Original Paper

The Effect of Combined Treatment with the (Pro)Renin Receptor Blocker HRP and Quinapril in Type 1 Diabetic Rats

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Key Words

Diabetic nephropathy • (pro)Renin receptor • Decoy peptide • Podocyte • ACE inhibitor

Abstract

Background/Aims: Diabetic nephropathy remains a major clinical problem. The effects of prorenin might be adverse, but the literature data are controversial. We compared the renal effects of the (pro)renin receptor ((P)RR) blockade and angiotensin converting enzyme (ACE) inhibition on the progression of diabetic nephropathy in rats. **Methods:** Diabetes (DM) was induced by ip. streptozotocin administration in adult male Sprague-Dawley rats, followed by eight weeks of treatment with the (P)RR blocker „handle region” decoy peptide (HRP, 0.1 mg/kg/day) or with the ACE inhibitor Quinapril (Q, 50 mg/kg/day) and grouped as follows: 1. Control (n=10); 2. DM (n=8); 3. DM+HRP (n=6); 4. DM+Q (n=10); 5. DM+Q+HRP (n=10). Renal functional parameters, histology and gene expressions were evaluated. **Results:** HRP reduced glomerulosclerosis and podocyte desmin expression, but did not affect proteinuria and tubular ERK(1/2) phosphorylation. Both Q and Q+HRP treatment reduced proteinuria, glomerular and tubular damage, tubular TGF-β1 expression and ERK(1/2) phosphorylation to the same extent. **Conclusion:** The effects of HRP were partially beneficial on diabetic kidney lesions as HRP reduced damage but did not improve tubular damage and failed to reduce ERK(1/2) phosphorylation in rats. The combination of HRP with Quinapril had no additive effects over Quinapril monotherapy on the progression of diabetic nephropathy.

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Introduction

End stage renal disease (ESRD) due to diabetic nephropathy is an increasing clinical problem. Treatment protocols targeting the renin-angiotensin-aldosterone system (RAAS) (angiotensin-converting enzyme inhibitors (ACEi), Ang II receptor blockers (ARB), aldosterone antagonists) have been widely used to reduce renal damage [1, 2]. However, even high doses of ACEi monotherapies fail to stop the progression. Recently, positive results were reported with the combination of ACEi with renal sympathetic denervation, ARB or renin inhibition [3-7]. It also has been recently postulated that renin and prorenin, referred to as (pro)renin have an Ang II independent direct fibrogenic effect in the kidney [8, 9]. This might explain why RAAS blockers have not yet been able to achieve complete remission. Contrary to renin and Ang II, prorenin is constitutively secreted, and may reach significantly higher blood levels than renin [10, 11].

Nguyen and colleagues cloned the highly conservative (pro)renin receptor ((P)RR) in 2002 [12]. The (P)RR binds both renin and prorenin. The (P)RR non-proteolytically activates (pro)renin, which then activates the renin-angiotensin pathway. However, (P)RR stimulation can also directly upregulate profibrotic genes, independent of the angiotensin pathway [9, 12, 13]. (P)RR binds to (pro)renin at the „handle” region of its prosegment. A decoy peptide, which is an epitope of the prosegment, was introduced to competitively block the binding of (pro)renin to the (P)RR [14]. Increased glomerular and tubular expression of (P)RR was reported in diabetic rats [15], suggesting a potential role for prorenin in the pathogenesis of diabetic nephropathy. Previously, (P)RR-blockade by the "handle" region peptide (HRP) prevented diabetic nephropathy and reduced cardiac hypertrophy in hypertensive rats [16-18]. In cultured mesangial cells, both the AT1 receptor blockade and downregulation of (P)RR expression with silencing RNA (siRNA) were reported to suppress the high glucose-induced TGF- β overexpression and their combination had additive effects [19]. In contrast, the combination of ACEi or ARB and HRP had no additive effects on reducing renal TGF- β expression compared to monotherapies in diabetic rats. Individual HRP and ACEi or ARB treatments achieved similar results [17, 19]. Other studies also debated the significant role of prorenin in progressive renal disease models [20, 21].

The controversial results raised the question whether or not the effects of the (P)RR blockade depend on the experimental model, or on the targeted renal cell types. Given the reported profibrotic effects of (pro)renin, one might also ask whether the (P)RR blockade could beneficially affect diabetic nephropathy similar to ACEi. Furthermore, the effects of HRP administration on diabetic tubular injury have not yet been investigated. Although ACEi is approved as an effective therapy to reduce diabetic podocyte injury, albuminuria and the progression of nephropathy, it increases renal expression of renin that can counteract the antifibrotic effects of ACEi by stimulating the (P)RR [5]. We aimed to study whether combined treatment with the putative (P)RR blocker HRP and the ACEi Quinapril delays more the progression of nephropathy than either monotherapy in type-1 diabetic rats. We focused our study on the markers of podocyte injury and tubulointerstitial fibrosis.

Materials and Methods

Animals

Eight-week old male Sprague-Dawley rats weighing 250 to 300g (Charles River, Sulzfeld, Germany) were housed in a room at a constant temperature of 22 $\pm$ 2°C with 12h light/dark cycle, and had access to standard rodent chow and water ad libitum.

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures and handling of animals during the investigations were reviewed and approved by the Food Chain Safety and Animal Health Directorate of Pest County under licence number 22.1/4268/003/2009 and by the local Ethical Committee for Animal Experimentation of Semmelweis University.

Induction of diabetes mellitus

Type-1 diabetes mellitus (DM) was induced with a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg), freshly dissolved in citrate buffer (0.1 mol/l). Control animals received only buffer. One week after treatment glucose concentration of blood samples obtained from the tail were determined using a digital blood glucose meter and test strips (Accu-Chek®, Roche Inc., Mannheim, Germany). Animals with blood glucose levels above 25 mmol/l were considered diabetic and were included in the study.

Experimental groups, treatment protocol

Untreated rats, injected only with citrate buffer, served as non-diabetic controls (Control, n=10). Diabetic rats were randomly assigned into 4 groups. Untreated diabetic rats served as positive controls (DM, n=10). Three groups of animals were treated for 8 weeks as follows: (P)RR-inhibitor, „handle region” decoy peptide (HRP) was infused from osmotic minipumps (ALZET Model 2004, Cupertino, CA, USA) changed once after 28 days (DM+HRP, 0.1mg/kg/day, n=8) in one group; the ACEi Quinapril was administered in the drinking water (DM+Q, 50mg/kg/day, n=10) in another group, and the third group was treated with the combination of Quinapril and HRP (DM+Q+HRP, n=10). During follow-up, 20% of untreated DM rats and 25% of DM+HRP rats died before harvest. The daily water intake was registered and the concentration of Quinapril was adjusted daily to deliver the desired dose. Body and kidney weights were measured at the time of harvest. All rats were anesthetized with isoflurane during osmotic minipump implantations, blood samplings and at the termination of the study.

Perfusion harvest, serum and urine chemistry tests

The day before harvest the urine was collected for 24h in diuresis cages (Tecniplast, Buguggiate, Italy). Blood samples were taken at harvest under isoflurane narcosis from the abdominal aorta and serum, and plasma (with EDTA as anticoagulant) was prepared. After blood sampling, the rats were perfused via the abdominal aorta with cold saline solution for 10-15 minutes at constant pressure of 120 mmHg. The excised left kidneys were snap-frozen in liquid nitrogen and stored for RNA and protein analysis, the right kidneys were fixed in 4% buffered formalin overnight before paraffin embedding. Serum and urine glucose, as well as serum and urine creatinine concentrations were determined photometrically using commercial kits (Diagnosztikum Zrt, Budapest, Hungary). Urine protein concentration was measured (BCA Protein Assay, Pierce Thermo Scientific, Rockford, USA), and urine protein/creatinine ratios were calculated.

Plasma renin activity

Plasma renin activity was measured using GammaCoat® Plasma Renin Activity ¹²⁵I RIA Kit (DiaSorin, Stillwater, Minnesota, USA) following the manufacturer's protocol.

Renal histology and immunochemistry

Formalin fixed paraffin embedded kidney sections were cut and stained with periodic-acid Schiff (PAS) reagent (PAS staining kit, Merck Chemicals, Darmstadt, Germany) to evaluate glomerular and tubulointerstitial damage, as follows: glomerular damage was scored according to the system of El Nahas [22]: grade 0, no change, healthy glomerulus; grade 1, lining of capillaries to Bowman's capsule; grade 2, accumulation of mesangial matrix in 25 % of the glomerulus; grade 3, accumulation of mesangial matrix in 50% of the glomerulus and capillary obliteration; grade 4, accumulation of mesangial matrix in >75% of the glomerulus. Using light microscopy at a magnification of x400, the glomerular and tubular scores of each animal were derived as the arithmetic mean of 60-80 glomeruli or 15-20 observation fields, respectively. The tubulointerstitial damage scores were assessed using the following scoring system (score 0-5) at a magnification of x100: grade 0, no change; grade 1-5 was assigned based on the number of the following criteria: tubular dilatation, tubular atrophy, hyaline in tubular lumen, interstitial infiltration of mononuclear cells, and interstitial fibrosis.

Immunohistochemistry was performed on paraffin sections using the avidin-biotin method as previously described [23]. Briefly, after antigen retrieval in citric buffer the slides were incubated overnight at 4°C with rabbit polyclonal anti-fibronectin (1:1000, Sigma-Aldrich Budapest, Hungary), monoclonal mouse anti-desmin (Dako #M0760, Farnk Diagnosztika, Budapest, Hungary) or rabbit polyclonal anti-TGF-β1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Secondary antibodies (SuperSensitive Link) were purchased from BioGenex (San Ramon, CA, USA). The slides were developed with Liquid Permanent Red (Dako).

Immunohistochemical staining was examined at a magnification of x400 using the following semiquantitative scoring; 0: no staining, 1: weak, 2: mild, 3: strong, 4: very strong staining, as described previously [23]. All samples were evaluated in a blinded manner.

Indirect immunofluorescence for renin was performed on paraffin-embedded kidney sections in the dark. Briefly, blocking solution (10% donkey serum in 1% BSA-PBS) was applied for 30 minutes, and then the slides were incubated overnight at 4°C with primary chicken anti-renin antibody (a kind gift from Prof. A. Kurz, Regensburg, Germany) at a 1:200 dilution. After washing the slides in 1% BSA-PBS, secondary antibody (donkey anti-chicken-TRITC (Jackson ImmunoResearch, West Grove, PA, USA)) was applied (1:200) for 2 hours at room temperature, and then the slides were mounted. The intensity of renin immunofluorescence was measured on the transversal sections of afferent arterioles using Image Pro Plus software (Media Cybernetics Inc, Bethesda, MD, USA).

Immunoblot

Kidney samples (20 mg) were homogenized in RIPA lysis buffer containing complete protease inhibitor cocktail (Roche). Protein concentration was determined by the BCA Assay. Samples were mixed with 2x Laemmli buffer and boiled. Equal amounts of protein (40 µg) were separated on 12% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and blocked with 5% skim milk in Tris-buffered saline (TBS), containing 0.1% Tween-20. Membranes were incubated overnight at 4°C with goat polyclonal Lcn2 antibody (1:500, R&D Systems, Minneapolis, MN, USA), rabbit polyclonal phospho-ERK(1/2) (1:1000, Cell Signaling, Danvers, MA, USA), rabbit polyclonal total-ERK(1/2) (1:2000, Cell Signaling) or mouse monoclonal α -tubulin antibody (1:10000, Sigma-Aldrich, Budapest, Hungary), washed and incubated with peroxidase-conjugated secondary antibody (anti-mouse IgG or anti-goat IgG, 1:5000, Cell Signaling.). Blots were visualized by ECL detection kit (Pierce/Thermo Scientific, Rockford, IL, USA).

Quantitative RT-PCR

100 mg of whole kidneys were homogenized and total RNA was isolated according to the manufacturer's protocol (SV Total RNA kit, Promega, Madison, WI, USA). 2 µg RNA was reverse transcribed (High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Foster City, CA, USA) using random primers. PCR reactions were performed on a BioRad CFX thermal cycler (BioRad, Hercules, CA, USA) using the Power SYBR Green PCR Master Mix (Applied Biosystems). Specificity and efficiency of the PCR reaction was confirmed with melting curve and standard curve analysis, respectively. Duplicate samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression. Mean values are expressed with the formula $2^{-\Delta\Delta Ct}$. Primer sequences were as follows: *Tgfb1* forward 5-ACCATCCATGACATGAACC-3; *Tgfb1* reverse 5-TCATGTTGGACAACCTGCTCC-3; *Lcn2* forward: 5-ACGTCACTTCCAT CCTCGTC-3; *Mmp2* forward: 5-GCTGATACTGACACTGGTACTG-3; *Mmp2* reverse: 5-CACTGTCCGCCAATAAACCC-3; *Mmp9* forward: 5-CTTGAAGTCTCAGAAGGTGGATC-3; *Mmp9* reverse: 5-CGCCAGAAGTATTTGTCATGG-3; *Timp1* forward: 5-TTCTTGCAACTCGGACCTG-3; *Timp1* reverse: 5-ACAGCGTCGAATCCTTTGAG-3; *Timp2* forward: 5-TCGAATTTATCTACACGGCCC-3; *Timp2* reverse: 5-GGCACAATAAAGTCACAGAGGG-3; *Timp3* forward: 5-AAGGCAAGATGTACACAGGG-3; *Timp3* reverse: 5-TGGAGGTCACAAAGCAAGG-3; *Nphs1* forward: 5-GCCTCTTGACCATCGCTAATG-3; *Nphs1* reverse: 5-GACAACCTTCAGTCCCAGTG-3; *Nphs2* forward: 5-TCCCTTTTCCATCTGGTTCTG-3; *Nphs2* reverse: 5-CTTGTGATAGGTGTCCAGGC-3; *Gapdh* forward: 5-CAATGACCCCTTCATTGACC-3; *Gapdh* reverse: 5-CGCCAGTAGACTCCACAACA-3.

Statistics

All data are presented as mean $\pm$ SD. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was applied to compare the control group to all diabetic groups. Two-way ANOVA was further performed to compare the treated diabetic groups to each other followed by Tukey's multiple comparisons test. The level of significance was set to $p < 0.05$ (GraphPad Prism v5 (GraphPad Software, Inc., San Diego, CA, USA)).

Results

Metabolic parameters were unaffected by treatment protocols

In our study functional metabolic alterations, such as increased serum glucose level and

Table 1. Basic metabolic and renal parameters at harvest

	Plasma glucose (mg/dl)	Body weight (grams)	Daily water intake (ml/day)	Kidney weight (grams)	Relative kidney weight (mg kidney weight/g body weight)	Proteinuria (mg protein/24h/g body weight)	Protein/creatinine (mg/mg)
Control	256.2 ± 76	519.5 ± 41	18 ± 6	2.0 ± 0.2	4.0 ± 0.4	0.22 ± 0.03	6.4 ± 0.6
DM	911.1 ± 239*	304.0 ± 46*	36 ± 3*	2.6 ± 0.3*	7.8 ± 1.2*	0.99 ± 0.23*	51.3 ± 8.0*
DM+HRP	902.6 ± 101*	287.7 ± 32*	44 ± 9*	2.3 ± 0.3	8.0 ± 0.5*	1.04 ± 0.1*	54.7 ± 12.5*
DM+Q	900.7 ± 291*	265.8 ± 43*	37 ± 7*	2.0 ± 0.2#	8.0 ± 0.8*	0.75 ± 0.12*#	31.4 ± 10.3*#
DM+Q+HRP	996.3 ± 306*	251.8 ± 49*	37 ± 8*	1.9 ± 0.4#	8.1 ± 2.6*	0.79 ± 0.11*#	38.7 ± 6.5*#

Data are presented as mean ± SD, *p < 0.05 vs. Control (one-way ANOVA followed by Dunnett's multiple comparisons test). # p < 0.05 vs. DM (two-way ANOVA followed by Tukey's multiple comparisons test). Sample numbers for each group were: Control n=10, DM n=8, DM+HRP n=6, DM+Q n=10, DM+Q+HRP n=10.

daily water intake showed successful diabetes induction (Table 1). None of the treatments influenced these parameters, which means that the obtained results on kidney function were independent of metabolic state.

HRP had no effect on renal hypertrophy and proteinuria

In the present study, diabetic rats had an increased wet kidney weight as well as kidney weight/body weight ratio, representing renal hypertrophy. Neither HRP nor Quinapril treatment attenuated renal hypertrophy (Table 1). Proteinuria is an early sign of diabetic nephropathy [24]. At harvest all diabetic groups exhibited significant proteinuria (expressed as urinary protein/creatinine ratio) as compared to controls. Proteinuria was not reduced in DM+HRP group, but was significantly lower in DM+Q and DM+Q+HRP groups (Table 1).

HRP reduced glomerulosclerosis but had only a mild effect on tubular injury

The kidneys of untreated DM rats showed significant glomerular damage: a tuft adhesion to Bowman's capsule, collapse of capillary loops and mild mesangial expansion (Fig. 1a,b). Glomerular damage was significantly reduced to a similar extent by both Q and HRP, but co-treatment did not have additive effects. Tubulointerstitial damage also developed in all diabetic groups as shown by tubular dilatation, hyaline deposition, tubular atrophy and interstitial matrix expansion (Fig. 1a,c). Interestingly, HRP reduced tubular damage only by about 30%, while DM+Q and DM+Q+HRP treatments were much more effective.

We also evaluated the extent of distal tubular damage by lipocalin-2 protein expression [25]. Renal lipocalin-2 expression more than 2-fold increased in untreated diabetic rats (Fig. 1d). According to histology, HRP monotherapy reduced renal lipocalin-2 expression by 20%. On the other hand, lipocalin-2 expression returned to the control level after Q and Q+HRP administrations.

Plasma renin activity, renal renin and (P)RR expression

As compared to controls, the plasma renin activity (PRA) tended to be higher in untreated diabetic rats and in the DM+HRP group, but Q and Q+HRP markedly increased PRA (Fig. 2d). Renal renin mRNA expression analysis showed similar results (Fig. 2c). Immunofluorescent staining localized renin production mainly to the afferent arterioles, where the staining intensity was very scarce in controls (Fig. 2a), untreated DM and in DM+HRP rats, but was prominent in DM+Q and DM+Q+HRP kidneys.

The renal mRNA expression of (P)RR was markedly increased in DM rats, as compared to non-diabetic controls. Treatment with HRP slightly reduced (P)RR gene expression, while (P)RR expression was similar to that in the control group both after Q and Q+HRP administration (Fig. 2e).

Diverse effects of HRP on glomerular and tubular TGF-β1 expression

Significantly increased TGF-β1 immunostaining was observed in all diabetic groups vs. the control group both in the glomeruli and the tubulointerstitium (Fig. 3a-c). Glomerular TGF-β1 immunostaining was reduced to the same extent by all treatments. However, tubular TGF-β1 immunoreactivity was reduced only by Q and Q+HRP treatments. Evaluation of TGF-β

Fig. 1. Markers of glomerular and tubular damage. Representative photomicrographs of PAS stained kidneys (a) show normal glomerular structure in control, mild mesangial expansion, collapse of capillary loops and tuft adhesion to Bowman's capsule in DM and almost normal structure in DM+HRP, DM+Q, and DM+Q+HRP groups (400x magnification, bar scale represents 50 μ m). Glomerulosclerosis (b) and tubular injury scores (c) for each group are shown. Representative immunoblot and densitometric analysis (d) of renal lipocalin-2 (Lcn2) showed increased expression in DM, which was mildly reduced in DM+HRP group but completely repressed in DM+Q and DM+Q+HRP groups. Data are presented as mean $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test was applied to compare the control group to all diabetic groups. Two-way ANOVA was further performed to compare the treated diabetic groups to each other followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Sample numbers for each group were: Control $n = 10$, DM $n = 8$, DM+HRP $n = 6$, DM+Q $n = 10$, DM+Q+HRP $n = 10$.

mRNA expression in whole kidney homogenates revealed a two-fold higher expression in untreated DM rats as compared to controls (Fig. 3d). TGF- β 1 overexpression was markedly reduced by Q and Q+HRP treatments, and to a lesser extent by HRP monotherapy as well, in parallel to the observed histology and lipocalin-2 expression (Fig. 1c,d).

Glomerular fibronectin immunoreactivity increased by 50% in untreated DM rats, which was reduced to the control level by all treatments (Fig. 3e).

Diverse effects of HRP on renal MMP and TIMP expression

As a part of the diabetes-induced renal fibrosis, we observed a marked decrease in the renal expression of matrix metalloproteases MMP-2 and MMP-9 (Fig. 4a,b). Although HRP had no effect on renal MMP-2 expression, treatment with Q and Q+HRP restored MMP-2 expression to the control level (Fig. 4a). However, none of the treatments were able to restore MMP-9 mRNA expression (Fig. 4b). Gene expression of tissue inhibitor of metalloprotease-1 (TIMP-1) was increased by 60% in untreated diabetic rats, and this increase was inhibited by all treatments (Fig. 4c). TIMP-2 expression was not influenced by diabetes or by any of the

Fig. 2. Renin and (P)RR

expression. Representative photomicrographs (630x magnification, bar scale = 50 μ m) of immunofluorescent staining for renin (a) depict afferent arterioles (aa) adjacent to a glomerulus (g). Relative renin staining intensity was scarce in controls, DM and DM+HRP kidneys, but it was marked in DM+Q and DM+Q+HRP kidneys – summarized in (b). Renin mRNA expression (c) and plasma renin activity (d) were increased in DM+Q and DM+Q+HRP groups. Prorenin receptor mRNA analysis (e) shows significant (P)RR overexpression in DM rats, which was significantly reduced by HRP and completely repressed by Q and Q+HRP treatments. Data are presented as mean $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test was applied to compare the control group to all diabetic groups. Two-way ANOVA was further performed to compare the treated diabetic groups to each other followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Sample numbers for each group were: Control $n = 10$, DM $n = 8$, DM+HRP $n = 6$, DM+Q $n = 10$, DM+Q+HRP $n = 10$.

treatments (Fig. 4d). Diabetes also induced TIMP-3 overexpression, which was effectively inhibited only by Q (Fig. 4e) in monotherapy or in combination.

HRP failed to reduce ERK(1/2) phosphorylation

Extracellular signal regulated kinase ERK(1/2) phosphorylation was increased 3-fold in untreated diabetic rats, which was reduced in the Q and Q+HRP groups only, but was not affected by HRP (Fig. 5a,b). This observation indicates that, in contrast to Q, HRP failed to reduce ERK(1/2) phosphorylation in monotherapy.

HRP effectively reduced diabetic podocyte injury

Glomerular desmin immunostaining increased in all diabetic kidneys as compared to the non-diabetic controls (Fig. 6a,b), which was reduced by all treatments, indicating similar

Fig. 3. Immunostaining for profibrotic TGF- β_1 and fibronectin were performed in paraffin embedded kidney sections. Representative photomicrographs of TGF- β_1 stained glomeruli and tubulointerstitium (a) show no staining in control, strong glomerular TGF- β_1 expression in DM but only weak glomerular expression in DM+HRP, DM+Q and DM+Q+HRP groups (400x magnification, bar scale = 50 μ m). The significant tubulointerstitial TGF- β_1 expression in DM was reduced in DM+Q and DM+Q+HRP groups, but not in DM+HRP group. Glomerular (b) and tubulointerstitial (c) TGF- β_1 staining scores are shown for each group. Expression levels of the renal TGF- β_1 mRNA (d) show that the diabetes-induced TGF- β_1 overexpression was slightly reduced in DM+HRP but it was markedly ameliorated in DM+Q and DM+Q+HRP groups. All treatments reduced the diabetes induced fibronectin overexpression (e). Data are presented as mean $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test was applied to compare the control group to all diabetic groups. Two-way ANOVA was further performed to compare the treated diabetic groups to each other followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Sample numbers for each group were: Control $n = 10$, DM $n = 8$, DM+HRP $n = 6$, DM+Q $n = 10$, DM+Q+HRP $n = 10$.

beneficial effects of both HRP and Quinapril on podocyte injury. Interestingly, the combination of Q and HRP was additive as compared to HRP monotherapy (Fig. 6b). Diabetes reduced nephrin and podocin mRNA expression by 30% in DM rats as compared to controls. HRP did not change nephrin, but increased podocin expression. In contrast, Q and Q+HRP treatments prevented the diabetes-induced loss of both nephrin and podocin expression (Fig. 6c,d).

Fig. 4. Renal mRNA expression levels of matrix metalloproteinases MMP-2 (a), MMP-9 (b) and their inhibitors TIMP-1 (c), TIMP-2 (d) and TIMP-3 (e) are shown. Compared to controls, MMP-2 (a) was reduced in DM and DM+HRP groups, but it was normalized in DM+Q and DM+Q+HRP groups. MMP-9 (b) was reduced in all diabetic groups regardless of treatment. TIMP-1 expression (c) increased in DM kidneys, but it was normalized by all treatments. TIMP-2 expression (d) was similar in all groups. TIMP-3 expression (e) increased in DM kidneys. HRP did not affect TIMP-3, while Q and Q+HRP treatment reduced TIMP-3 expression. Data are presented as mean $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test was applied to compare the control group to all diabetic groups. Two-way ANOVA was further performed to compare the treated diabetic groups to each other followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Sample numbers for each group were: Control $n = 10$, DM $n = 8$, DM+HRP $n = 6$, DM+Q $n = 10$, DM+Q+HRP $n = 10$.

Discussion

The present study demonstrates that (P)RR blockade by HRP exerts diverse effects on glomerular and tubular pathology in the streptozotocin rat model of diabetes. While glomerular lesions were reduced by HRP as effectively as by Quinapril or by their combination, HRP failed to restore the tubulointerstitial MMP/TIMP balance, the tubular TGF- β expression or the tubular ERK1/2 phosphorylation. Furthermore, treatment with both HRP and the ACEi Quinapril had no synergistic effects on the progression of diabetic nephropathy.

The importance of prorenin has been recently postulated in the progression of chronic renal diseases. First, prorenin is a constitutively secreted protein that may reach 100-fold higher circulating levels than renin [10, 11]. Second, Ang II-independent direct profibrotic effects of prorenin have been reported [8, 26].

Fig. 5. Renal ERK(1/2) phosphorylation. Immunostaining of paraffin embedded kidney sections (a) depicted no staining in control, positive tubules in DM and in DM+HRP, but only weak tubular positivity in DM+Q and DM+Q+HRP groups (200x magnification, bar scale = 100 μ m). Densitometric analysis and a representative blot (b) shows accordingly that ERK(1/2) phosphorylation was increased in DM, repressed in DM+Q and DM+HRP+Q but it was not affected in DM+HRP group. Data are presented as mean $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test was applied to compare the control group to all diabetic groups. Two-way ANOVA was further performed to compare the treated diabetic groups to each other followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Sample numbers for each group were: Control $n = 10$, DM $n = 8$, DM+HRP $n = 6$, DM+Q $n = 10$, DM+Q+HRP $n = 10$.

Although both *in vivo* and *in vitro* studies demonstrated the efficacy of individual HRP therapy in the treatment of diabetic nephropathy [16-19], the effects of HRP on diabetic tubular lesions have not yet been investigated. Several studies have questioned the beneficial effect of (P)RR blockade, leading to controversial data. Feldt and colleagues reported that HRP failed to prevent (pro)renin signaling in rats overexpressing the human renin and angiotensin genes [20]. In another study, HRP failed to prevent hypertensive nephrosclerosis and to decrease renal Ang II levels [21]. The pathophysiological importance of prorenin has also been questioned. In the study of Mercure and colleagues, a considerable increase in circulating prorenin had no effect on cardiac fibrosis or renal glomerular injury in mice [27]. At this point, they have not measured (P)RR expression, which should have decreased due to elevated prorenin levels as described by Schefe and colleagues [28].

Diabetes is characterized by elevated (pro)renin levels [29-31] and (P)RR overexpression in podocytes [15, 32], which might exacerbate podocyte injury. Damaged podocytes show loss of nephrin and podocin but increased desmin expression [33]. Activation of (P)RR not only leads to intracellular Ang II production and concomitant podocyte injury, but also induces ERK1/2 phosphorylation [26]. However, the downstream pathophysiologic pathways remain obscure in podocytes. Both HRP and Quinapril reduced podocyte damage in our study, but their effects were not additive. In contrast, only ACE inhibition could ameliorate ERK1/2 phosphorylation. It has been reported that HRP might also exert partial agonist effect on (P)RR, apart from the receptor blockade [34]. This could explain why HRP did not influence the diabetes-induced ERK phosphorylation in our study.

During renal damage and remodeling, extracellular matrix (ECM) components accumulate driven by the overexpression of the profibrotic TGF- β [35, 36]. Mesangial cells respond to (pro)renin-(P)RR activation by ERK mediated TGF- β overexpression as well as by fibronectin production [8]. TGF- β influences ECM turnover by repressing matrix metalloproteinases (MMPs) and by inducing their tissue inhibitors (TIMPs) [37]. HRP has been reported to increase MMP-2 expression in rat mesangial cells [38]. In the present study, however, diabetic repression of MMP-2 was not inhibited by HRP. In contrast, Quinapril (alone

Fig. 6. Markers of podocyte damage. Desmin immunostaining was performed in paraffin embedded kidney sections. Representative photomicrographs of desmin stained glomeruli (a) show no staining in control, markedly positive podocytes in DM but only weak podocyte staining in DM+HRP, DM+Q and DM+Q+HRP groups (400x magnification, bar scale = 50 μ m). Glomerular desmin staining scores (b) are shown for each group. Renal expression levels of nephrin mRNA (c) show reduced expression in DM and DM+HRP as compared to controls, but it was normalized in DM+Q and DM+Q+HRP groups. Diabetes led to reduced podocin mRNA expression (c), which was normalized by all treatments. Data are presented as mean $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test was applied to compare the control group to all diabetic groups. Two-way ANOVA was further performed to compare the treated diabetic groups to each other followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Sample numbers for each group were: Control $n = 10$, DM $n = 8$, DM+HRP $n = 6$, DM+Q $n = 10$, DM+Q+HRP $n = 10$.

Diabetes led to reduced podocin mRNA expression (c), which was normalized by all treatments. Data are presented as mean $\pm$ SD. One-way ANOVA with Dunnett's multiple comparisons test was applied to compare the control group to all diabetic groups. Two-way ANOVA was further performed to compare the treated diabetic groups to each other followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Sample numbers for each group were: Control $n = 10$, DM $n = 8$, DM+HRP $n = 6$, DM+Q $n = 10$, DM+Q+HRP $n = 10$.

or in combination) significantly increased renal MMP-2 expression. Among the inhibitors of MMPs, elevated TIMP-1 levels have been reported in human diabetic nephropathy [39]. In our study, both TIMP-1 and TIMP-3 were upregulated in diabetic rats. While all treatments similarly reduced renal TIMP-1 gene expression, TIMP-3 overexpression was inhibited only by Quinapril (both in monotherapy and in combination). Thus, HRP monotherapy failed to inhibit TIMP-3 overexpression, despite its effect on TIMP-1. The lack of additive effects of HRP and ACEi in our study corroborates with previous reports. Possible add-on effects of HRP on

renal TGF- β expression have been investigated in combination with ARB [19] or with the ACEi imidapril [17]. Similarly to our observations, HRP failed to reduce tubular damage and albuminuria in transgenic rats overexpressing human renin and Ang II genes [20]. However, renin can also stimulate the (P)RR [12], and renin level is markedly increased after treatment with an ARB or ACEi. Therefore, although high (pro)renin levels can downregulate the (P)RR [28] the (P)RR likely remained sufficiently activated after treatment with Quinapril to counteract the effects of concomitant HRP administration.

Our study shows that HRP reduced diabetic tubular lesions significantly less than Quinapril. In contrast, HRP reduced proteinuria to the same extent as treatments with Q or Q+HRP. We postulate that the reduced protein reabsorption rate of injured tubules led to the observed proteinuria level in the HRP group, despite the presumably reduced glomerular protein filtration rate due to reduced podocyte damage. To guard against the idea that the lack of tubular effect by HRP is due to inappropriate dosage, we used a reportedly effective HRP dose of 0.1 mg/kg [9, 17, 31]. Interestingly, similar results were presented by Huang and colleagues even at a higher (0.2 mg/kg) dose of HRP [19]. In tubules, both (P)RR expression and the tubular injury marker lipocalin-2 have been localized mainly to collecting ducts [40, 41]. Our observation that HRP, as compared to Quinapril, did not significantly reduce tubular TGF- β expression and had only small influence on lipocalin-2 expression suggests that elevated renin with potentially higher (P)RR expression might have counteracted the effects of (pro)renin blockade in the tubules. In fact, HRP decreased (P)RR mRNA expression to a lesser extent than treatment with Q or Q+HRP.

In summary, we observed that the putative (pro)renin receptor blocker HRP exerts different effects on glomerular and tubular pathology in type-1 diabetic rats. Furthermore, the combination of HRP and the ACE inhibitor Quinapril had no further benefit on the progression of diabetic nephropathy over Quinapril monotherapy.

Conclusion

In our streptozotocin model of diabetic nephropathy, treatment with the (pro)renin receptor blocker HRP reduced podocyte damage but did not improve diabetic tubular lesions as compared to Quinapril treatment. The partial agonist effect of HRP on the (pro)renin receptor, as shown by maintained tubular ERK phosphorylation, might explain the weak tubular effects of HRP. Furthermore, the combination of HRP with Quinapril had no additive effects over ACE inhibitor monotherapy on the progression of diabetic nephropathy.

Abbreviations

ACEi: ACE inhibitor; ERK: Extracellular signal regulated kinase; ESRD: end stage renal disease; HRP: "handle" region decoy peptide; PAS: Periodic-Acid Schiff; (P)RR: (pro)renin receptor; STZ: streptozotocin.

Disclosure Statement

The authors declare no competing interest and nothing to disclose.

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