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

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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. 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 (AngII) 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

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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 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.

Cannulation of the abdominal aorta, kidneys were perfused with 100 mM 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 NAHD (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 mM; 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 MyiqTM Single-Color Real-Time PCR Detection System (Bio-Rad, Venendaal, 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 10 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. Heparanase 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 IgG2b Alexa-488 antibody (1:200) was incubated in PBA for 30 min. Subsequently, the cells were washed and resuspended in PBA. Fluorescence was measured using a FACSScan (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.
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′-GTGGGCCGCTCTAGGACACAA-3′</td>
<td>539 bp</td>
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<tr>
<td></td>
<td>5′-CTCTTTGAGTGTCAGGACATTG-3′</td>
<td></td>
</tr>
<tr>
<td>RPII</td>
<td>5′-GACGCCACACTCCAATGATTGAG-3′</td>
<td>265 bp</td>
</tr>
<tr>
<td></td>
<td>5′-GATCGTGCTGCTGGCTTCCAAGG-3′</td>
<td></td>
</tr>
<tr>
<td>HPSE-1</td>
<td>5′-GACGCGGAACTCCCGATGATAC-3′</td>
<td>217 bp</td>
</tr>
<tr>
<td></td>
<td>5′-GATCCGAAATGAGCTGCTTGAAGG-3′</td>
<td></td>
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<tr>
<td>SGK-1</td>
<td>5′-CTGTCTTACATCTGCAAGAG-3′</td>
<td>328 bp</td>
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<tr>
<td></td>
<td>5′-CCGTAGACATCTGCTACAGG-3′</td>
<td></td>
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<tr>
<td>MR</td>
<td>5′-GTGGACAGTCCTTCTACTCCAG-3′</td>
<td>285 bp</td>
</tr>
<tr>
<td></td>
<td>5′-TGACACCAGAAGCCTCTCCT-3′</td>
<td></td>
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</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>64 ± 287</td>
<td>289 ± 218*</td>
<td>569 ± 284</td>
<td>187 ± 190±†</td>
</tr>
<tr>
<td>Urinary MDA (nmol/24 h)</td>
<td>40 ± 8</td>
<td>64 ± 306‡</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.

Parameters at the end of the study are shown.

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, *P < 0.05 versus CON.

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, 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 all tended to reduce urinary MDA levels 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
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

![Fig. 1. Effect of ACEi and/or spironolactone on HS and heparanase expression in adriamycin nephropathy. (A) Immunofluorescence staining of rat kidney sections revealed a linear expression of HS in the GBM of control rats, whereas HS expression was decreased in animals with AN treated with vehicle (VEH). Treatment with either ACEi (LIS: 75 mg/L drinking water), spironolactone (SPIR: subcutaneous slow release 3.3 mg/day) or a combination (LIS + SPIR) resulted in restoration of HS expression. In addition, the decrease of HS in rats with AN was associated with an increased glomerular heparanase expression, which could be downregulated after treatment with LIS, SPIR and the combination therapy. (B) Quantification of the scores revealed a significant decrease of GBM HS in VEH compared to CON, which was partially, but significantly restored after treatment with LIS, SPIR or the combination. (C) Quantification of the scores revealed a significant increased glomerular expression of heparanase in VEH compared to CON. After treatment with LIS, SPIR or the combination, glomerular heparanase expression was significantly reduced. Kruskal–Wallis and Mann–Whitney U-tests: *$p < 0.05$ versus CON, †$P < 0.05$ versus VEH, ‡$P < 0.01$ for trend.

![Fig. 2. Correlations between proteinuria, expression of HS in the GBM and glomerular heparanase expression. (A) The reduction of HS expression in the GBM strongly correlated with the increased glomerular heparanase expression taking into account the different groups (■ VEH, ▲ LIS, ▼ SPIR, ◆ LIS+SPIR) ($R^2 = 0.59, P < 0.01$). (B) Furthermore, the degree of proteinuria correlated with the decreased expression of HS in the GBM ($R^2 = 0.37, P < 0.01$).]
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 aldosterone stimulation combined with SPIR (Figure 3). In addition, we determined the mRNA expression of SGK-1 as a positive control, since aldosterone is known to induce SGK-1 [21]. Indeed, SGK-1 expression was upregulated ~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 ~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 extracellular radicals such as superoxide and hydrogen peroxide, revealed an increased heparanase mRNA (A) and protein (B) expression. Also, exposure for 18 h to 5 mM NADH, inducing intracellular generation of reactive oxygen species, resulted in an increased heparanase mRNA (A) and protein (B) expression. Mann–Whitney U-test: (A) **P < 0.005 versus control, ***P < 0.0001 versus control, (B) #P < 0.05 versus control.

**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 superiority 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...
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 differ-
entiated 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 di-
rectly 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 ex-
pression could be reversed by ARB, suggesting the involve-
ment 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 re-
ceptor blockade. This demonstrates that AngII-induced
heparanase expression by podocytes is mediated via the
AngII type 1 receptor, which corresponds with the re-
ported 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 expres-
sion, suggesting a role for ROS in the regulation of hep-
aranase 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/OX, which generates
superoxide and hydrogen peroxide extracellularly, or with
NADH (intracellularly generated ROS), an increased hep-
aranase mRNA and protein expression was observed. No-
tably, 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 aldos-
terone 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. Fu-
ture studies should reveal whether ROS or other down-
stream pathways are common denominators in AngII- and

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 regu-
lation 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 ex-
crated. We have tried to measure the heparanase protein
and/or activity in the cell culture supernatant directly or
after (immuno)precipitation with anti-heparanase antibod-
ies or heparin-coated beads. However, we did not succeed
to measure heparanase protein and/or activity in the cul-
ture 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 protein-
uria in the nephritic 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 ex-
clude 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 frag-
ments, 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 mecha-
nisms may be critically involved in the development of pro-
teinuria 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 hep-
aranase expression are not sufficient to ameliorate protein-
uria. This finding may be explained by the fact that in ad-
tion to a restored HS expression in the GBM, a reduction
of blood pressure is required to achieve a reduction on pro-
teinuria, as we did observe with lisinopril and the combined
lisinopril/SPiR treatment regimes. Therefore, it is of inter-
est to evaluate the effect of blood pressure lowering drugs
other than ACE inhibitors, ATR blockers or aldosterone re-
ceptor 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.

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Conflict of interest statement. None declared.

References


Mechanical forces and TGFβ1 reduce podocyte adhesion through α3β1 integrin downregulation

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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 beta 1 (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