Regulation of glomerular heparanase expression by aldosterone, angiotensin II and reactive oxygen species

Mabel J. van den Hoven1,∗, Femke Waanders2,3,∗, Angelique L. Rops1,∗, Andrea B. Kramer2,3, Harry van Goor3, Jo H. Berden1, Gerjan Navis2 and Johan van der Vlag1

1Nephrology Research Laboratory, Nijmegen Centre for Molecular Life Sciences, Department of Nephrology, Radboud University Nijmegen Medical Centre, Nijmegen, 2Department of Nephrology and 3Department of Pathology and Laboratory Medicine, University Medical Centre Groningen, University of Groningen, Groningen, The Netherlands

Correspondence and offprint requests to: Johan van der Vlag; E-mail: J.vanderVlag@nier.umcn.nl

∗Contributed equally.

Abstract

Background. Inhibition of the renin–angiotensin–aldosterone system (RAAS) provides renoprotection in adriamycin nephropathy (AN), along with a decrease in overexpression of glomerular heparanase. Angiotensin II (AngII) and reactive oxygen species (ROS) are known to regulate heparanase expression in vivo. However, it is unknown whether this is also the case for aldosterone. Therefore, we further assessed the role of aldosterone, AngII and ROS in the regulation of glomerular heparanase expression.

Methods. Six weeks after the induction of AN, rats were treated with vehicle (n = 8), lisinopril (75 mg/L, n = 10), spironolactone (3.3 mg/day, n = 12) or the combination of lisinopril and spironolactone (n = 14) for 12 weeks. Age-matched healthy rats served as controls (n = 6). After 18 weeks, renal heparanase and heparan sulfate (HS) expression were examined by immunofluorescence staining. In addition, the effect of aldosterone, AngII and ROS on heparanase expression in cultured podocytes was determined.

Results. Treatment with lisinopril, spironolactone or their combination significantly blunted the increased glomerular heparanase expression and restored the decreased HS expression in the GBM. Addition of aldosterone to cultured podocytes resulted in a significantly increased heparanase mRNA and protein expression, which could be inhibited by spironolactone. Heparanase mRNA and protein expression in podocytes were also significantly increased after stimulation with AngII or ROS.

Conclusions. Our in vivo and in vitro results show that not only AngII and ROS, but also aldosterone is involved in the regulation of glomerular heparanase expression.

Keywords: glomerular basement membrane; heparan sulfate; heparanase; proteinuria; renin–angiotensin–aldosterone system

Introduction

The renin–angiotensin–aldosterone system (RAAS) is a potent mediator in the initiation and progression of chronic kidney disease. Functional blockade of RAAS with angiotensin-converting enzyme inhibition (ACEi) and/or angiotensin II type 1 receptor blockade (ARB) is currently common practice for both the prevention and treatment of chronic kidney disease. Many reports have shown that AngII, the primary mediator of the RAAS, mediates progressive renal damage. However, recent studies suggest that the mineralocorticoid hormone aldosterone, involved in sodium and potassium homeostasis, is an additional factor in the development and progression of renal disease itself [1–3]. In line with this notion, administration of an aldosterone receptor blocker (spironolactone) on top of ACEi exerts an added renoprotective effect compared to ACEi alone [4–6].

The mechanisms of the adverse effects of aldosterone on the kidney are incompletely known. In experimental and human glomerular diseases, a decreased expression of heparan sulfate (HS) in the GBM is commonly present [7]. This loss of HS could be attributed to an increased expression of heparanase, which is an endo-β(1–4)-D-glucuronidase that cleaves HS side chains. In several experimental and human glomerular diseases, such as passive Heymann nephritis, puromycin-induced nephrosis, anti-GBM nephritis, diabetic nephropathy, adriamycin-induced nephrosis (AN) and minimal change disease, an increased expression of glomerular heparanase was observed, which correlated with a decreased expression of HS in the GBM [8].

Recently, we demonstrated that in AN, a model for chronic proteinuria-induced renal damage, ARB and scavengers of hydroxyl radicals reduced or prevented glomerular heparanase expression and restored loss of HS in the GBM [9]. This suggests that AngII and reactive oxygen species (ROS) are involved in the regulation of heparanase.
expression in vivo in AN. Furthermore, it is known that both aldosterone and AngII may induce ROS as mediators of renal injury [10–12]. It is unknown whether aldosterone is able to induce glomerular heparanase expression. Therefore, in the present study, we evaluated the effects of aldosterone receptor blockade by spironolactone, ACEi by lisinopril, or a combination of spironolactone and lisinopril on the glomerular expression of heparanase and HS in rats with AN. During proteinuric diseases, glomerular heparanase expression is mainly upregulated in podocytes and to a much lesser extent in glomerular endothelial cells [8,9,13–15]. Therefore, we evaluated whether aldosterone, AngII and ROS were able to directly regulate heparanase expression in cultured mouse podocytes.

Subjects and methods

Animals and experimental design

Fifty male Wistar rats (HsdCpb: Wu; Harlan Inc., Zeist, The Netherlands) weighing 250–275 g were used in this study. The animals were housed in a temperature-controlled room with a 12 h light–dark cycle. Animals had free access to food and water. All surgical procedures took place under isoflurane anaesthesia in N2O/O2.

Adriamycin nephrosis (AN) was induced by injection of 1.75 mg/kg adriamycin (Doxorubicin®) into the tail vein (n = 44). After 6 weeks, when proteinuria had stabilized, rats were stratified according to proteinuria and received treatment for 12 weeks. Vehicle-treated rats received normal saline and removed. All experimental procedures were approved by the local Animal Ethics Committees.

Clinical parameters

During the study, body weight and systolic blood pressure (SBP) were measured weekly in metabolic cages with measurement of water intake. Urinary protein was measured in 24-h urine samples by the Jaffé method, analysed on a multi-test analyser system (Merck Mega, Darmstadt, Germany).

Histological assessment of renal damage

Pieces of kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 μm) were stained with periodic acid–Schiff (PAS) and examined by light microscopy in a blinded fashion to evaluate focal glomerulosclerosis (FGS). We semi-quantitatively scored 50 glomeruli on a scale of 0–4. FGS was scored positive when collapsed capillary lumens, mesangial matrix expansion, hyalinosis and adhesion formation were present in the same quadrant. If 25% of the glomeruli were affected, a score of 1 was given, 50% was scored as 2, 75% as 3 and 100% as 4. The FGS scores presented in Table 2 are the median FGS scores per 50 glomeruli per experimental group, multiplied by 100 (score in arbitrary units from 0 to 400).

Immunofluorescence staining

Expression of heparanase, heparan sulfate (HS) and agrin was determined by indirect immunofluorescence staining on 2 μm cryostat kidney sections as previously described [9,13]. The sections were double-stained with a rabbit anti-heparanase antibody, diluted 1:100 (Prospectany, Rehovot, Israel), and a mouse monoclonal anti-HS antibody, JM403, which recognizes HS in the GBM, diluted 1:300, or double-stained with a monoclonal hamster anti-agrin antibody, M91, diluted 1:800 and JM403. The blinded sections were examined with a Zeiss Axioskop microscope by two independent observers. Agrin and HS expression was scored in 25 glomeruli per rat on a scale of 0–10 (in arbitrary units (AU)) for linearity of the GBM. Heparanase expression was scored on a scale of 0–5 for staining intensity [9]. Scoring between both observers was consistent, since analysis by the Bland and Altman method showed little interobserver variability with mean differences and 95% limits of agreement of 0.036 (−0.03/0.11) for agrin, −0.13 (−0.29/0.03) for HS and −0.24 (−0.30/−0.18) for heparanase.

Cell culture

Mouse podocytes (MPC-5) derived from the conditionally immortalized mouse (H-2Kb) with a temperature-sensitive variant of the SV40 large T antigen (tsA58) were cultured as reported [18]. To evaluate the effects of aldosterone, AngII and ROS on heparanase expression, podocytes were seeded on flasks pre-coated with 10% collagen A (Biochrom AG, Berlin, Germany) and cultured for 14 days at 37°C in an atmosphere of 5% CO2. Hereafter, the culture medium was replaced by RPMI 1640 containing 0.5% FCS for 24 h. Subsequently, podocytes were stimulated for 18 h with 100 nM aldosterone (Sigma, Zwijndrecht, The Netherlands), 1 μM AngII (Sigma), 50 μM xantine with 50 μM xantine oxidase (Sigma) to generate extracellular ROS, or 5 mM NADH (Roche Diagnostics, Penzberg, Germany), to generate intracellular ROS. Antagonists were added in a fresh medium with 0.5% FCS for 1 h at 37°C prior to the addition of AngII, aldosterone and generators of ROS, and all compounds were co-incubated for an additional 18 h. The aldosterone receptor was blocked with different concentrations of the non-specific mineralocorticoid receptor (MR) blocker spironolactone (1, 10 and 100 nM; Sigma). AngII receptor type 1 or 2 was inhibited by 5 μM L158,890 (MSD) or 5 μM P123.319 (Sigma), respectively. All experiments were performed at least in triplicate.

RNA isolation and real-time RT-PCR

Total RNA was extracted from podocytes using the RNeasy mini kit (Qiagen Benelux, Venlo, The Netherlands). RNA (1 μg) was reverse-transcribed into cDNA using first strand cDNA synthesis kit with oligo dT primers and Superscript reverse transcriptase (Invitrogen Life Technologies, Breda, The Netherlands). The mRNA levels of the MR, heparanase and serum glucocorticoid-regulated kinase 1 (SGK-1; positive control) were quantified by real-time PCR with the MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands). Each PCR reaction was performed with the use of Fast-start sybr green mix (Roche Diagnostics) and gene-specific primers (Biolegio BV, Malden, The Netherlands), as listed in Table 1. PCR conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 30 s, 56°C for 30 s and 72°C for 30 s. Expression of the housekeeping genes β-actin and/or RNA polymerase II was used to quantify heparanase, MR and SGK-1 expression by the delta–delta cycle time (Ct) method. No-RT and water as template were included as negative RT-PCR controls.

Flow cytometry

Podocytes were detached with 10 mM EDTA and washed with PBS. Protein expression was measured using Fix and Perm reagents (Sanbio, Uden, The Netherlands) as described [19]. Cells were incubated with Perm for 15 min and washed in PBA [PBS containing 0.5% BSA (Sigma)]. Subsequently, the cells were incubated with the monoclonal anti-heparanase antibody (HPA1; clone HP3/17) (1:100) (Tebu Bio BV, Heerhugowaard, The Netherlands) for 30 min in Fix/PBA and washed in PBA. The detecting goat-anti-mouse IgG2a Alexa-488 antibody (1:200) (Tebu Bio BV, Heerhugowaard, The Netherlands) was included as a negative control. All experiments were included as negative controls. Fluorescence was measured using a FACScan (BectonDickinson, San Jose, CA, USA).

Preparation of cell extracts, SDS-PAGE and western blot analysis

Podocytes, detached from about 400 cm² of culture, were lysed in a 500 μl lysis buffer [250 mM NaCl, 0.1% NP-40, 0.5 mM dithiothreitol (DTT), 50 mM HEPES pH 7.0, 5 mM EDTA with protease inhibitors (Roche, Almere, The Netherlands)] and sonicated twice on ice. Cellular debris was removed by centrifugation at 14 000 × g at 4°C for 10 min.
Regulation of glomerular heparanase expression by RAAS

Results

Clinical characteristics and renal structural damage

Clinical characteristics of the animals are described in detail elsewhere [20] and are summarized in Table 2. Briefly, 6 weeks after induction of nephrosis and before the start of treatment, the mean proteinuria was 214 ± 125 mg/24 h. In vehicle-treated rats, proteinuria progressed to 641 ± 287 mg/24 h at Week 18. Treatment with spironolactone (SPIR) monotherapy did not significantly reduce proteinuria, whereas treatment with lisinopril (LIS) and the combined treatment with SPIR and LIS significantly reduced proteinuria compared to vehicle-treated animals and animals treated with only SPIR. However, there was no significant difference between LIS monotherapy and the combined treatment with LIS/SPIR with regard to proteinuria and blood pressure. LIS and the combination therapy significantly reduced blood pressure compared to vehicle-treated animals and non-treated animals. Body weight and serum creatinine were not different between the groups.

To evaluate the effects of the different treatment modalities on the level of oxidative stress, we measured urinary MDA. Urinary MDA was significantly elevated in vehicle-treated animals compared to control. LIS, SPIR and the combined treatment all tended to reduce urinary MDA levels compared to vehicle, without differences between the groups (Table 2).

There was a significant increase in FGS in all adriamycin animals compared with control animals, with the highest values in vehicle, followed by SPIR, LIS and with the lowest FGS score in the LIS+SPIR group (P < 0.05 for trend). The FGS score was significantly reduced in the

### Table 1. Mouse primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5'-GTGGGCGCTCTAGGACACAA-3'</td>
<td>539 bp</td>
</tr>
<tr>
<td>RPII</td>
<td>5'-CTCTTTGATGTCACGCCAGATTTC-3'</td>
<td></td>
</tr>
<tr>
<td>HPSE-1</td>
<td>5'-GAGGGACAGCAAATCGATAGTGGG-3'</td>
<td>265 bp</td>
</tr>
<tr>
<td>SGK-1</td>
<td>5'-GATCCAGAATTTGACCGTTCAGTTG-3'</td>
<td>217 bp</td>
</tr>
<tr>
<td>MR</td>
<td>5'-GTGGACAGCTTCATCTCAGG-3'</td>
<td>328 bp</td>
</tr>
<tr>
<td></td>
<td>5'-TGACACCAGAAGGCTCTCTC-3'</td>
<td>285 bp</td>
</tr>
</tbody>
</table>

RPII, RNA polymerase II; HPSE-1, heparanase 1; SGK-1, serum glucocorticoid-regulated kinase 1; MR, mineralocorticoid receptor.

### Table 2. Clinical features at Week 18

<table>
<thead>
<tr>
<th></th>
<th>CON (n = 6)</th>
<th>VEH (n = 8)</th>
<th>LIS (n = 10)</th>
<th>SPIR (n = 12)</th>
<th>LIS+SPIR (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>504 ± 52</td>
<td>474 ± 28</td>
<td>451 ± 28</td>
<td>455 ± 28</td>
<td>464 ± 30</td>
</tr>
<tr>
<td>Proteinuria (mg/24 h)</td>
<td>28 ± 9</td>
<td>641 ± 287</td>
<td>289 ± 218</td>
<td>569 ± 284</td>
<td>187 ± 190</td>
</tr>
<tr>
<td>Urinary MDA (mmol/24 h)</td>
<td>40 ± 8</td>
<td>64 ± 30</td>
<td>55 ± 8</td>
<td>50 ± 9</td>
<td>51 ± 8</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>137 ± 8</td>
<td>169 ± 33</td>
<td>118 ± 25</td>
<td>150 ± 14</td>
<td>101 ± 25</td>
</tr>
<tr>
<td>Serum creatinine (µmol/L)</td>
<td>55 ± 2</td>
<td>69 ± 14</td>
<td>65 ± 11</td>
<td>67 ± 15</td>
<td>69 ± 13</td>
</tr>
<tr>
<td>FGS (0–400)</td>
<td>2 ± 3</td>
<td>57 ± 84</td>
<td>18 ± 31</td>
<td>40 ± 38</td>
<td>12 ± 12</td>
</tr>
</tbody>
</table>

CON, healthy control; VEH, vehicle; LIS, lisinopril; SPIR, spironolactone; LIS+SPIR, combination of lisinopril and spironolactone; MDA, malondialdehyde; FGS, focal glomerulosclerosis.

All values are expressed as mean ± SD.

ANOVA with the bonferroni post hoc test for body weight, blood pressure and serum creatinine (normally distributed); Kruskal–Wallis and Mann-Whitney U-tests for proteinuria, urinary MDA and FGS (not normally distributed): *P < 0.05 versus VEH, †P < 0.05 versus SPIR, ‡P < 0.05 versus LIS, §P < 0.05 versus all groups.

The resulting extract was about 10-fold concentrated by a Centricon YM-30 column (Millipore, Amsterdam, The Netherlands) according to the manufacturers’ instructions. The protein concentration was determined by the bicinchoninic acid assay (Sigma) with BSA as a standard.

Exactly 50 µg of total protein was applied in each lane. As a positive control, 15 ng of purified recombinant heparanase was included (kindly provided by Dr I. Vlodavsky). The proteins were then resolved by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with the monoclonal anti-heparanase antibody (HPA1; clone HPS3-17; dilution 1:200), detected after incubation with an IRDye800-conjugated anti-mouse IgG antibody (Li-Cor Biosciences, Bad Homburg, Germany) and scanned with the Odyssey® (Li-Cor Biosciences).

Statistical analysis

Data were tested for normality, and when non-normal distribution was present differences between groups were detected by Kruskal–Wallis and Mann–Whitney U-tests; otherwise ANOVA with bonferroni post hoc tests was used. Spearman’s Rho correlation coefficients were calculated. Linear regression was performed to calculate the association between proteinuria, HS and heparanase. Statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was assumed at the 5% level.

### Results

Clinical characteristics and renal structural damage

Clinical characteristics of the animals are described in detail elsewhere [20] and are summarized in Table 2. Briefly,
SPIR+LIS group compared to the vehicle and SPIR groups (Table 2).

Treatment with ACEi and/or spironolactone restores HS expression in the GBM and reduces glomerular heparanase expression

To evaluate the effects of treatment with ACEi (LIS), SPIR or a combination (LIS+SPIR) on HS and heparanase expression in AN, we performed immunofluorescence stainings on rat kidney cryosections (Figure 1). Figure 1A shows a linear HS staining of the GBM and no glomerular heparanase expression in control rats (CON), whereas in diseased adriamycin nephrotic animals (VEH) HS expression was decreased and associated with an increased glomerular heparanase expression. Monotherapy with LIS or SPIR and combination therapy with LIS+SPIR partially restored HS expression, whereas the combination of LIS+SPIR therapy seems to be the most effective therapy for restoration of HS in the GBM (Figure 1A and B). Additionally, glomerular heparanase expression was significantly reduced after treatment with LIS, SPIR or LIS+SPIR (Figure 1C). The combined treatment with ACEi and SPIR also appeared to be superior in the suppression of glomerular heparanase expression. These data strongly suggest that both aldosterone and AngII are involved in the increased glomerular heparanase expression in AN. Figure 2 shows that there was a clear correlation between the decreased expression of HS in the GBM and the glomerular heparanase expression, all groups taken into account ($R^2 = 0.59, P < 0.01$; Figure 2A).

Furthermore, a significant association was found between
urinary protein excretion and expression of HS in the GBM

\[ \text{aldosterone} + 100 \text{nM spironolactone} \]

which was unaffected by the addition of 100 nM aldosterone or 100 nM aldosterone + 1, 10 or 100 nM SPIR. White bars represent heparanase expression. Grey bars represent SGK-1 expression. Black bars represent MR expression. Mann–Whitney U-test: \(^* P < 0.01\) versus control, \(^a P < 0.05\) versus 100 nM Aldo.

**Aldosterone-induced heparanase mRNA and protein expression by podocytes are attenuated by spironolactone**

To extend the findings of SPIR on glomerular heparanase expression in AN from our in vivo study, we evaluated the effect of aldosterone on heparanase expression by cultured podocytes. We first determined whether the podocytes were able to respond to aldosterone by measuring the expression of the MR. Indeed, there was considerable expression of MR (Ct = 27.0), whereas no differences could be observed in expression of MR after aldosterone stimulation or aldoterone stimulation combined with SPIR (Figure 3). In addition, we determined the mRNA expression of SGK-1 as a positive control, since aldosterone is a known inducer of SGK-1 [21]. Indeed, SGK-1 expression was upregulated \(\sim 5\)-fold in podocytes after stimulation with aldosterone, which could be inhibited by SPIR (Figure 3). Exposure of podocytes to 100 nM aldosterone increased heparanase mRNA and protein expression about 2- to 3-fold (Figures 3 and 4). Treatment of the cells with SPIR prior to stimulation with aldosterone prevented heparanase mRNA expression completely at the lowest dose tested, i.e. 1 nM SPIR, which was also observed at the protein level (Figures 3 and 4A). These data reveal that the MR is constitutively expressed in mouse podocytes and that aldosterone is directly able to induce SGK-1 and heparanase expression in these cells.

**Angiotensin II-induced heparanase expression is mediated via the angiotensin II type 1 receptor**

Previously, it has been demonstrated that this podocyte cell line expresses both type 1 and 2 receptors for AngII [18]. Exposure of podocytes to AngII resulted in an \(\sim 3\)-fold increased heparanase protein expression as measured with flow cytometry (Figure 4A; \(P < 0.05\)). Western blot analysis of heparanase expression by cultured mouse podocytes appeared not to be easy and frequently gave negative results (no bands). However, we succeeded to visualize heparanase protein expression in podocytes cultured...
without or with AngII, which also revealed a 3-fold increase in heparanase protein expression after exposure to AngII (Figure 4B). Furthermore, western blot analysis revealed expression of the 50 kDa active form of heparanase and not of the 65 kDa pro-form. Similar results were obtained at the mRNA level (not shown). AngII-induced heparanase expression could be inhibited by ARB (Figure 4A), whereas blockade of AngII type 2 receptor had no effect, implicating that AngII-induced heparanase expression in podocytes is mediated via the AngII type 1 receptor (data not shown).

Aldosterone-induced heparanase expression by cultured podocytes is not affected by an AngII receptor blocker and AngII-induced heparanase expression is not affected by spironolactone

Since AngII- and aldosterone-induced signalling pathways may interact, we performed some interference experiments. First, we did not observe an additive effect in upregulation of heparanase expression by podocytes when AngII and aldosterone were added together (data not shown). Furthermore, when we combined AngII addition with SPIR addition, and vice versa aldosterone addition with ARB, we did not observe an inhibition of AngII- or aldosterone-induced expression of heparanase by podocytes (Figure 4A). Furthermore, we did not observe an additive effect in inhibition of heparanase expression when ARB and SPIR addition were combined (Figure 4A).

These data suggest that AngII-induced heparanase expression by cultured podocytes is not further enhanced by prior and simultaneously ligation of the aldosterone receptor and vice versa that aldosterone-induced heparanase expression is not amplified by prior and simultaneously ligation of the AngII type 1 receptor.

Role for reactive oxygen species in the regulation of heparanase expression

The actions of AngII and aldosterone may involve ROS [10,12], whereas we have demonstrated in AN that ROS are able to induce glomerular heparanase expression [9]. Therefore, we tested the effects of intracellularly or extracellularly generated ROS on heparanase expression by cultured podocytes. Exposure of podocytes to xanthine/xanthine oxidase (X/XO), which produces extracellularly generated superoxide and hydrogen peroxide, induced a significantly increased heparanase mRNA and protein expression (Figure 5A and B). Moreover, intracellularly generated radicals, which are produced after incubation of the cells with NADH, induced also a significantly increased heparanase mRNA and protein expression. These data reveal the involvement of ROS in the regulation of heparanase expression by cultured podocytes.

Discussion

Our in vivo and in vitro data strongly suggest that aldosterone induces glomerular heparanase expression, which can be blocked by SPIR. Moreover, we demonstrated that AngII and ROS directly induce glomerular heparanase expression in vitro in podocytes.

Consistent with our previous study [9], a decreased HS expression in the GBM was associated with an increased glomerular heparanase expression in AN. These effects are attenuated by treatment with SPIR, suggesting that aldosterone, at least in part, mediates these effects. This is supported by the in vitro data, showing that aldosterone directly induces heparanase expression in podocytes, which could be blocked by SPIR. ACEi also reduced heparanase expression and restored HS expression in vivo, suggesting involvement of AngII in the regulation of heparanase expression as well. Combining both treatments tended to superiorities in normalizing HS and heparanase expression in AN, as compared to monotherapy with either ACEi or SPIR.

Glomerular heparanase expression in proteinuric models is mainly localized in podocytes [9,13,14], suggesting that podocytes are responsible for the regulation of heparanase expression. Glomerular endothelial cells do express heparanase to a much lesser extent. However, heparanase expression in our glomerular endothelial cell line is not upregulated by AngII (unpublished data), in contrast to
Regulation of glomerular heparanase expression by RAAS 2643

pro-inflammatory cytokines [19]. Aldosterone binds to the MR in the cytoplasm, which after its translocation to the nucleus, results in transcription of target genes involved in controlling blood pressure and volume status, like SGK-1 [21]. We demonstrated that the MR is expressed by differentiated mouse podocytes and that aldosterone highly up-regulated SGK-1 expression by these cells, which could be inhibited by SPIR. Most importantly, aldosterone-induced heparanase mRNA and protein expression in podocytes could be attenuated by pre-treatment of the cells with SPIR. These data strongly suggest that aldosterone is directly involved in the induction of heparanase expression by podocytes.

Previously, we showed in vivo in AN that reduction of HS in the GBM and the increased glomerular heparanase expression could be reversed by ARB, suggesting the involvement of AngII in the regulation of heparanase expression [9]. We now investigated whether AngII can directly induce heparanase expression in vitro. Stimulation of podocytes with AngII resulted in the induction of heparanase, which could be inhibited by ARB, but not by AngII type 2 receptor blockade. This demonstrates that AngII-induced heparanase expression by podocytes is mediated via the AngII type 1 receptor, which corresponds with the reported AngII-induced HSPG reduction in human podocytes [11,22].

HS expression in rats with AN after treatment with SPIR and/or ACEi was not completely restored to the level of healthy control rats. This phenomenon could possibly be attributed to the presence of ROS. We previously reported that scavenging of hydroxyl radicals in this model partially prevented loss of HS and suppressed heparanase expression, suggesting a role for ROS in the regulation of heparanase expression [9,23]. Now we show that urinary MDA, as a marker for oxidative stress, was elevated in AN, and lower after treatment with SPIR and/or ACEi. When we stimulated podocytes in vitro with X/XO, which generates superoxide and hydrogen peroxide extracellularly, or with NADH (intracellularly generated ROS), an increased heparanase mRNA and protein expression was observed. Notably, AngII and aldosterone also may induce ROS leading to renal injury [10,12], which implies that the regulation of heparanase expression in podocytes by RAAS may occur at several interacting levels. Recently, it was shown that uni-nephrectomized rats, continuously infused with aldosterone and fed a high-salt diet, developed a progressive proteinuria, which could be prevented by treatment with eplerenone, an aldosterone receptor blocker or the ROS scavenger tempol [21]. In the same study, it was shown that addition of aldosterone to cultured rat podocytes induced SGK-1, which could be prevented by the addition of tempol. In our current in vitro study, we could not show that AngII-induced heparanase expression by cultured podocytes is influenced by aldosterone or vice versa that aldosterone-induced heparanase expression is amplified by AngII. Future studies should reveal whether ROS or other downstream pathways are common denominators in AngII- and aldosterone-induced heparanase expression [11].

We have demonstrated that AngII, aldosterone and ROS are able to induce heparanase expression at the mRNA and protein levels in cultured podocytes. In addition to regulation at the mRNA and protein levels, the regulation of heparanase activity involves post-translational processing of the native protein. Heparanase may occur in an inactive pro-form of 65 kDa and in an active form as a heterodimer of a 50 kDa and 8 kDa subunit [8], which may be excrated. We have tried to measure the heparanase protein and/or activity in the cell culture supernatant directly or after (immuno)precipitation with anti-heparanase antibodies or heparin-coated beads. However, we did not succeed to measure heparanase protein and/or activity in the culture medium. This may be explained by the fact that the 18 h period of stimulation was too short to get sufficient accumulation of detectable amounts of heparanase protein and/or activity in the medium.

SPIR monotherapy did not significantly reduce proteinuria in the nephrotic rats, despite significantly reduced glomerular heparanase expression and partially restored HS expression in the GBM. This observation is in line with recent data that question the primary role of HS in charge-selective glomerular permeability. The loss of HS in the GBM in podocyte-specific agrin or EXT-1 knock-out mice or in a mouse model with transgenic overexpression of heparanase did not lead to severe proteinuria [8,24–26]. However, it should be noted that these studies do not exclude a role for heparanase and/or heparanase-mediated HS loss in the complex pathogenesis of proteinuria. Heparanase action may lead to the chronic loss of bioactive HS fragments, HS-bound factors, such as growth factors, cytokines and chemokines, the chronic disturbance of HS-dependent glomerular cell-GBM interactions and cellular responses induced by direct binding of the heparanase protein to glomerular cells. All these heparanase-mediated mechanisms may be critically involved in the development of proteinuria and its downstream effects on renal damage [8,24].

The absence of an anti-proteinuric effect in the SPIR-treated rats supports the pharmacological specificity of the reduction of heparanase and improvement of HS expression by SPIR, as it excludes the possibility that these effects are the non-specific consequences of proteinuria (reduction), i.e. heparanase expression is not induced by proteinuria, which is an important conclusion that could not be drawn from previous studies. In line with the current data in the lisinopril group, we previously showed that proteinuria in AN was reduced during antihypertensive intervention by RAAS blockade [9,27]. Our current data in the SPIR group show that in this model, apparently, partial restoration of HS expression in the GBM and reduction of glomerular heparanase expression are not sufficient to ameliorate proteinuria. This finding may be explained by the fact that in addition to a restored HS expression in the GBM, a reduction of blood pressure is required to achieve a reduction on proteinuria, as we did observe with lisinopril and the combined lisinopril/SPIR treatment regimes. Therefore, it is of interest to evaluate the effect of blood pressure lowering drugs other than ACE inhibitors, ATR blockers or aldosterone receptor blockers, like for example hydralazine. On the other hand, lowering of blood pressure will not always result in an improvement of proteinuria and/or renal damage.

During the review process of this paper, another study was published that addressed the effects of ACE inhibition and aldosterone receptor blockade in AN using
about a 3-fold higher concentration of Adriamycin [28], with results partially in line with our current data. In this study, the effects of monotherapy with eplerenone, a selective aldosterone antagonist, and of the ACE inhibitor enalapril and their combination were evaluated. Monotherapy with eplerenone and enalapril, as well as the combination, reduced daily and cumulative protein excretion ~2-fold, without an effect on blood urea nitrogen. However, there was no significant effect of either monotherapy on the ratio of daily urinary protein to creatinine. Interestingly, the combination of eplerenone and enalapril was superior, and significantly reduced the urinary ratio of protein to creatinine. These authors non-quantitatively evaluated the glomerular expression of nephrin and podocin, which seemed to be partially preserved by eplerenone and/or enalapril treatment, like we found for glomerular HS in our study. In addition, in rat transplant arteriopathy, we recently reported that SPIR protects against vasculopathy, with a trend towards lower proteinuria, but without effect on creatinine [3]. So, SPIR can exert specific beneficial renal effects without clear-cut effects on renal function within the time frame of the study. A plausible explanation could be that a possible renoprotective effect is not directly apparent from renal function due to induction of volume depletion by the diuretic effect of SPIR.

In conclusion, our in vivo and in vitro results show that aldosterone, AngII and ROS are critically involved in the regulation of glomerular heparanase expression. Furthermore, in AN, the most effective reduction in glomerular heparanase expression, and restoration of HS in the GBM, was achieved by the combination of LIS and SPIR, suggesting that a combination of ACE inhibition and aldosterone receptor blockade is promising in treatment of proteinuric diseases.

Acknowledgements. This study was supported by the Dutch Diabetes Research Foundation (grant 2001.00.048) and the Dutch Kidney Foundation (grant C05.2152). Lisinopril and L158,809 were kind gifts from Merck Sharp and Dohme Research Laboratories (Rahway, NJ, USA). We thank Pieter Klok, Casandra van Bavel, Wim Tamboer and Marian Bulthuis for skilled (bio)technical assistance, Peter Mundel (Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029, USA) for providing the MPC-5 podocyte cell line and Israel Vlodavsky (Rappaport Faculty of Medicine, Technion, Haifa, Israel) for providing purified recombinant active heparanase.

Conflict of interest statement. None declared.

References

Mechanical forces and TGFβ1 reduce podocyte adhesion through α3β1 integrin downregulation

Cecile Dessapt1, Marc Olivier Baradez2, Anthea Hayward1, Alessandra Dei Cas1, Stephen M. Thomas1, Giancarlo Viberti1 and Luigi Gnudi1

1 Cardiovascular Division, School of Medicine, King’s College London, London and 2 School of Life Science, Kingston University, Kingston-upon-Thames, UK

Correspondence and offprint requests to: Luigi Gnudi; E-mail: luigi.gnudi@kcl.ac.uk

Abstract

Background. Podocyturia is a marker of diabetic nephropathy, a possible determinant of its progression and a powerful risk factor for cardiovascular disease. A reduction in podocyte adhesion to the glomerular basement membrane (GBM) via downregulation of α3β1 integrin expression, the main podocyte anchoring dimer to the GBM, may represent one of the mechanisms of podocyturia in glomerular disease. This study investigated the role of mechanical forces and transforming growth factor beta1 (TGFβ1) in podocyte adhesion and integrin expression.

Methods. Conditionally immortalized murine podocytes were exposed to mechanical stretch and/or TGFβ1 for 48 h. Podocyte adhesion, apoptosis and α3β1 integrin expression were assessed.

Results. Stretch and TGFβ1 significantly reduced podocyte adhesion and α3β1 integrin expression, events paralleled by increased apoptosis. Blockade of β1 integrin, with a specific antibody, demonstrated a reduced podocyte adhesion indicating that β1 integrin downregulation was required for the loss of podocyte adhesion. This was linked to an increase in podocyte apoptosis. The role of apoptosis in podocyte adhesion was further investigated using caspase-3 inhibitors. Podocyte apoptosis inhibition did not affect stretch- and TGFβ1-mediated integrin downregulation and the loss of podocyte adhesion, suggesting that α3β1 integrin downregulation is sufficient to alter cell adhesion. Although stretch significantly increased podocyte TGFβ type I, II and III receptors but not podocyte TGFβ1 secretion, the combination of stretch and TGFβ1 did not show any additive or synergistic effects on podocyte adhesion and α3β1 integrin expression.

Conclusions. These results suggest that downregulation of α3β1 integrin expression, by mechanical forces or TGFβ1, is per se sufficient to reduce podocyte adhesion. Apoptosis may represent a parallel important determinant of the podocyte loss from the GBM.

Keywords: cell adhesion; podocyte; stretch; TGFβ1; α3β1 integrin

Introduction

Glomerular capillary stability depends on the co-operative function of endothelial cells, mesangial cells and podocytes along the glomerular basement membrane (GBM). The histopathological changes observed in the glomerulus of chronic nephropathies are characterized by alteration of the filtration barrier (predominantly podocytes and GBM) and by excessive extracellular matrix deposition [1]. Podocytes are terminally differentiated epithelial cells with a unique structure and function. They are crucial in preserving glomerular capillary integrity, regulating the synthesis of GBM extracellular matrix proteins [2], and maintaining specific restriction to plasma protein filtration [3]. In glomerulopathies, podocyte injury may lead to foot process fusion and podocytopaenia. Podocyte dysfunction and podocyte loss have been linked to the pathogenesis of proteinuria and, ultimately, to glomerulosclerosis [4–8]. The exact aetiology for podocyturia remains unclear but two mechanisms have been suggested: apoptosis and reduced adhesion to the GBM [9]. Podocyte apoptosis has been