Angiotensin II Type 1–Receptor Mediated Changes in Heparan Sulfate Proteoglycans in Human SV40 Transformed Podocytes

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Abstract. In patients with diabetic nephropathy, glomerular staining for heparan sulfate proteoglycans (HSPG) side chains and for agrin is decreased. In the present study, the influence of angiotensin II (AngII) on the production of HSPG in SV40 transformed podocytes was investigated. SV40 transformed human podocytes were cultivated with or without 1 μM AngII, and HSPG production was measured by sequential DEAE-anion exchange chromatography and HPLC-DEAE separation. Expression of agrin was studied by indirect immunofluorescence and Western blot analysis using specific mono- and polyclonal antibodies. DEAE separation of total glycosaminoglycans (GAG) revealed a significant increase of GAG in the culture supernatant and decrease in the cell and matrix layer when podocytes were cultured for 72 h in the presence of AngII. This was particularly found for HS-GAG. Qualitative analysis of HSPG, using gel filtration of HNO_2-treated fractions, showed that AngII treatment decreased N-sulfation of HS-GAG side chains. Indirect immunofluorescence staining with anti-agrin polyclonal antibody was strongly decreased after AngII stimulation. A reduction in agrin expression in cell extracts could also be detected in Western blot analysis using an mAb. No changes in agrin mRNA were found after AngII stimulation. It is concluded from this study that AngII decreases the amount of HSPG on the cell surface and in the extracellular matrix of podocytes. Because HSPG play a fundamental role in the permselectivity of the glomerular basement membrane, these results thus may explain at least partially the antiproteinuric effects of angiotensin-converting enzyme inhibition in patients with diabetic nephropathy.

Diabetic nephropathy (DN) is characterized by mesangial matrix expansion, thickening of the glomerular basement membrane (GBM), and a concomitant loss of heparan sulfate proteoglycans (HSPG) (1–4). HSPG consist of a core protein to which one or more HS glycosaminoglycan (GAG) side chains are attached. To date, three HSPG core proteins—perlecan, agrin, and collagen XVIII (5–7)—have been identified in the GBM.

The permselectivity of the GBM is partly due to the high negative charge of HSPG and their interaction with other matrix components (8–10). Most studies on the decrease of glomerular HSPG in patients with DN have suggested that a selective dysregulation in sulfation of HSPG is underlying the observed reduction in HSPG expression (11–13). However, we recently provided *in vivo* and *in vitro* evidence that under hyperglycemic conditions, the expression of the core protein of agrin in the GBM of these patients and in cultured podocytes is also affected (14).

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In patients with DN, an initial increase in the GFR is followed by a linear decrease in GFR over time (15). It is believed that in these patients, autoregulation of renal blood flow is impaired and, consequently, systemic pressure is transferred to the glomerular capillary loops, resulting in hyperfiltration (15,16). Several clinical studies have demonstrated that antihypertensive drugs reduce the rate of decline of renal function in DN (17–19). In addition, evidence is accumulating that the use of angiotensin-converting enzyme (ACE) inhibitors or angiotensin II (AngII) receptor antagonists as antihypertensive therapy have an additional renoprotective effect beyond its action on BP (20–25). In a recent publication by Wapstra *et al.* (26), it could be demonstrated that ACE inhibition preserved HSPG and, to a smaller extent, agrin in the GBM of rats with established adriamycin-induced nephropathy. It thus seems that AngII might influence the production of HSPG in glomerular cells. Compatible with this are studies from van Det *et al.* (27) demonstrating that AngII inhibits HSPG production and stimulates TGF-β production in cultured human mesangial cells. Because the GBM is mainly produced by glomerular endothelial cells and podocytes, we addressed in the present study whether AngII is able to modulate the production of HSPG in cultured human podocytes.

Materials and Methods

Cell Culture

Human SV40 transformed podocytes were used to investigate the influence of AngII on the production of GAG and agrin. SV40

transformed podocytes were provided by Prof. Rondeau (Hopital Tenon, Paris, France) and characterized as described previously (28). The cells were cultivated in uncoated culture flasks in DMEM/HAM F12 medium (PromoCell, Germany) supplemented with 10% FCS (Greiner, Germany), insulin-transferrin selenium (all in concentrations of 5 ng/ml), epidermal growth factor (5 ng/ml), and penicillin/streptomycin (10 U/ml). The medium was refreshed every 3 d, and the cells were subcultured upon confluence.

RNA Isolation and Reverse Transcription–PCR

Total RNA was extracted from cells using RNA-Trizol (Life Technologies BRL, Eggenstein, Germany) and finally dissolved in diethylpyrocarbonate–treated water. For excluding amplification of contaminated genomic DNA, DNAse I (Roche, Mannheim, Germany) treatment was performed on all samples before cDNA synthesis. Total RNA (0.5 μg) was reverse transcribed in cDNA following the instructions of SuperScript TM II Preamplification System (Life Technologies, Karlsruhe, Germany). Briefly, first-strand cDNA was synthesized using 10 U of Super Script II Reverse Transcriptase (Life Technologies BRL), 6.25 ng of random hexamers, 1.25 μM Oligo(dT) 16 Primer (Perkin Elmer, Weiterstadt, Germany), and 0.5 μg of total RNA in a total volume of 20 μl. PCR reactions for AngII type 1 receptor (AT1R), AT2R, agrin, and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) were performed using specific primers that were constructed from cloned human cDNA of human AT1R, AT2R, agrin, and GAPDH, respectively. The sequence for the forward and reverse primers were as follows: forward (AT1R) ACCGCCCCTCAGATAATGTA, reverse (AT1R) GCTCTTGGACCTGTGATGTG; forward (AT2R) TGCGGTAGACCCGACATAGA, reverse (AT2R) GGTGAACAATAGCCAGGTATCG; forward (agrin) GGAGGCTGCCTATGTGTGCCTGT, reverse (agrin) GGGAACCTTCCCTCTTGCTCCCTAT; and forward (GAPDH) GTCTTCACCACCATGGAGAAA, reverse (GAPDH) ATCCACAGTCTTCTGGGTGG. Each reaction consisted of 1 μl of cDNA, 2.5 mM of each dNTP, 2.5 U of *Taq*DNA polymerase, 20 pmol of each primer, 1.0 mM Tris-HCl (pH 8.8), 0.15 mM $MgCl_2$, and 7.5 mM KCl in a total volume of 50 μl. After 3 min of denaturation at 94°C and 2 min of annealing at 50°C, amplification was initiated using 25 cycles of primer extension (72°C, 1.5 min), denaturation (94°C, 1 min), and primer annealing (50°C, 1 min). PCR products were separated on 1.5% agarose gel.

Western Blot Analysis

Podocytes were harvested with trypsin EDTA, and subsequently the cell pellet was washed twice in ice-cold PBS. The cells were lysed adding 100 μl of lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 12.5 μl of 10% Igpal (all from Sigma, St. Louis, MO), followed by centrifugation (12,000 $\times$ *g*). Both the membrane fraction (pellet) and the cytoplasmic fraction (supernatant) were analyzed for AT1R expression. Protein concentration of the cytoplasmic fraction was determined by the Coomassie Blue assay. The membrane fraction was directly resuspended in 20 μl of SDS loading buffer. For the detection of agrin, proteoglycans were extracted from the cell matrix using 4 M of guanidine HCl and dialyzed against DEAE buffer (listed below). Proteoglycans were isolated using DEAE-Sepharose. Protein concentration was determined by Coomassie Blue. A fixed amount of protein (10 μg) for both AngII–stimulated and unstimulated cells was loaded on the gel. The positive control was a GBM extract and contained only 1 μg. The gel was stained after blotting using a sensitive silver staining technique according to the manufacturer's manual (Amersham, Freiburg, Germany).

The samples were then separated on a 10% SDS-PAGE or 4 to 20% gradient gel according to Laemmli (29) and semidry blotted on a polyvinylidene fluoride membrane. The membrane was incubated overnight in 5% milk powder in TBS (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) solution. Thereafter, the blot was incubated for 1 h with polyclonal rabbit anti–AT1R-1 antibodies (Alexis, Gruenberg, Germany) or JM72 for detection of agrin. After washing, the blot was incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibodies for 30 min. Antibody binding was visualized by chemiluminescence on a BIO-MAX film.

Metabolic Labeling

Podocytes were cultured in 75-cm^2 flasks until confluence and either stimulated or not for 3 d with 1 μM AngII. Concentration and time of stimulation were chosen on the basis of our previous findings (27). On the second day, the medium was replaced by sulfate-free DMEM (2% FCS), supplemented with 1 μM AngII, depending on whether the cells were initially stimulated with AngII, and on the third day, 100 μCi/ml of both $Na^{35}SO_4$ and ^{3}H-glucosamine (NEN Dupont, Boston, MA) was added to all cultures. In some experiments, losartan (10 nM), PD123.390 (10 nM), or N-Acetylcysteine (Nac, 500 μg/ml) was added to the cells during AngII stimulation to block AngII–mediated signaling or, in the case of Nac, to prevent depolymerization of HSPG by scavenging of reactive oxygen species (ROS) (30). Then the cell supernatant was collected and the cells were washed three times with ice-cold PBS. Cells were scraped into extraction buffer containing 4 M guanidine HCl and protease inhibitors (10 mM EDTA, 5 mM PMSF, 2 mM benzamidine, 50 mM ϵ-aminocaproic acid, 0.5 U/ml aprotinin, and 5 mM iodoacetamide; all from Sigma) and incubated for 24 h at 4°C. Both cell supernatants and cell extracts were dialyzed extensively at 4°C against 0.5 M sodium acetate (pH 5.8) supplemented with the same protease inhibitors. Finally, the fractions were dialyzed against DEAE buffer (6 M urea, 50 mM Tris, 0.2% CHAPS, and protease inhibitors) and first separated on a 5-ml DEAE anion exchange chromatography column. After extensive washing of the column with DEAE buffer, bound material was eluted with DEAE buffer containing 1 M NaCl. The eluted material was dialyzed against DEAE buffer and stored until use. For the second and final purification of the different types of proteoglycans, the labeled material was separated using HPLC with a DEAE anion exchange 75 $\times$ 7.7-mm Bio-Gel TSK DEAE-5PW HPLC column (Biorad Richmond, CA). Bound material was eluted using a linear (0.1 to 1 M) gradient of NaCl. The peaks were dialyzed against distilled water, freeze-dried, and stored at −20°C. Characterization of the different types of proteoglycans was performed as described previously (27). Briefly, each of the pooled fractions was treated with 0.75 M NaOH and 20 mM sodium borohydride ($NaBH_4$) for 1 h at 72°C followed by separation on a G50 size exclusion column run in 0.5 M sodium acetate buffer containing 0.2% CHAPS and protease inhibitors. The G50 column was pretreated with 1 mg/ml heparin to avoid sticking of labeled GAG to the column. The void volume was tested for sensitivity to Chondroitinase ABC (2.5 mU), Chondroitinase AC (2.5 mU), and nitrous acid (HNO_2) pH 1.5. Identification of the labeled GAG was determined by the following criteria: (*1*) molecules with sensitivity to HNO_2 without sensitivity to Chondroitinase ABC or AC are designated as HS, (*2*) molecules with sensitivity to Chondroitinase ABC without sensitivity to Chondroitinase AC or HNO_2 were designated as dermatan sulfate, and (*3*) molecules equally sensitive to both Chondroitinase ABC and AC without sensitivity to HNO_2 were designated as chondroitin sulfate.

N-Sulfation of HS-GAG

$^{35}S/^{3}H$-labeled HS-GAG was isolated from the supernatant and cell extracts of AngII–stimulated and unstimulated cells as described above. The fractions were treated with papain (1 mg/ml) for 18 h at 55°C and subsequently heat-inactivated. The material was centrifuged to spin down the papain, and the supernatants were collected and subjected to HNO_2 treatment. Thereafter, the material was loaded on a G25 Sephadex separation column in 0.5 M NH_4HCO_3 to separate di-, tetra-, and oligosaccharides fractions.

Indirect Immunofluorescence

To detect changes in agrin expression in podocytes that were cultured for 7 d in the presence of various concentrations of AngII, we performed indirect immunofluorescence (IIF). To this end, the cells were washed extensively with ice-cold PBS and subsequently fixed in methanol. The cells were incubated with one of the following monoclonal and polyclonal antibodies: JM72 (1:400, mouse monoclonal IgG1) for the detection of agrin and AS46 (1:200, rabbit polyclonal) for the detection of the C-terminal part of agrin. Incubation with the primary antibodies was performed for 1 h at room temperature. After three washes with ice-cold PBS, the appropriate secondary antibody conjugated to FITC (all from Dako, Glostrup, Denmark) was added as recommended by the manufacturer. All dilutions were made in PBS/BSA (1% wt/vol). All slides were photographed, coded, and independently evaluated by three people.

Statistical Analyses

For statistical analysis, Fisher exact test and Mann-Whitney *U* test (Wilcoxon rank sum test) were applied. $P < 0.05$ was considered to be significant.

Results

Expression of AngII Receptors in SV40 Transformed Podocytes

The expression of both AT1R and AT2R was studied by means of Western blot and reverse transcription–PCR (RT-PCR) analysis. It was found that the AT1R was constitutively expressed by these cells. In contrast, no expression of the AT2R could be observed (Figure 1).

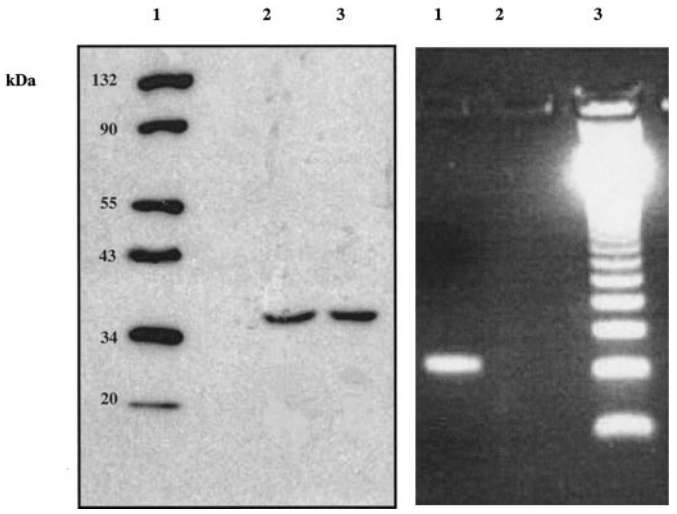

Figure 1. Expression of angiotensin II (AngII) type 1 receptor (AT1R) by Western blot analysis and reverse transcription–PCR. (Left) Detection of AT1R protein in the cytoplasmic (lane 2) and membrane fraction (lane 3) of cultured SV40 transformed podocytes. Lane 1, molecular weight marker in kD. (Right) Expression of AT1R (lane 1) but not AT2R (lane 2) mRNA in SV40 transformed podocytes. Lane 3, DNA ladder.

Modulation of GAG Production by AngII

To study the influence of AngII on GAG production, we cultured podocytes for 3 d in the presence of 1 μM AngII. Although the absolute amount of GAG produced in the culture supernatant and extracellular matrix (ECM) did not differ between unstimulated and AngII–stimulated cells, it was found that AngII stimulation resulted in a relative decrease of GAG in the ECM, concomitantly with an increase in the culture supernatant. Thus, based on both ^{3}H-glucosamine and ^{35}S incorporation of extracted GAG, the ECM contained significantly less GAG after AngII stimulation compared with unstimulated cells. In addition, in supernatants of AngII–stimulated cells, significantly more GAG were found compared with supernatants of unstimulated cells ($P < 0.05$, medium *versus* AngII; Figure 2). This was not due to the release of GAG from the ECM, because AngII stimulation of ^{3}H-glucosamine and ^{35}S prelabeled podocytes did not result in this effect (data not shown). The effect of AngII on the modulation of GAG production was specific for the AT1R as it could be inhibited by losartan but not by the AT2R specific antagonist PD123.319. Although AngII is able to stimulate the production of ROS in an AT1R-dependent manner, changes in GAG production were not mediated via ROS, because addition of N-acetyl cysteine to AngII–stimulated cells had no effect (Figure 3).

We next investigated whether AngII stimulation resulted in the modulation of specific proteoglycans. To this end, podocytes were stimulated or not for 3 d with AngII and metabolically labeled with ^{3}H-glucosamine and $Na^{35}S$ during the last 24 h of culture. Proteoglycans in the ECM and culture supernatant were separated by DEAE-HPLC anion-exchange chromatography. On the basis of the conductivity, two and three peaks were identified in the ECM and culture supernatant, respectively, and were pooled for further analysis (Figure 4). Whereas in the ECM, peak I was >95% susceptible to HNO_2 treatment, peak II was equally susceptible to Chondroitinase ABC and AC treatment. In the culture supernatant, HNO_2 susceptibility was found in peak I to a small extent (25%) and in peak II to a larger extent (80%). Peak III was susceptible to Chondroitinase ABC and AC but not to HNO_2 treatment (82 *versus* 8%). No influence of AngII stimulation was observed on the susceptibility either toward HNO_2 or toward Chondroitinase ABC and AC (data not shown). Because HSPG are susceptible only to HNO_2 treatment, the percentage of HSPG in the isolated GAG from the ECM and culture supernatant could be calculated. AngII stimulation resulted in a relative decrease and increase of HSPG in the ECM and in the culture supernatant, respectively (Table 1).

Influence on N-Sulfation of HSPG by AngII

HSPG in the ECM of unstimulated and AngII–stimulated podocytes were further analyzed with respect to the amount of

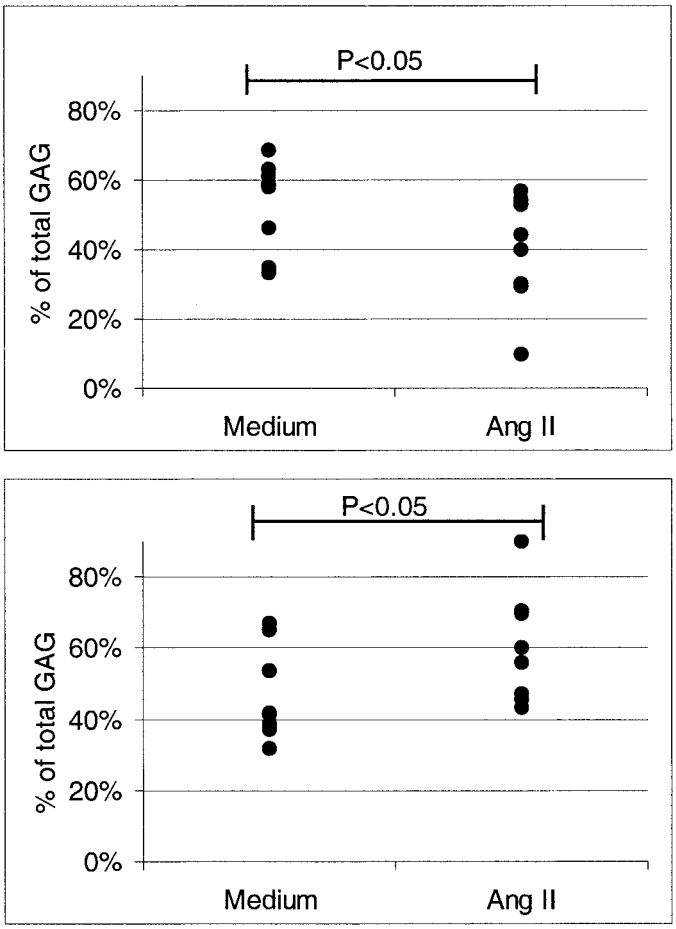

Figure 2. Relative distribution of total glycosaminoglycans (GAG) in the extracellular matrix (ECM; top) and supernatant (bottom) of unstimulated (medium) and AngII–stimulated cells based on ^{3}H-glucosamine incorporation. A significant decrease in GAG expression in the ECM was found upon AngII stimulation concomitantly with an increase of GAG in cell supernatants. GAG were isolated as described in Materials and Methods. The results of seven different experiments are depicted.

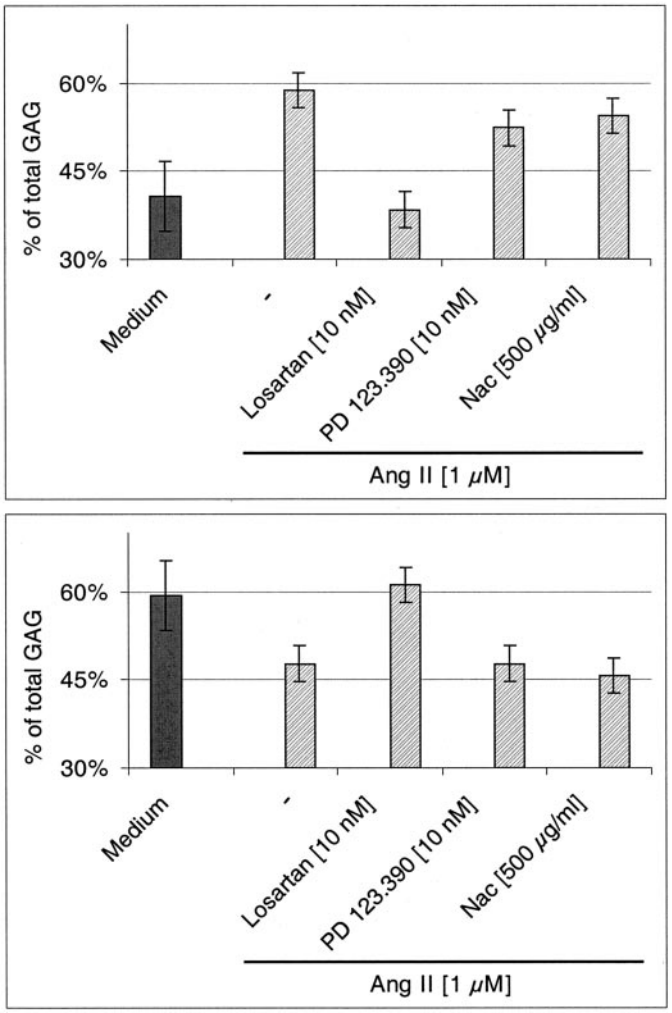

Figure 3. Modulation of GAG production by AngII is mediated via AT1R. Podocytes were either not stimulated (■) or stimulated with 1 μM of AngII for 3 d (▨) in the absence (−) or presence of 10 nM losartan, 10 nM PD123.319, or 500 μg/ml N-acetyl cysteine (Nac). The distribution of total GAG in the ECM (bottom) and supernatant (top) was calculated as in Figure 2. The mean percentage of total GAG ± SD of three different experiments is depicted.

N-sulfated glucosamine saccharides. To this end, HNO_2 susceptible GAG from the ECM were subjected to G25 Sepharose size exclusion chromatography. In comparison with unstimulated cells, there was a relative decrease in the amount of N-sulfated glucosamines upon AngII stimulation. This was reflected by a relative increase in N-sulfated poly- and tetrasaccharides (fractions A and B, respectively) and a decrease in N-sulfated disaccharides (fraction C; Figure 5).

Influence of AngII on the Expression of Agrin

Because it was found that there was a decrease in the amount of HSPG in the ECM in AngII–stimulated podocytes, we next questioned whether the major HSPG expressed by podocytes, *i.e.*, agrin, was also affected. Podocytes were cultured for 7 d in the presence or absence of 1 μM AngII and stained with polyclonal (AS46) and monoclonal (JM72) antibodies. Whereas AS46 is raised against a recombinant fragment of the C-terminal part of agrin, JM72 recognizes a more N-terminal epitope. A striking reduction in staining with AS46 in AngII–stimulated cells (Figure 6) was observed, which could be reversed by addition of losartan during AngII stimulation. Staining with JM72 did not reveal these changes; however, in Western blot analysis using this antibody, agrin was detected in lower amounts in podocyte extracts after AngII stimulation (Figure 7). Because both IIF and Western blot analysis showed a decreased agrin expression after AngII stimulation, RT-PCR was performed to elucidate whether agrin mRNA expression was also influenced by AngII. No changes in agrin mRNA expression were found between unstimulated and AngII–stimulated podocytes (data not shown).

Discussion

Several studies have demonstrated that the use of ACE inhibitors or AngII receptor blocking agents have an antipro-

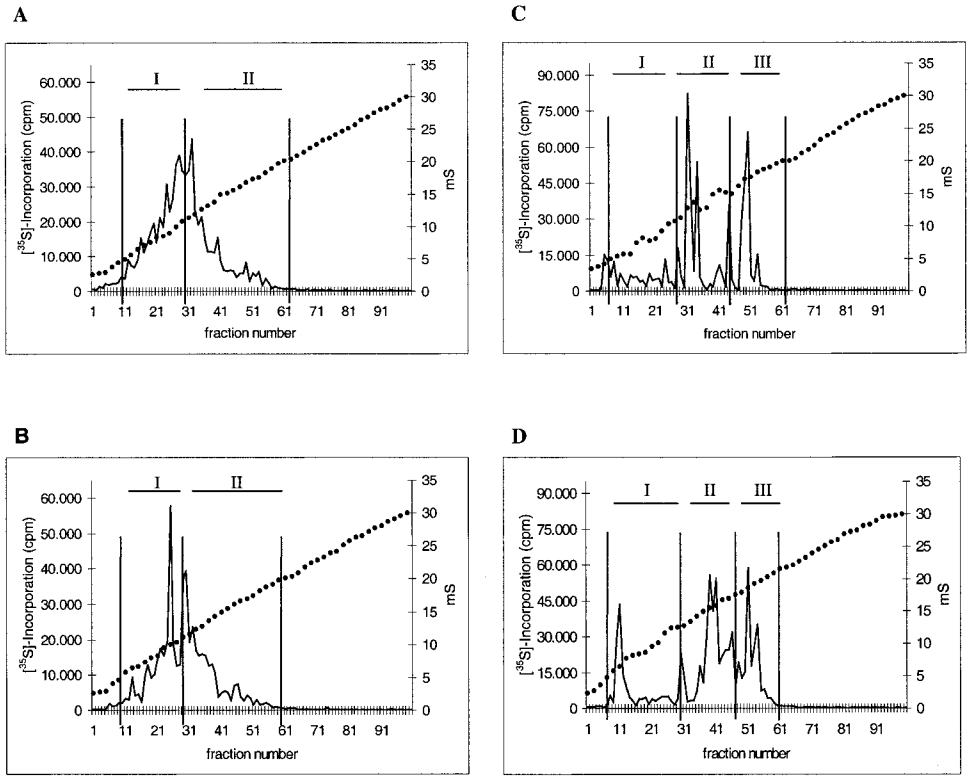

Figure 4. Separation of proteoglycans by HPLC-DEAE anion exchange. Podocytes were either cultured for 3 d in medium (A and C) or in medium supplemented with 1 μM AngII (B and D). Proteoglycans in the ECM (A and B) and supernatants (C and D) were separated and pooled in fractions I to III for further analysis. The HPLC profile of a representative experiment ($n = 3$) is depicted.

teinuric effect in human and experimental renal diseases and prevent progressive loss of renal function, partially independent of its hemodynamic action (20–25). Ample evidence also exists that AngII induces quantitative and qualitative changes in proteoglycans (27,31,32). In human mesangial cells, AngII not only decreases the absolute amount of HS but also leads to a decreased N-sulfation of glucosamine saccharides (27). As N-sulfation is an important event in further sulfation of HS (33), decreases in this process may have a large impact on the overall sulfation of HS and thus might influence the negative charge in the GBM. The aim of the present study was to investigate whether AngII can influence proteoglycan expression in SV40 transformed podocytes.

Experimental data, accumulating over the past decade, have now demonstrated that the AT1R is expressed *in vivo* and *in vitro* in rat podocytes (34,35), but data on human podocytes are still lacking. Therefore, we first studied by means of Western blot and RT-PCR analysis whether the AngII receptors were expressed in these cells. Whereas the AT2R could not be detected, expression of both AT1R mRNA and protein was observed, thus making these cells suitable to address our specific question. AngII stimulation of podocytes resulted in relative decrease of GAG in the ECM of the cells, concomitantly with an increase in the cell culture supernatant. This was specifically found in the HSPG fraction of GAG. Losartan but not the AT2R-specific receptor antagonist PD123.319 could block this response, suggesting an AT1R-mediated process (36).

Table 1. Influence of angiotensin II (AngII) stimulation on glucosaminoglycan composition

	ECM		Culture Supernatant	
	Medium	AngII	Medium	AngII
HSPG	74.0 ± 2.7[a]	66.3 ± 2.3	42.9 ± 4.4	45.2 ± 7.6
Non-HSPG	26.0 ± 2.7	37.7 ± 2.3	57.1 ± 4.4	54.8 ± 7.6

The results of three independent experiments are depicted as mean % of total glycosaminoglycans (GAG) ± SD. Percentages were calculated from the total amount of GAG, based on ^{35}S-incorporation, that was susceptible to HNO_2 treatment. Significant differences between medium and AngII in the mean % of heparan sulfate proteoglycans (HSPG) were found in the extracellular matrix (ECM) ($P < 0.05$) but not culture supernatant.

AngII has multiple effects on cardiomyocytes and renal cells, including vasoconstriction, cell growth, and induction of proinflammatory cytokines (37,38). Recent studies suggest that AngII can induce oxidative stress, resulting in the generation of ROS, such as superoxide and, via the generation of hydrogen peroxide, hydroxyl radicals (39). It is believed that this is mediated via the multi-enzyme complex NADPH oxidase (39,40). Upregulation of several subunits of this complex occurs after stimulation with AngII in an AT1R-dependent manner. This is an important finding because ROS are able to depolymerize glomerular HS and may degrade several other

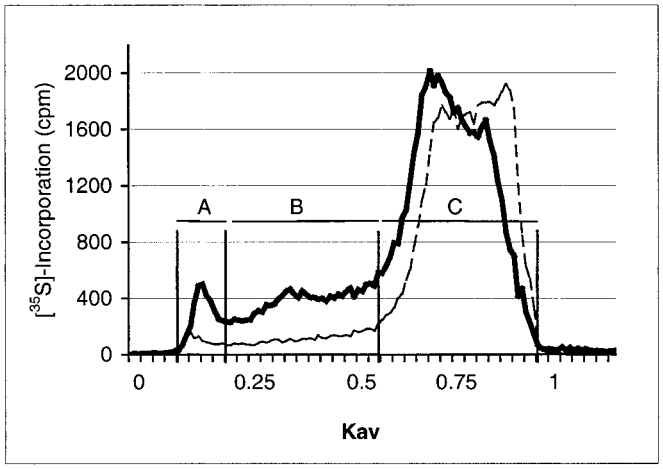

Figure 5. N-sulfation of heparan sulfate–GAG in AngII–stimulated and unstimulated cells. HNO_2-susceptible fractions were further analyzed by means of G25 size exclusion chromatography. The more that N-linked sulfation occurs, the smaller the stretches of unsubstituted N-acetyl glucosamine will be after HNO_2 treatment of heparan sulfate proteoglycans. The profiles obtained from the HNO_2-susceptible material of the ECM of AngII–stimulated (—) and unstimulated (---) cells are depicted. On the basis of the Kav, the profile was divided into three regions: A, polysaccharides; B, tetra-saccharides; and C, disaccharides.

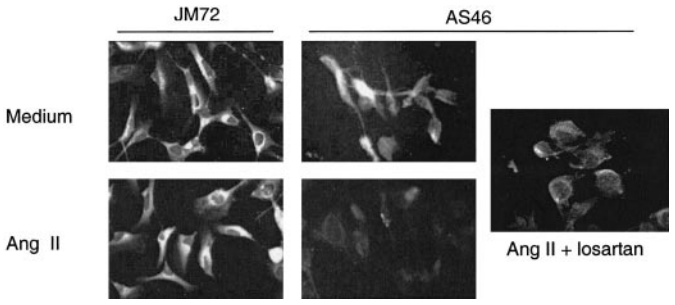

Figure 6. Indirect immunofluorescence staining for agrin. Podocytes were cultured for 7 d in the absence or presence of 1 μM AngII. In addition, losartan was added (10 nM) to some cultures during AngII stimulation. The cells were fixed and stained for agrin using JM72 mAb and AS46 polyclonal antibody. The result of a representative experiment ($n = 3$) is depicted.

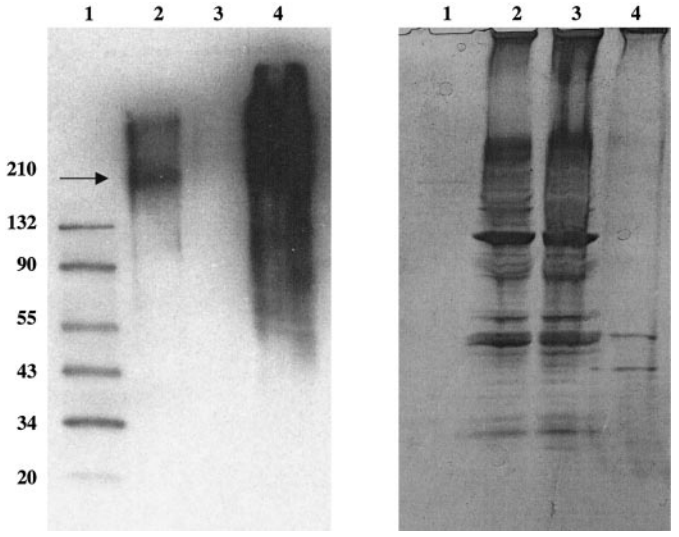

Figure 7. Downregulation of agrin expression in ECM of cultured podocytes after AngII stimulation. (Left) Western blot analysis for agrin using JM72. Proteoglycans were extracted from the ECM of unstimulated (lane 2) and AngII–stimulated (3 d, 1 μM; lane 3) podocytes using DEAE-Sepharose as described in Materials and Methods. A guanidine/HCl extract of the GBM (lane 4) was used as positive control. (Right) The corresponding gel was stained using a silver staining technique to ensure equal loading of the extracts from stimulated and unstimulated cells. The result of a representative experiment ($n = 4$) is depicted. The molecular weight marker is depicted in lane 1 in kD.

proteoglycans (30,41). Although we observed that the modulation in GAG expression in SV40 transformed podocytes was AT1R dependent, this was not due to the production of ROS, because addition of N-acetyl cysteine did not influence the effect of AngII stimulation. It can be argued that we did not test the NAPD/NAPDH oxidase inhibitor diphenylene iodonium or other scavengers such as catalase, both of which have been shown to reduce the oxidative mediated effects of AngII simulation (42,43). It must be stressed, however, that N-acetyl cysteine has been used successfully, in similar concentrations as we have, in other studies on AngII–induced oxidative stress, demonstrating that N-acetyl cysteine *per se* is able to inhibit the production of ROS mediated by AngII (44).

Our data are in striking contrast to those obtained with vascular smooth muscle cells (31,32). In these studies, it was shown that AngII stimulates the production of proteoglycans in a dose- and time-dependent manner. Moreover, it has been demonstrated that AngII stimulates the mRNA expression of the HSPG core proteins versican, biglycan, and perlecan. Other publications (27), however, using human mesangial cells, have reported results compatible with our own data in that a reduction in HSPG was found. This was paralleled by a decrease in perlecan expression. It thus seems that AngII influences proteoglycan expression differently in renal and nonrenal cells.

The influence of AngII on perlecan expression was not studied by us, because agrin is more abundantly expressed than perlecan in the GBM and in podocytes (6). AngII did not influence agrin mRNA expression, although staining for agrin was diminished, using a polyclonal antibody that was raised against a recombinant fragment of agrin. This was not found when mAb JM72, recognizing a more N-terminal part of agrin, was used. It must be stressed, however, that in Western blot analysis, detection of agrin was also decreased with JM72 in cellular extracts of podocytes stimulated with AngII. Because losartan was able to reverse the effect of AngII on agrin expression, it seems that changes in agrin expression can be mediated via AT1R stimulation. Whether this also occurs *in vivo*, in the GBM of patients with DN, is not known. There is, however, compelling evidence from an animal model (26) of adriamycin nephropathy that the reduction in agrin expression can be prevented by ACE inhibition, thus suggesting that AngII also influences agrin expression *in vivo*.

It also remains to be elucidated why AngII decreases the expression of HSPG in the ECM while increasing its expression in the cell culture supernatant. The release of HSPG from the ECM into the supernatant can be excluded because AngII stimulation did not result in the release of prelabeled GAG into the supernatant. Breakdown of the HSPG core protein could theoretically lead to the release of HS-GAG side chains in the supernatant. This explanation, however, is unlikely, at least for agrin, because neither in the supernatant nor in the ECM were breakdown products detected in Western blot analysis. It therefore seems plausible that the effect of AngII on GAG expression is posttranslational at the level of targeting proteoglycans either to the supernatant or to the ECM, although formal proof for this is lacking. In addition, our data indicate that the relative amount of N-sulfation of HS-GAG is decreased after AngII stimulation. This was also found for mesangial cells (27). The first step in HSPG sulfation is mediated by an enzyme called N-deacetylase/N-sulfotransferase. This enzyme conducts two activities: it deacetylates N-acetyl glucosamine first and subsequently transfers a sulfate group to the glucosamine molecule. Only when this step is completed can further sulfation of HSPG, *i.e.*, O-sulfation, occur. Thus, the amount of N-linked sulfation determines the overall sulfation and therefore the negative charge of HSPG. A reduced N-linked sulfation as a result of stimulation with AngII therefore may contribute to the reduced negative charge barrier in the GBM seen in patients with DN.

N-linked sulfation is not equally distributed on HSPG. Stretches of N-sulfate glucosamine containing disaccharides alternate with stretches of unsubstituted N-acetyl glucosamine–containing disaccharides. The more that N-linked sulfation occurs, the smaller the stretches of unsubstituted N-acetyl glucosamine will be. This is of high biologic relevance because the pattern of sulfation of HSPG determines binding and biologic activity of a number of humoral factors (45,46).

In conclusion, our data demonstrate that AngII reduces the expression of HSPG in the ECM of human SV40 transformed podocytes. Because podocytes and glomerular endothelial cells are predominantly responsible for the production of the GBM, our study may at least partially explain the beneficial effect of ACE inhibitors on the preservation of HSPG in the GBM in animal models of renal diseases. Moreover, as HSPG play a fundamental role in the permselectivity of the GBM, our data may also explain the antiproteinuric effect of ACE inhibitors.

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