT (Abcam, Cambridge, MA, USA), ACE (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), AT1R, AT2R (Abcam), or β-actin (Sigma Chemical Co., St Louis, MO, USA). The membrane was then washed once for 15 min and twice for 5 min 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 donkey anti-goat IgG (Amersham Life Science, Inc., Arlington Heights, IL, USA). 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).

8. Measurement of renin activity

Renin activity was determined as the formation of AI in the presence of excess substrate, AGT. Samples were incubated with or without porcine AGT (1 μM) (Sigma Chemical Co.) at 37°C for 1 hr, followed by the measurement of AI levels using a commercial ELISA kit (DB Biosciences, San Diego, CA, USA).

9. ELISA for AI and AII

The levels of AI and AII were measured in conditioned culture media using a commercial ELISA kit (Peninsula Laboratories, Belmont, CA, USA) according to the manufacturer's protocol.

10. Immunofluorescent microscopy

Confluent GECs on cover slips were washed with cold PBS, fixed with 70% methanol for 30 min at 4℃, washed again with cold PBS, and then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After rinsing with PBS, nonspecific binding sites were blocked with 5% goat serum in PBS for 1 hr at room temperature. Primary antibodies for VE-cadherin and heparan sulfate glycosaminoglycans (HS-GAG) (Abcam, Cambridge, MA, USA) (prediluted in 5% goat serum, 4% bovine serum albumin, and 0.2% Triton X-100 in PBS) were applied at 4℃ overnight. Antigen-antibody complexes were visualized by incubation with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 hr at room temperature. The cover slips were washed with PBS and mounted on glass slides using Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA). Images were obtained by using digital camera attached to fluorescence microscopy (BX51; Olympus Co., Tokyo, Japan).

11. Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) permeability assay

The permeability of GECs to macromolecules was assessed by measuring the passage of FITC-BSA (Sigma Chemical Co.) across the monolayer. Polycarbonate micropore membranes with a diameter of 24 mm and a pore size of 0.4 μm (Corning Costar, Cambridge, MA, USA), which were coated with

Quick Coating Solution, were used. GECs ($1x10^3$ in ENDO-Growth medium) were seeded onto the membranes, and were cultured for 5-7 days to allow them to become confluent. After serum restriction for 24 hr, the cells were cultured under different experimental conditions for 24 hr. After then, the medium in the insert was replaced with ENDO-Growth medium with 0.5 mg/ml FITC-BSA, and an aliquot of medium from the upper chamber was collected at 24 hr, and the fluorescence of the aliquot was measured using a fluorospectrometer at the wavelength of 492 and 520 nm.

12. Scanning electron microscopy

Cells cultured under different experimental conditions for 24 hr on chamber slide and isolated glomeruli from each animal were fixed with 2% glutaraldehyde-paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 2 hr at 32℃, washed three times for 30 min in 0.1 M PB, postfixed in 1% OsO4 for 2 hr at 4℃, and dehydrated by treatment with a graded series of ethanol (5 min each in 50, 60, 70, 80, 90, and 95% and twice in 100%). After then, the specimens were infiltrated with propylene oxide followed by isoamyl acetate, and subjected to Critical Point Dryer (HCP-2, Hitachi, Japan). They were coated with gold by ion sputter (IB-3, Eiko, Japan), and examined and photographed in a random and unbiased manner with a scanning electron microscopy (S-800-FE-SEM, Hitachi, Japan) at the acceleration voltage of 20 kV.

13. Morphometric measurement of GEC fenestrae

Photographs taken of 10 GECs from 3 different experiments in each group and 10 glomeruli from 3 rats in each group were used to determine the size of fenestrae. From each photograph, 20 fenestrae were randomly selected, and their areas were constructed and measured using hand-free tool of Image J software v1.60 (NIH, Bethesda, MA, USA; online at http://rsbweb.nih.gov/ij).

14. Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the statistical package SPSS for Windows Ver. 13.0 (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 further confirmed by the Mann-Whitney U-test. P values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Cell culture studies

A. AGT, ACE, renin, AT1R, and AT2R mRNA expression

We first examined the changes in the mRNA expression of the RAS components in cultured GECs. There was a significant increase in AGT mRNA expression in GECs exposed to HG medium. The ratio of AGT mRNA to 18s rRNA was 4.9-fold higher in HG-stimulated GECs compared to NG cells ($P < 0.01$). In contrast, there were no significant differences in ACE, renin, AT1R, and AT2R mRNA expression between the HG and NG groups (Figure 1). On the other hand, mannitol had no effect on the mRNA expression of the RAS components in cultured GECs.

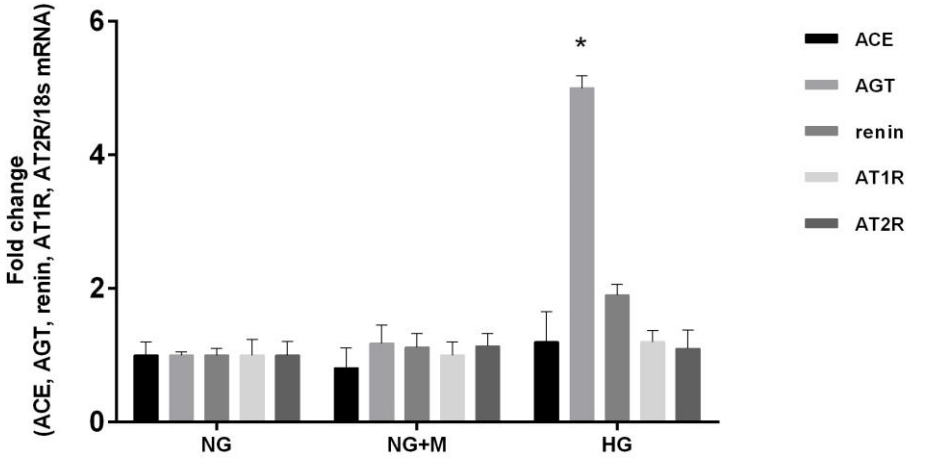

Figure 1. mRNA expressions of ACE, AGT, renin, AT1R and AT2R in GECs exposed to 5.6 mM glucose (NG), NG + 24.4 mM mannitol (NG+M), and 30 mM glucose (HG). There was a significant increase in AGT mRNA expression in GECs exposed to HG medium. In contrast, there were no significant differences in ACE, renin, AT1R, and AT2R mRNA expression among the groups. On the other hand, mannitol had no effect on the mRNA expression of the RAS components in cultured GECs. *; $P < 0.01$ vs. NG and NG+M groups

B. AGT, ACE, AT1R, and AT2R protein expression

Similar to the mRNA expression, the protein expression of AGT was also significantly higher in GECs cultured under HG conditions. Densitometric quantitation revealed an average 3.4-fold increase in AGT protein expression in HG-stimulated GECs compared to NG cells ($P < 0.01$). In contrast, there were no significant differences in ACE, AT1R, and AT2R mRNA expression between the HG and NG groups (Figure 2). Meanwhile, mannitol had no effect on the protein expression of the RAS components in cultured GECs.

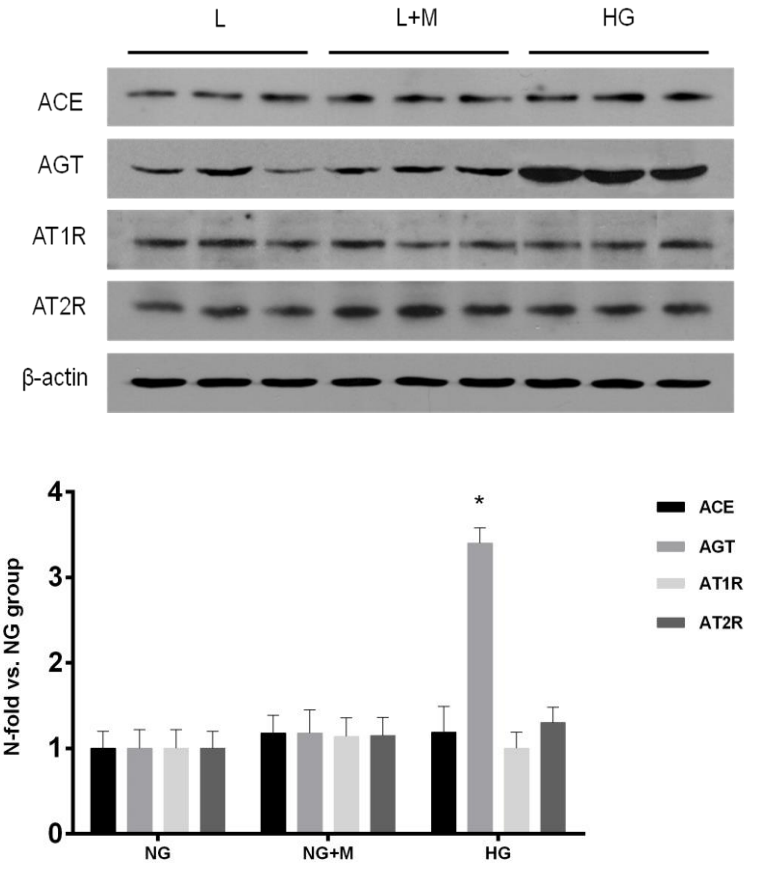

Figure 2. Protein expressions of ACE, AGT, AT1R and AT2R in GECs exposed to 5.6 mM glucose (NG), NG + 24.4 mM mannitol (NG+M), and 30 mM glucose (HG). Similar to the mRNA expression, the protein expression of AGT was also significantly higher in GECs cultured under HG conditions. In contrast, there were no significant differences in ACE, AT1R, and AT2R mRNA expression among the groups.

Meanwhile, mannitol had no effect on the protein expression of the RAS components in cultured GECs. *; $P < 0.01$ vs. NG and NG+M groups

C. Renin activity

Renin activity in cultured GECs, which was determined by measuring AI levels in the presence of excess exogenous AGT, was comparable among the groups (data not shown).

D. AI and AII levels in conditioned culture media

The concentrations of AI and AII in HG-conditioned cultured media (AI, 362.5 ± 35.7 pg/μl; AII, 118.7 ± 15.7 pg/μl) were significantly higher than those in NG media (AI, 110.3 ± 14.6 pg/μl; AII, 70.8 ± 10.7 pg/μl) ($P < 0.05$) (Figure 3).

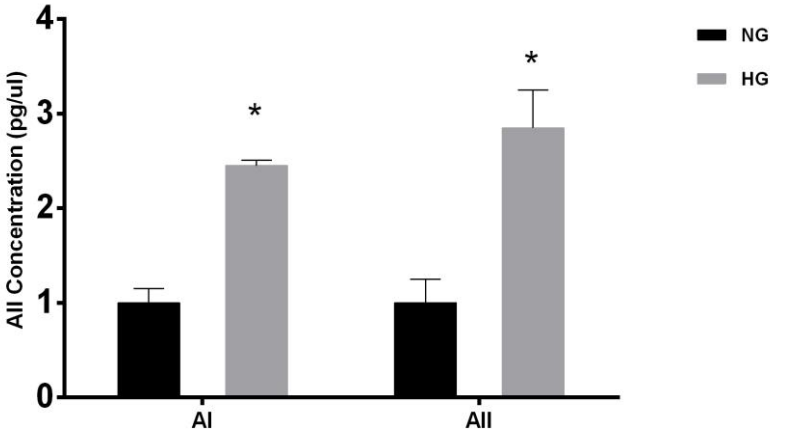

Figure 3. AI and AII levels in 5.6 mM glucose (NG) and 30 mM glucose (HG)-conditioned culture media. The concentrations of AI and AII in HG-conditioned cultured media were significantly higher than those in NG media. *; $P < 0.05$ vs. NG group

E. VE-cadherin and HS-GAG protein expression

Immnunofluorescent staining revealed that there was no change in VE-cadherin protein expression, while HS-GAG protein expression was significantly decreased in GECs exposed to HG medium compared to NG cells ($P < 0.01$). In addition, the decrease in HS-GAG protein expression in HG-stimulated GECs was significantly attenuated by L-158,809 ($P < 0.05$) (Figure 4).

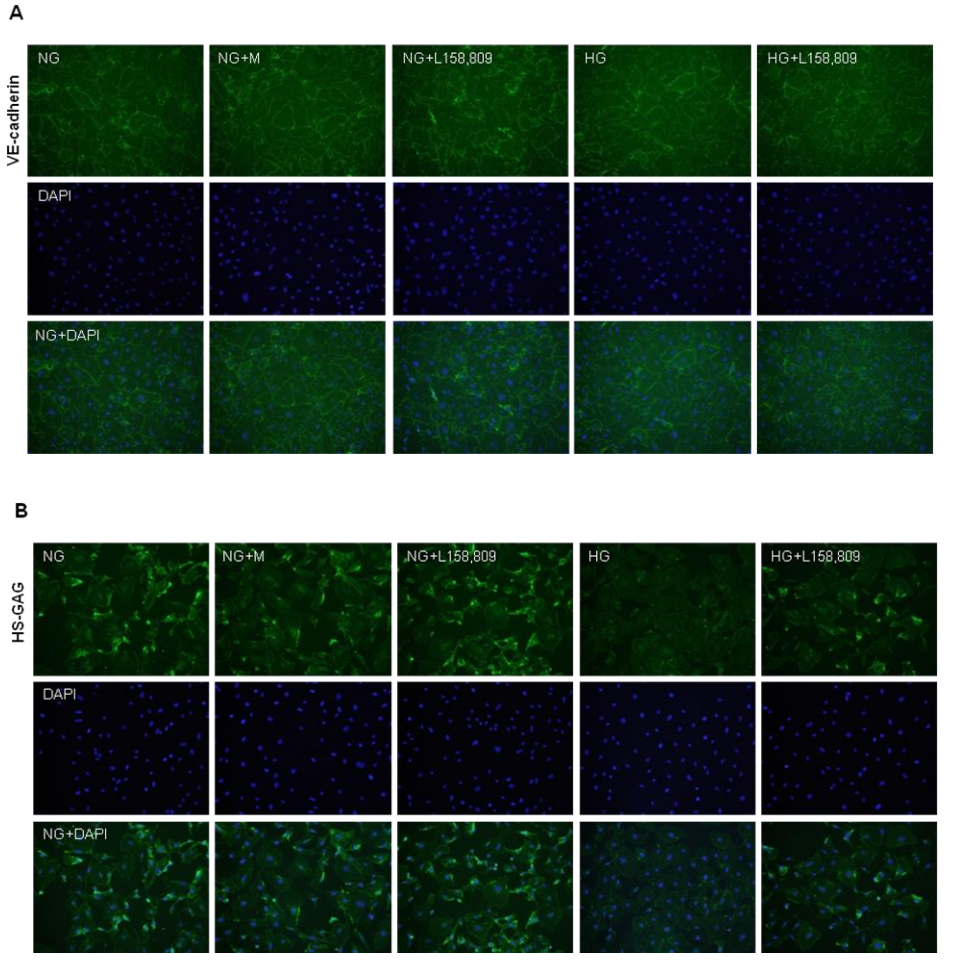

Figure 4. VE-cadherin (A) and HS-GAG (B) protein expression, assessed by immnunofluorescent staining in GECs exposed to 5.6 mM glucose (NG), NG + 24.4 mM mannitol (NG+M), NG + 10^{-7} M L-158,809, and 30 mM glucose (HG), and HG + 10^{-7} M L-158,809. There was no change in VE-cadherin protein expression, while HS-GAG protein expression was significantly decreased in GECs exposed to HG medium compared to NG cells. In addition, the decrease in HS-GAG protein expression in HG-stimulated GECs was significantly attenuated by L-158,809 (×200).

F. FITC-BSA permeability assay

Compared to NG-stimulated cells, the permeability to albumin determined by FITC-BSA permeability assay was significantly higher in GECs exposed to HG medium ($P < 0.05$), and L-158,809 treatment significantly abrogated the increase in albumin permeability in HG cells ($P < 0.05$) (Figure 5).

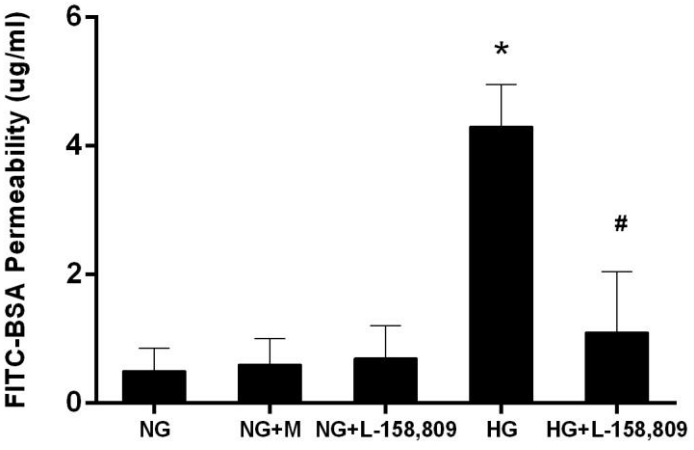

Figure 5. FITC-BSA permeability assay in GECs exposed to 5.6 mM glucose (NG), NG + 24.4 mM mannitol (NG+M), NG + 10-7 M L-158,809, and 30 mM glucose (HG), and HG + 10-7 M L-158,809. Compared to NG-stimulated cells, the permeability to albumin determined by FITC-BSA permeability assay was significantly higher in GECs exposed to HG medium, and L-158,809 treatment significantly abrogated the increase in albumin permeability in HG cells. *; $P < 0.05$ vs. NG, NG+M, and NG+L-158,809 groups, $^{\#}$; $P < 0.05$ vs. HG group

G. Morphological change of fenestrae in cultured GECs

The size of GEC fenestrae was assessed using scanning electron microscopy. The mean size of fenestrae was significantly greater in GECs cultured under HG conditions than that in NG cells ($P < 0.01$), and the enlarged fenestrae in HG-stimulated cells were signifiantly ameliorated by L-158,809 ($P < 0.05$) (Figure 6).

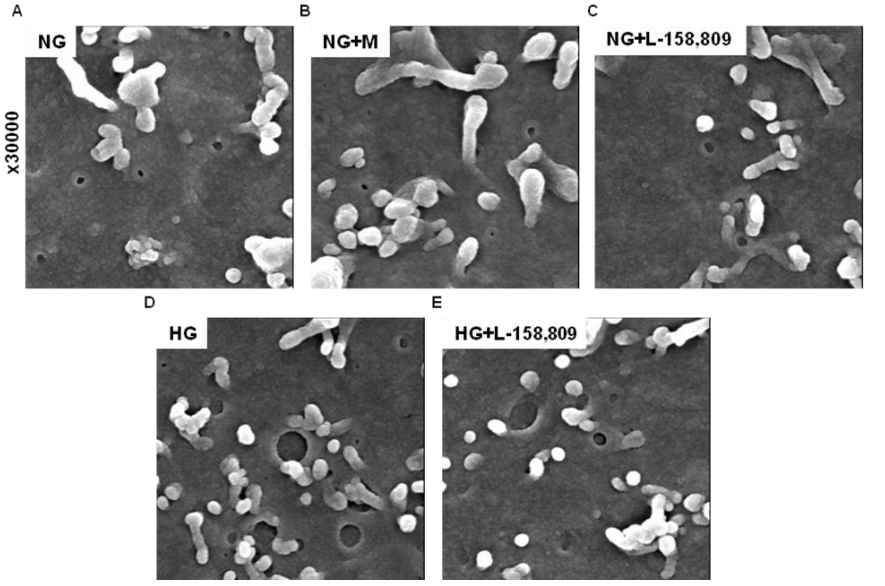

Figure 6. SEM images of GECs exposed to 5.6 mM glucose (NG), NG + 24.4 mM mannitol (NG+M), NG + 10-7 M L-158,809, and 30 mM glucose (HG), and HG + 10-7 M L-158,809. The mean size of fenestrae was significantly greater in GECs cultured under HG conditions than that in NG cells, and the enlarged fenestrae in HG-stimulated cells were signifiantly ameliorated by L-158,809 (×30K).

2. Animal Studies

A. Animal data

All animals gained weight over the 3-month experimental period, but weight gain was significantly higher in C rats compared to DM rats ($P < 0.001$). The ratio of kidney weight to body weight in DM rats ($1.54 \pm 0.13\%$) was significantly higher than that in C rats ($0.53 \pm 0.04\%$) ($P < 0.01$), and this increase in DM rats was significantly attenuated by the administration of L-158,809 ($P < 0.05$). The mean blood glucose levels in DM and DM+ARB rats were significantly higher compared to C and C+ARB rats ($P < 0.001$). Compared to the C (0.40 ± 0.06 mg/day) and C+ARB groups (0.41 ± 0.07 mg/day), 24-hr urinary albumin excretion was significantly higher in DM rats (1.99 ± 0.22 mg/day) ($P < 0.01$), and L-158,809 treatment significantly reduced albuminuria in DM rats ($P < 0.05$) (Table 1).

Table 1. **Animal data**

	C (n=8)	C+ARB (n=8)	DM (n=8)	DM+ARB (n= 8)
Body weight (Bwt, g)	579.53±14.93	570.1±16.7	344.73±14.3*	316.9±17.13*
Blood glucose (mg/dl)	3.17±0.09	3.06±0.10	3.39±0.12	3.18±0.14
Kidney Wt/Bwt (%)	0.53±0.04	0.59±0.06	1.54±0.13**	0.84±0.07**
24 hr UAE (mg/day)	0.40±0.06	0.41±0.07	1.99±0.22**	1.04±0.19#

*; $P < 0.001$ vs. C and C+ARB groups, **; $P < 0.01$ vs. C and C+ARB groups, #; $P < 0.05$ vs. DM group

Wt, weight; UAE, urinary albumin excretion

B. Morphological change of fenestrae in glomerular capillary wall

The change in the size of fenestrae in cultured GEC exposed to HG was further verified in experimental diabetic rats. As seen in Figure 7, the size of fenestrae was significantly greater in isolated glomeruli from DM rats compared to C glomeruli ($P < 0.05$), and the increase in the size of fenestrae in glomerular capillary wall was significantly inhibited in DM rats treated by L-158,809 ($P < 0.05$).

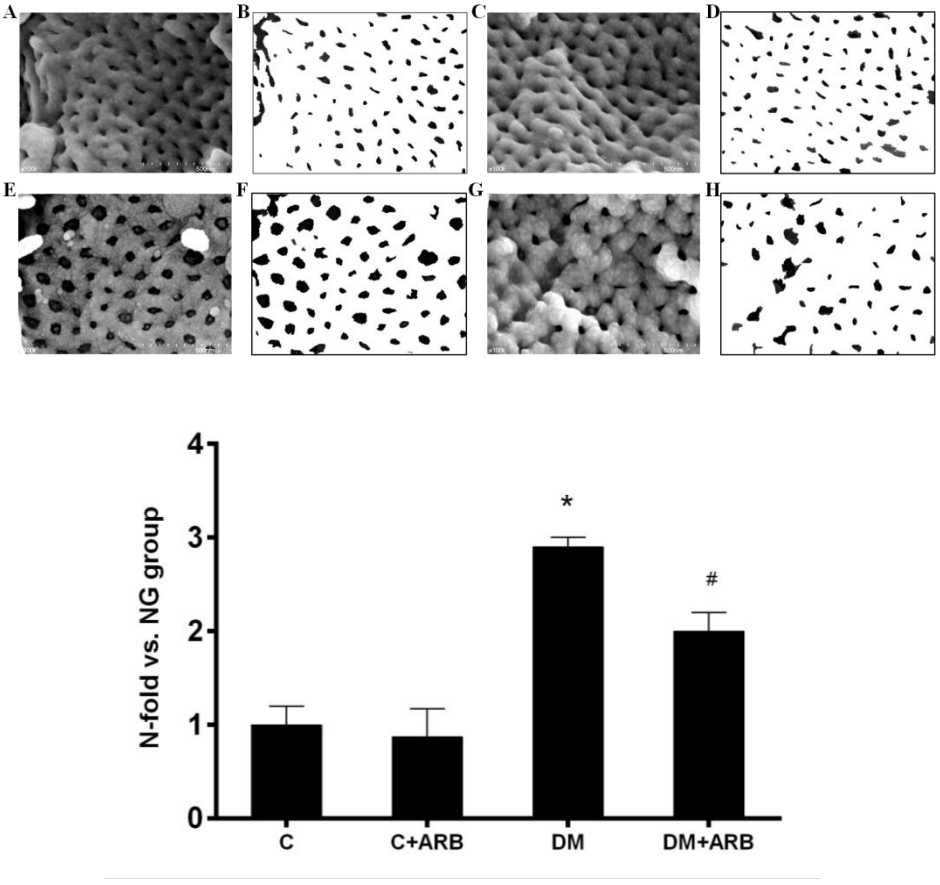

Figure 7. SEM images of GECs in control (C), C + L-158,809 (ARB), diabetes (DM), and DM + L-158,809 rats. The size of fenestrae was significantly greater in isolated glomeruli from DM rats compared to C glomeruli, and the increase in the size of fenestrae in glomerular capillary wall was significantly inhibited in DM rats treated by L-158,809 (×100K). *; $P < 0.05$ vs. C and C + L-158,809 groups, #; $P < 0.05$ vs. DM group

IV. DISCUSSION

Even though previous studies have shown that local RAS within proximal tubular cells, mesangial cells[14], and podocytes[17,18] are activated under diabetic conditions and that this activated local RAS in these cells play a pivotal role in the pathogenesis of diabetic nephropathy[19-22], it has never been explored in GECs. In addition, recently, accumulating evidence has indicated that the changes in GECs are associated with the development of proteinuria[23] in diabetic nephropathy. In this study, I demonstrate for the first time that AII concentrations are increased in high glucose-stimulated GECs, and the increase in AII levels under diabetic conditions is attributed to an increase in AGT expression which results in an increase in the AII substrate, rather than more conversion of AGT to AI or increased conversion of AI to AII. Moreover, ARB treatment is found to significantly abrogate the increase in albumin permeability in GECs exposed to HG medium. Based on these findings, I suggest that locally activated RAS within GECs under diabetic conditions is partly responsible for albuminuria in diabetic nephropathy.

Diabetic nephropathy, the leading cause of ESRD worldwide, is characterized pathologically by cellular hypertrophy and increased extracellular matrix accumulation and clinically by proteinuria[24,25]. Even though the molecular and cellular mechanisms responsible for these disease characteristics remain incompletely resolved, previous studies have suggested that the diabetic

milieu per se, hemodynamic changes, and local growth factors such as AII are involved in the pathogenesis of diabetic nephropathy[9, 26]. Among these potential mediators, the RAS has emerged as an important role player in the overall pathophysiology of diabetic nephropathy[27] based on the results of innumerable previous clinical and experimental studies showing that blockade of the RAS not only reduce proteinuria but also inhibit the progression of diabetic nephropathy irrespective of the blood pressure[28-30]. Furthermore, plasma renin activity is known to be suppressed in diabetes[31]. Based on these findings, it is suggested that the beneficial effect of RAS blockers in diabetic nephropathy may be attributed to its direct effect on various renal cells.

Many investigators including my colleagues demonstrated that AII mediated apoptosis, stimulated transforming growth factor-β1 production and signaling pathways such as mitogen-activated protein kinase, and contributed to the development of hypertrophy and matrix expansion in mesangial cells and podocytes. In addition, local RAS was found to exist and to be activated in tubular epithelial cells, mesangial cells, and podocytes under diabetic conditions. In contrast to mesangial cells and podocytes, the RAS in GECs have only recently drawn scientific attention. A recent study by Velez et al showed that human GECs possess an endogenous cleavage capacity of angiotensin peptides in human GECs by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and isotope-labeled peptide quantification[5]. They also demonstrated that ACE activity was prominent, while

AII-metabolizing activity was modest in GECs. Moreover, prolylendopeptidase, prolylcarboxypeptidase, aminopeptidase N, and aspartyl aminopeptidase were revealed to be contained in GECs that participate in membrane-bound angiotensin peptide cleavage. Furthermore, high glucose and AII were found to induce the expression of a dysfunctional endothelial nitric oxide synthase (eNOS) as well as to stimulate the production of reactive oxygen species (ROS) from NADPH oxidase in cultured GECs. Cyclooxygenase-2 expression and prostacyclin synthesis were also upregulated in these cells by high glucose and AII[32]. These findings suggest that not only high glucose but also AII are involved in the regulation of glomerular microcirculation under diabetic conditions. Despite a few investigations showing the presence of ACE and the effect of AII in GECs, it has not yet been clarified whether the whole components of the RAS exist in GECs and whether their expression are changed under diabetic conditions. In the current study, I demonstrate for the first time that AGT, ACE, ATIR, and AT2R are all present in cultured GECs and that AII levels are increased in high glucose-stimulated GECs, which is mainly attributed to an increase in the expression of ANG.

Since GEC layer is a component of glomerular filtration barrier along with GBM and podocytes, GECs have also been supposed to contribute to the development of proteinuria in various kidney diseases, including diabetic nephropathy[33]. Previous studies showed an increase in albumin permeability across cultured human umbilical vein endothelial cells and bovine aortic

endothelial cells after 2 days and 4 days incubation with high glucose medium, respectively[34]. Similarly, Nitta et al found that the permeability of fluorescein-labeled albumin was significantly increased in bovine GECs monolayer exposed to high glucose medium and that increased albumin permeability by high glucose was significantly ameliorated by H-7, a protein kinase C (PKC) inhibitor[35]. Based on these findings, they suggested that high glucose concentrations activated PKC, resulting in an increase in albumin permeability across GECs. The results of the present study also demonstrated that the permeability of albumin was significantly increased in cultured human GECs cultured under high glucose conditions, which was concordant with aforementioned previous studies. In this study, I also found that a PKC inhibitor significantly attenuated the increase in AII levels in GECs incubated with high glucose medium and that the increase in albumin permeability across high glucose-stimulated GECs monolayer was significantly abrogated by ARB treatment. Taken together, it is inferred that locally activated RAS is involved in albumin permeability in GECs under diabetic conditions partly via the PKC pathway.

GECs are covered by 200-400 nm thickness of glycocalyx, which covers both fenestral and inter-fenestral area[6, 36]. The glycocalyx, which is composed of proteoglycan core proteins, GAG chains, and sialoglycoproteins, is covalently bound to the GEC membrane and connected with the outer cell coat by charge-charge interaction. In addition, it is known to serve as a charge-selective

glomerular filtration barrier, especially attributed to the anionic charge characteristics of HS-GAG. Mounting evidence has shown that this GEC glycocalyx is altered under diabetic conditions. Singh et al demonstrated that the biosynthesis of GAG, especially HS-GAG, was significantly reduced in cultured GECs under high glucose conditions, while the expression of proteoglycan core proteins remained unchanged[6]. Moreover, they found a significant increase in the passage of albumin across high glucose-stimulated GECs monolayer without any changes in intercellular junctions, which was reproduced by enzymatic removal of heparan sulfate from the GEC glycocalyx. ROS, an important mediator in the pathogenesis of diabetic nephropathy, also caused a significant decrease in heparan sulfate staining from the surface of cultured GECs along with a significant increase in albumin permeability, but the effect of ROS was not mediated via the GAG biosynthetic pathway. Based on these findings, it is surmised that excessive ROS generation under high glucose conditions contributes to the development of albuminuria by altering the property of glycocalyx in GECs. In the current study, a significant decrease in the expression of heparin sulfate was also observed in GECs exposed to high glucose medium, which was in accordance with the previous studies. In contrast, a previous study by Wijnhoven et al failed to show a significant increase in proteinuria in rats treated with heparanase III, in which heparan sulfate was successfully removed from the glomeruli. Furthermore, in transgenic mice overexpressing human heparanase, biochemical analysis of heparan sulfate

isolated from adult tissues revealed a profound decrease in the size of heparin sulfate chains along with an increase in urinary protein content. Compared to control mice, however, the increase in proteinuria in transgenic mice was less than 2-fold (0.277 vs. 0.155 mg/ml) in spite of extensive foot process effacement. These conflicting results led to a question whether heparin sulfate GAG in the glycocalyx is solely responsible for the permselectivity exerted by the glomerular endothelium or some other changes in GECs are involved in this phenomenon.

Another characteristic finding of GECs is the presence of numerous transcellular fenestrations with a diameter of 60-100 nm in normal state[33]. However, the functional role of GECs fenestrations in glomerular filtration barrier has not been fully elucidated in various kidney diseases. Especially in diabetic nephropathy, to date, only a few studies have examined the changes in GECs fenestrations. Toyoda et al studied 18 patients with type 1 diabetes (7 normoalbuminuric, 6 microalbuminuric, and 5 proteinuric) and 6 normal control subjects, and investigated the structural characteristics of podocytes and endothelial cells in diabetic nephropathy[37]. Using stereological measurements with overlapping digital electron microscopic images, the proportion of fenestrated GECs were demonstrated to be significantly lower in diabetic patients even with normoalbuminuria, and the extent of reduction in GECs fenestration were found to significantly correlate inversely with mesangial fractional volume per glomerulus ($r = -0.57$, $P = 0.01$) and podocyte foot

process width (r = -0.58, P = 0.01). Similar findings were also observed in type 2 diabetic patients. A recent study by Weil et al included 37 Pima Indians with type 2 diabetes (11 normoalbuminuric, 16 microalbuminuric, and 10 macroalbuminuric), and showed that the mean percentage of GECs fenestration was significantly lower in diabetic patients with macroalbuminuria (19.3%) compared to patients with normoalbuminuria (27.4%) or microalbuminuria (27.2%)[38]. In addition, there were inverse correlations of the percent of fenestrated GECs with albuminuria, glomerular basement membrane thickness, and fractional mesangial area, and a positive correlation between the percentage of GECs fenestration and the glomerular filtration rate. In the present study, I demonstrated an increase in the size of GECs fenestrae rather than a decrease not only in vivo but also in vitro. The duration of diabetes in both previous human studies was more than 10 years. Moreover, the scanning electron microscopic images were not provided in those studies. These different experimental conditions and subjects may contribute to an opposite findings on the size of GECs fenestrae between mine and previous studies. Based on the results of this study, collectively, I presumed that a decrease in heparin sulfate GAG along with enlarged fenestrae in GECs under diabetic conditions contributed to an increase in albumin permeability.

In conclusion, the current study found that all components of the RAS were present in GECs and local RAS was activated under diabetic conditions. Furthermore, diabetes-induced RAS activation in GECs was involved in

albumin permeability. Therefore, the inhibitory effect of RAS blockade on proteinuria in diabetic nephropathy seems to be partly attributed to its direct impact on GECs.

V. CONCLUSION

In this study, I investigated whether AGT activity was significant increase mRNA expression and protein expression in GECs cultured under HG conditions. Also albumin permeability was significantly higher in GECs exposed to HG medium. In addition, I found the change in the size of fenestrae in cultured GECs and STZ-induced diabetic rats. We examined the changes suggest that the local RAS is activated in GECs under high glucose condition and activated RAS may play an important role in the development of albuminuria in diabetic nephropathy.

1. Angiotensinogen mRNA and protein expression were significantly increased in HG-stimulated GECs compared to NG cells.

2. AI and AII concentrations were also significantly higher in HG-conditioned media. In contrast, there were no differences in renin activities, and angiotensin converting enzyme and AII type 1 and type 2 receptor expression among the groups.

3. Immunofluorescence staining for HS-GAG and VE-cadherin revealed that HS-GAG protein expression was significantly decreased, while there was no change in VE-cadherin protein expression in GECs exposed to HG medium.

4. Transwell assay revealed that FITC uptake in filtered media through HG-stimulated GECs were 3.3-fold higher compared to NG cells and L-158,809 treatment significantly abrogated the increase in albumin permeability in HG

cells.

5. On SEM examination, the diameter of fenestrae in HG-stimulated GECs was significantly greater compared to NG cells. Also the number of fenestrae tended to be increased in GECs exposed to HG, but did not reach statistical significance.

6. In vivo, compared to the C group, 24 hr urinary albumin excretion was significantly higher in the DM group, and ARB treatment significantly reduced albuminuria in DM rats.

7. The increase in fenestrae diameter and enhanced albumin permeability through GECs under HG conditions were significantly abrogated by L-158,809 pretreatment.

In conclusion, these findings suggest that Local RAS within GECs was activated under HG conditions and this locally activated RAS seemed to be associated with the alteration in GEC fenestration along with a decrease in HS-GAG, resulting in the development of albuminuria in diabetic nephropathy.

REFERENCES

1. Miner JH. Glomerular filtration: the charge debate charges ahead. Kidney Int. 2008;74:259-61.

2. Deen WM, Lazzara MJ, Myers BD. Structural determinants of glomerular permeability. Am J Physiol Renal Physiol. 2001;281:F579-96.

3. Ziyadeh FN. The extracellular matrix in diabetic nephropathy. Am J Kidney Dis. 1993;22:736-44.

4. Mundel P, Shankland SJ. Podocyte biology and response to injury. J Am Soc Nephrol. 2002;13:3005-15.

5. Velez JC, Ierardi JL, Bland AM, Morinelli TA, Arthur JM, Raymond JR, et al. Enzymatic processing of angiotensin peptides by human glomerular endothelial cells. Am J Physiol Renal Physiol. 2012;302:F1583-94.

6. Singh A, Friden V, Dasgupta I, Foster RR, Welsh GI, Tooke JE, et al. High glucose causes dysfunction of the human glomerular endothelial glycocalyx. Am J Physiol Renal Physiol. 2011;300:F40-8.

7. Dronavalli S, Duka I, Bakris GL. The pathogenesis of diabetic nephropathy. Nat Clin Pract Endocrinol Metab. 2008;4:444-52.

8. Ravid M. Dual blockade of the renin-angiotensin system in diabetic nephropathy. Diabetes Care. 2009;32 Suppl 2:S410-3.

9. Leehey DJ, Singh AK, Alavi N, Singh R. Role of angiotensin II in diabetic nephropathy. Kidney Int Suppl. 2000;77:S93-8.

10. Weir MR, Dzau VJ. The renin-angiotensin-aldosterone system: a specific target for hypertension management. Am J Hypertens. 1999;12:205S-13S.

11. Correa-Rotter R, Hostetter TH, Rosenberg ME. Renin and angiotensinogen gene expression in experimental diabetes mellitus. Kidney Int. 1992;41:796-804.

12. Anderson S, Jung FF, Ingelfinger JR. Renal renin-angiotensin system

in diabetes: functional, immunohistochemical, and molecular biological correlations. Am J Physiol. 1993;265:F477-86.

13. Zhang SL, To C, Chen X, Filep JG, Tang SS, Ingelfinger JR, et al. Essential role(s) of the intrarenal renin-angiotensin system in transforming growth factor-beta1 gene expression and induction of hypertrophy of rat kidney proximal tubular cells in high glucose. J Am Soc Nephrol. 2002;13:302-12.

14. Singh R, Singh AK, Alavi N, Leehey DJ. Mechanism of increased angiotensin II levels in glomerular mesangial cells cultured in high glucose. J Am Soc Nephrol. 2003;14:873-80.

15. Vidotti DB, Casarini DE, Cristovam PC, Leite CA, Schor N, Boim MA. High glucose concentration stimulates intracellular renin activity and angiotensin II generation in rat mesangial cells. Am J Physiol Renal Physiol. 2004;286:F1039-45.

16. Durvasula RV, Petermann AT, Hiromura K, Blonski M, Pippin J, Mundel P, et al. Activation of a local tissue angiotensin system in podocytes by mechanical strain. Kidney Int. 2004;65:30-9.

17. Liebau MC, Lang D, Bohm J, Endlich N, Bek MJ, Witherden I, et al. Functional expression of the renin-angiotensin system in human podocytes. Am J Physiol Renal Physiol. 2006;290:F710-9.

18. Yoo TH, Li JJ, Kim JJ, Jung DS, Kwak SJ, Ryu DR, et al. Activation of the renin-angiotensin system within podocytes in diabetes. Kidney Int. 2007;71:1019-27.

19. Chawla T, Sharma D, Singh A. Role of the renin angiotensin system in diabetic nephropathy. World J Diabetes. 2010;1:141-5.

20. Schjoedt KJ. The renin-angiotensin-aldosterone system and its blockade in diabetic nephropathy: main focus on the role of aldosterone. Dan Med Bull. 2011;58:B4265.

21. Siragy HM, Carey RM. Role of the intrarenal renin-angiotensin-aldosterone system in chronic kidney disease. Am J Nephrol. 2010;31:541-50.

22. Ruggenenti P, Cravedi P, Remuzzi G. The RAAS in the pathogenesis and treatment of diabetic nephropathy. Nat Rev Nephrol. 2010;6:319-30.

23. Haraldsson B, Nystrom J, Deen WM. Properties of the glomerular barrier and mechanisms of proteinuria. Physiol Rev. 2008;88:451-87.

24. Adler S. Structure-function relationships associated with extracellular matrix alterations in diabetic glomerulopathy. J Am Soc Nephrol. 1994;5:1165-72.

25. Mogensen CE, Christensen CK, Vittinghus E. The stages in diabetic renal disease. With emphasis on the stage of incipient diabetic nephropathy. Diabetes. 1983;32 Suppl 2:64-78.

26. Wolf G, Ziyadeh FN. The role of angiotensin II in diabetic nephropathy: emphasis on nonhemodynamic mechanisms. Am J Kidney Dis. 1997;29:153-63.

27. Remuzzi G, Perico N, Macia M, Ruggenenti P. The role of renin-angiotensin-aldosterone system in the progression of chronic kidney disease. Kidney Int Suppl. 2005:S57-65.

28. Kalaitzidis R, Bakris GL. Effects of angiotensin II receptor blockers on diabetic nephropathy. J Hypertens Suppl. 2009;27:S15-21.

29. Siamopoulos KC, Kalaitzidis RG. Inhibition of the renin-angiotensin system and chronic kidney disease. Int Urol Nephrol. 2008;40:1015-25.

30. Sachetelli S, Liu Q, Zhang SL, Liu F, Hsieh TJ, Brezniceanu ML, et al. RAS blockade decreases blood pressure and proteinuria in transgenic mice overexpressing rat angiotensinogen gene in the kidney. Kidney Int. 2006;69:1016-23.

31. Gordon MS, Price DA, Hollenberg NK. Blunted suppression of plasma renin activity in diabetes. J Renin Angiotensin Aldosterone Syst. 2000;1:252-6.

32. Jaimes EA, Hua P, Tian RX, Raij L. Human glomerular endothelium: interplay among glucose, free fatty acids, angiotensin II, and oxidative stress. Am J Physiol Renal Physiol. 2010;298:F125-32.

33. Satchell SC, Braet F. Glomerular endothelial cell fenestrations: an integral component of the glomerular filtration barrier. Am J Physiol Renal Physiol. 2009;296:F947-56.

34. Yamashita T, Mimura K, Umeda F, Kobayashi K, Hashimoto T,

Nawata H. Increased transendothelial permeation of albumin by high glucose concentration. Metabolism. 1995;44:739-44.

35. Nitta K, Horiba N, Uchida K, Horita S, Hayashi T, Kawashima A, et al. High glucose modulates albumin permeability across glomerular endothelial cells via a protein kinase C-dependent mechanism. Nihon Jinzo Gakkai Shi. 1995;37:317-22.

36. A LWMMR, Loeven MA, van Gemst JJ, Eversen I, Van Wijk XM, Dijkman HB, et al. Modulation of heparan sulfate in the glomerular endothelial glycocalyx decreases leukocyte influx during experimental glomerulonephritis. Kidney Int. 2014.

37. Toyoda M, Najafian B, Kim Y, Caramori ML, Mauer M. Podocyte detachment and reduced glomerular capillary endothelial fenestration in human type 1 diabetic nephropathy. Diabetes. 2007;56:2155-60.

38. Weil EJ, Lemley KV, Mason CC, Yee B, Jones LI, Blouch K, et al. Podocyte detachment and reduced glomerular capillary endothelial fenestration promote kidney disease in type 2 diabetic nephropathy. Kidney Int. 2012;82:1010-7.

ABSTRACT(IN KOREAN)

고포도당으로 자극한 사구체 내피세포에서 국소 레닌-안지오텐신계의 활성이 알부민 투과성에 미치는 영향

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팽 지 선

배 경 : 당뇨병성 신병증은 말기 신부전증의 가장 흔한 원인질환으로 질병이 진행됨에 따라 고혈압과 단백뇨가 나타나며, 사구체 여과율의 저하가 발생된다. 이러한 당뇨병성 신병증의 발생 및 진행에는 여러 가지 인자가 관여하는 것으로 알려져 있으나, 특히 레닌-안지오텐신계(rennin-angiotensin system, RAS) 활성화에 의한 안지오텐신 II (angiotensin II, Ang II)가 중요한 인자로 여겨지고 있다.

한편, 사구체 내피세포 (glomerular endothelial cells)는 신장에서 족세포와 사구체 기저막과 더불어 사구체 여과장벽 (glomerular filtration barrier)의 구성 요소로, 족세포와 기저막 아래의 혈관 사이 세포를 지지하며, 혈액 및 조직 사이에서 매개 역할을 한다. 이러한 사구체

내피세포 표면에는 다른 내피세포와는 달리 직경 70~90 nm 의 천공(fenestrae)이 밀집 되어 있는데, 정상에서는 물이나 작은 용질만이 내피세포 천공을 통하여 사구체 미세혈관벽을 투과하게 되고, 크기가 큰 알부민 등은 투과하지 못하는 것으로 알려져 있다. 그러나, 당뇨병이 진행됨에 따라, 사구체 내피세포의 천공이 변형되고, 사구체 여과장벽의 손상이 나타나는데, 이로 인해 알부민 및 다른 단백질의 투과 증가가 나타나게 된다. 더 나아가, 지속된 단백뇨 배설 증가로 사구체 경화증 및 세뇨관간질 섬유화가 초래되며, 이로 인해 신부전이 진행되게 된다.

흥미롭게도 사구체의 내피세포 내에 국소적으로 레닌-안지오텐신계가 존재한다는 것이 최근 연구를 통하여 밝혀졌는데, 아직까지 당뇨병성 신병증 발병기전에 사구체 내피세포에서 레닌-안지오텐신계의 역할은 알려져 있지 않다. 이에 본 연구자는 사구체 내피세포에서 레닌-안지오텐신계의 존재 유무를 규명하고, 고포도당 자극에 따른 사구체 내피세포의 형태학적 변화에 대해 알아보고자 하였다.

결 과 :

1. 사구체 내피세포 내 AGT mRNA 와 protein 발현은 고포도당군에서 정상 포도당군에 비하여 의미있게 증가 되었다.

2. 사구체 내피세포 배양액 내 ＡⅠ 과 ＡⅡ 농도는 고포도당군에서 정상포도당군에 비하여 의미있게 증가하였다.

3. 면역형광염색상, 세포 내 HS-GAG 발현은 고포도당군에서 정상포도당군에 비하여 의미있게 감소하였으며, 이는 L-158,809 처치 후 고포도당군에 비해 유의하게 증가하였다. 반면, VE-cadherin 의 발현은 군간 차이를 보이지 않았다.

4. 사구체 내피세포의 알부민 투과성은 정상포도당군에 비하여 고포도당군에서 유의하게 증가하였으며, L-158,809 처치로 증가된 알부민 투과성이 의미있게 감소되었다.

5. 사구체 내피세포의 천공크기는 정상포도당군에 비하여 고포도당군에서 유의하게 증가하였으며, L-158,809 처치 후, 증가된 천공크기는 의미있게 감소되었다.

6. 24시간 뇨알부민 배설은 정상군에 비하여 당뇨군에서 유의하게 높았으며, 증가된 뇨알부민 배설이 L-158,809 투여 후 의미있게 감소하였다.

7. 사구체 내피세포 천공크기는 당뇨군에서 의미있게 증가하였으며, L-158,809 의 투여 후, 유의하게 감소되었다.

결 론 : 이상의 결과로 사구체 내피세포에서 고포도당에 의한 국소 RAS 의 활성을 확인할 수 있었으며, 이러한 활성은 사구체 내피세포내 HS-GAG 발현의 감소와 fenestrae 의 형태적 변화를 유도 하였을 것으로

사료되고, 이는 당뇨병성 신병증에서 발생되는 알부민뇨의 한 원인이 될 것으로 생각된다.

핵심 되는 말 : 사구체 내피세포, 레닌-안지오텐신, 천공, 당뇨병성 신병증