

Add-on blockade of (pro)renin receptor in imidapril-treated diabetic SHRsp

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1. ABSTRACT

To examine the involvement of (pro)renin receptor in the accelerated organ damage in streptozotocin-induced diabetic male SHRsp, the rats fed a high-salt diet were divided into 5 groups: a group treated with the vehicle, a group treated with 15 mg/kg/day of imidapril (ACEi), a group treated with 60 mg/kg/day of imidapril (High ACEi), a group treated with handle region peptide (HRP), and a group treated with both ACEi and HRP (ACEi+HRP). After 8 weeks, the arterial pressure was similar in the vehicle and HRP groups and decreased in the ACEi-treated groups. The renal angiotensin II content decreased similarly in the groups treated with ACEi and/or HRP. Urinary protein excretion also decreased in the ACEi, High ACEi, and HRP groups and significantly further decreased in the ACEi+HRP group. The heart weight of the ACEi+HRP group was significantly lower than that of any other groups, although the cardiac angiotensin II levels decreased similarly in the groups treated with ACEi and/or HRP. Thus, (pro)renin receptor contributes to the accelerated pathogenesis in the heart and kidneys of diabetic SHRsp.

2. INTRODUCTION

Diabetic patients reportedly have a high prevalence of hypertension, and the incidence of diabetic patients continues unabated; diabetes itself is associated with a risk of vascular complications, disability, and death (1). Diabetes and hypertension are commonly associated conditions, and the prevalence of hypertension among diabetic patients is reportedly higher than that among the general population (2). Hypertension further increases the already high risk of cardiovascular complications and chronic kidney diseases associated with diabetes (3).

Recent studies have shown that activated prorenin and the (pro)renin receptor can contribute to pathogenesis in the heart and kidneys of animal models with diabetes (4, 5) and genetic hypertension (6-8). *In vitro* studies have provided evidence that the binding of prorenin to the (pro)renin receptor activates the enzymatic activity of prorenin non-proteolytically and stimulates (pro)renin receptor-dependent, angiotensin II-independent intracellular signals (9, 10). Studies have also shown that the (pro)renin receptor binds to the “handle region” of

prorenin and that the handle region peptide (HRP) corresponding to the amino acid sequence of the “handle region” inhibits the binding of prorenin to the (pro)renin receptor competitively (11, 12). Since the *in vivo* long-term administration of HRP had beneficial effects on the heart and kidneys in type 1 diabetic rats (4), type 2 diabetic mice (5), spontaneously hypertensive rats (SHR) (8), and stroke-prone SHR (SHRsp) (6, 7), activated prorenin and the (pro)renin receptor seem to be involved in the pathogenesis of end-organ damage in diabetes or hypertension. However, whether the binding of prorenin to the (pro)renin receptor contributes to the development of end-organ damage in an animal model with both diabetes and hypertension has not yet been determined.

The present study was designed to examine the effects of HRP on the development of nephropathy and cardiac hypertrophy in streptozotocin-induced type 1 diabetic SHRsp. In addition, its effects were compared with the effects of angiotensin-converting enzyme (ACE) inhibitors to assess the possible contribution of (pro)renin receptor-dependent, angiotensin II-independent signals to the pathogenesis of end-organ damage.

3. METHODS

3.1. Animals

Male SHRsp (Sankyo Laboratory, Tokyo, Japan) were maintained in a temperature-controlled room, at 23°C with a 12:12-hour light-dark cycle. The rats were given free access to water and a high-salt diet of rat chow (8% NaCl, CE-2; Nihon Clea, Tokyo, Japan). At 5 weeks of age, all the rats were treated intraperitoneally (i.p.) with 10 mM of citrate buffer containing 65 mg/kg of streptozotocin (Wako Pure Chemical, Tokyo, Japan) and were divided into 5 groups: rats treated with saline as the vehicle, those treated with 15 mg/kg/day of the angiotensin-converting enzyme inhibitor imidapril (ACEi), those treated with 60 mg/kg/day of imidapril (high ACEi), those treated with 0.1 mg/kg/day of handle region peptide (HRP), and those treated with both 15 mg/kg/day of imidapril and 0.1 mg/kg/day of HRP (ACEi+HRP). The rats were then given imidapril hydrochloride at concentrations of 0.015% or 0.060% in their drinking water for 4 wks. The diabetic rats drank more than 15 mL of water every day, and thus ingested at least 15 or 60 mg/kg/day of imidapril, respectively. An osmotic minipump (model 2004 for 28-day use; Alzet, Cupertino, CA) containing saline or a decapeptide, NH₂-RILLKKMP₂SV-COOH, as the HRP, was subcutaneously implanted in each rat while the rat was under sodium pentobarbital anesthesia (50 mg/kg, i.p.). The Keio University Animal Care and Use Committee approved all the experimental protocols including the implantation of both the minipump and a radiotelemetry transmitter.

3.2. Experimental protocols

At 7 weeks of age, we implanted a telemetry transmitter probe (model TA11PA-C40; Data Sciences International) into rats while the rats were under sodium pentobarbital anesthesia (50 mg/kg, i.p.). The flexible tip of the probe was positioned immediately below the renal arteries. The transmitter was then surgically sutured into

the abdominal wall, and the incision was closed. The rats were returned to their home cages and allowed to recover for 4 days before starting any measurements. We monitored the conscious mean arterial pressure, heart rate, and activity in unrestricted and untethered animals using the Dataquest IV system (Data Sciences International), which consists of the implanted radiofrequency transmitter and a receiver placed under each cage. The output was relayed from the receiver through a consolidation matrix to a personal computer. Individual 10-s mean arterial pressures, heart rates, and activity waveforms were sampled every 5 minutes on day 21 after streptozotocin injection, and the daily average and standard deviation (SD) was then calculated. At 8 weeks of age, a 24-hour urine sample was collected using a metabolic cage, and the urinary protein excretion level was determined using a Micro TP test kit (Wako Pure Chemical, Osaka, Japan). Blood was obtained from the tail vein, and glucose was analyzed using the Accu-Chek comfort (Roche Diagnostics, Tokyo, Japan). After measuring the arterial pressure and sampling the urine and blood, the rats were decapitated and the heart and kidneys were removed.

3.3 Morphological evaluation

Parts of the heart and kidneys removed from each animal were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4); paraffin-embedded sections of the heart were stained using the Masson trichrome method, and those of the kidney were stained using the Periodic acid Schiff method.

Non-proteolytically activated prorenin in the kidneys was evaluated using immunohistochemical staining for the anti-rat “gate region” antibody, as described previously (7). Briefly, deparaffinized sections were pretreated with proteinase K, and after boiling the sections in citrate buffer with microwaves to unmask the antigenic sites, endogenous biotin was blocked with a Biotin Blocking System (X0590; DAKO Corp., Carpinteria, CA). Next, the sections were immersed in 0.3% H₂O₂ in methanol to inhibit endogenous peroxidase and then precoated with 1% non-fat milk in phosphate-buffered saline to block non-specific binding. The anti-rat “gate region” antibody was applied to the sections as the primary antibody. The sections were then incubated with a biotin-conjugated anti-rabbit IgG as the secondary antibody, and the antibody reactions were visualized using a Vectastain ABC Standard Kit (Vector Laboratories, Burlingame, CA) and an AEC Standard Kit (DAKO) according to the manufacturers’ instructions.

3.4. Measurements of angiotensin peptides

Parts of the removed cardiac ventricle and kidneys were weighed and placed in ice-cold methanol (10% wt/vol), homogenized with a chilled glass homogenizer, and centrifuged. The supernatant was then dried and reconstituted in 4 mL of 50 mM sodium phosphate buffer containing 1% albumin. Reconstituted samples were extracted using a Bond-Elut column (Analytichem, Harbor City, CA), and the eluents were evaporated to dryness and reconstituted in angiotensin peptide assay diluent. The angiotensin II content was

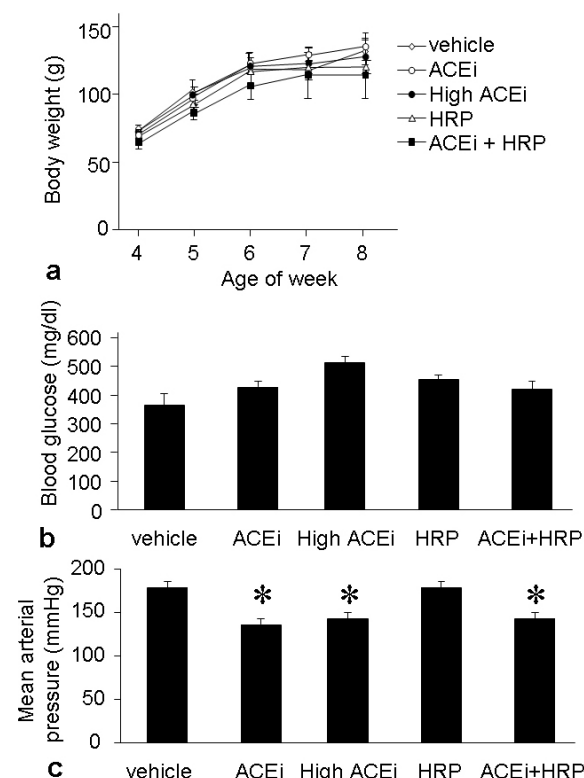


Figure 1. Changes in body weight (a) during the observation period, and the blood glucose (b) and mean arterial pressure (c) at 8 weeks of age in diabetic SHRsp treated with the vehicle (open diamond, $n=11$), 15 mg/kg/day of the angiotensin-converting enzyme inhibitor imidapril (ACEi, open circle, $n=9$), 60 mg/kg/day of imidapril (high ACEi, closed circle, $n=5$), 0.1 mg/kg/day of handle region peptide (HRP, open triangle, $n=10$), or combined therapy with ACEi and HRP (ACEi+HRP, closed square, $n=9$). * $P < 0.05$ versus rats treated with the vehicle.

quantitatively determined using a radioimmunoassay with rabbit anti-angiotensin II antiserum (Arnel, New York, NY), as previously reported (13).

3.5. Real-time Quantitative RT-PCR Analysis

We used the RNeasy Mini kit (Qiagen, Tokyo, Japan) to extract the total RNA from parts of the heart and kidneys removed from each animal and then performed real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using the TaqMan One-Step RT-PCR Master Mix Reagents kit and an ABI Prism 7700 HT Detection System (Applied Biosystems). The probes and primers used for the rat genes encoding collagen I, transforming-growth-factor beta (TGF β) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been previously described (6, 14).

3.6. Statistical Analyses

Within-group statistical comparisons were made using a one-way analysis of variance (ANOVA) for repeated measures followed by the Newman-Keuls post-

hoc test. Differences between groups were evaluated using a two-way ANOVA for repeated measures combined with the Newman-Keuls post-hoc test. $P < 0.05$ was considered significant. Data are reported as the mean \pm SD.

4. RESULTS

4.1. Body weight, blood glucose, and mean arterial pressure

As shown in Figure 1, the body weight of diabetic SHRsp treated with the vehicle, ACEi, high ACEi, HRP, or ACEi+HRP increased during the 4-week observation period, and the increases in body weight were similar among all the groups. At 8 weeks of age, the blood glucose levels of the diabetic SHRsp treated with the vehicle, ACEi, high ACEi, HRP, or ACEi+HRP averaged 350 mg/dL or more and were not statistically different among the groups. The mean arterial pressure of the 8-week-old diabetic SHRsp treated with the vehicle averaged 174 ± 6 mmHg. The 4-week treatments with ACEi, high ACEi, or ACEi+HRP significantly and similarly decreased the mean arterial pressure, but the treatment with HRP did not alter the mean arterial pressure. The mean arterial pressures of the 8-week-old diabetic SHRsp treated with ACEi, high ACEi, and ACEi+HRP averaged 134 ± 3 , 141 ± 7 , and 142 ± 5 mmHg, respectively, and were significantly lower than that of the 8-week-old diabetic SHRsp treated with the vehicle. The mean arterial pressure of the 8-week-old diabetic SHRsp treated with HRP averaged 173 ± 7 mmHg and was similar to that of the 8-week-old diabetic SHRsp treated with the vehicle.

4.2. Glomerular activated prorenin, proteinuria and renal morphology

Immunostaining for activated prorenin was enhanced in the kidneys of diabetic SHRsp, compared with that in Wyster Kyoto rats (WKY). This enhancement was unaffected by the ACEi treatment but was reduced by the HRP treatment (Figure 2).

As shown in Figure 3, the urinary protein excretion of the 8-week-old diabetic SHRsp treated with the vehicle averaged 83.2 ± 26.3 mg/day and was significantly and similarly reduced by the 4-week treatments with ACEi, high ACEi, or HRP. The urinary protein excretion levels of the diabetic SHRsp treated with ACEi, high ACEi, and HRP averaged 37.4 ± 16.4 , 38.1 ± 11.5 , and 36.9 ± 13.1 mg/day, respectively. The 4-week treatment with ACEi+HRP further decreased the urinary protein excretion level of the diabetic SHRsp. The urinary protein excretion level of the diabetic SHRsp treated with ACEi+HRP averaged 16.3 ± 4.9 mg/day and was significantly lower than those of the rats treated with the vehicle, ACEi, high ACEi, or HRP. A severe and diffuse glomerulosclerosis was observed in the kidneys of the 8-week-old diabetic SHRsp treated with the vehicle, whereas a moderate and segmental glomerulosclerosis was observed in the kidneys of the 8-week-old diabetic SHRsp treated with ACEi or HRP, and only a slight glomerulosclerosis was observed in the kidneys of the 8-week-old diabetic SHRsp treated with ACEi+HRP.

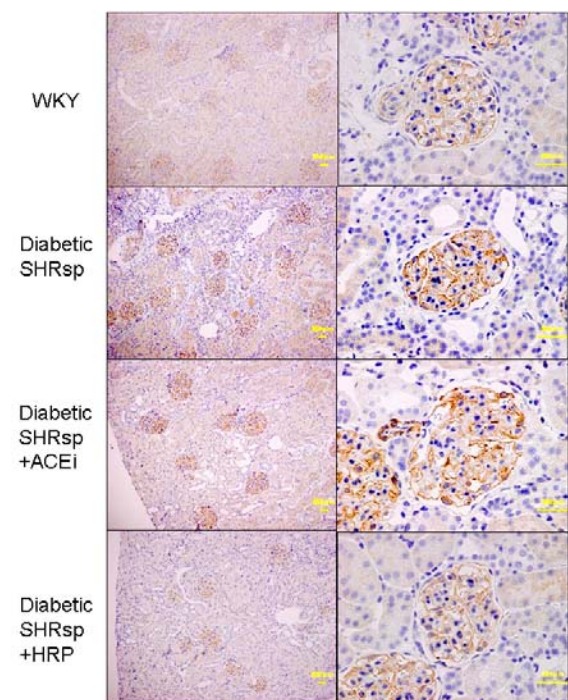


Figure 2. Activated prorenin assessed by immunostaining for anti-rat “gate region” antibody in the glomeruli of WKY rats, untreated diabetic SHRsp, diabetic SHRsp treated with 15 mg/kg/day of the angiotensin-converting enzyme inhibitor imidapril (ACEi), and diabetic SHRsp treated with 0.1 mg/kg/day of handle region peptide (HRP).

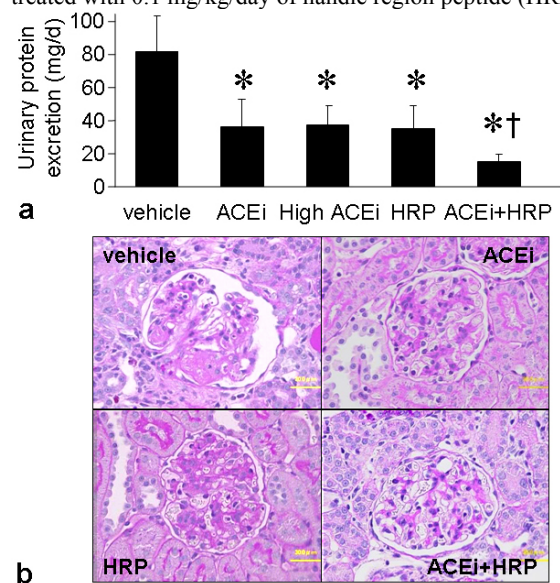


Figure 3. Urinary protein excretion (a) and representative glomerulosclerosis (b) in 8-week-old diabetic SHRsp treated with the vehicle (n=8), 15 mg/kg/day of the angiotensin-converting enzyme inhibitor imidapril (ACEi, n=10), 60 mg/kg/day of imidapril (high ACEi, n=7), 0.1 mg/kg/day of handle region peptide (HRP, n=10), or combined therapy with ACEi and HRP (ACEi+HRP, n=12). * $P < 0.05$ versus the rats treated with the vehicle. † $P < 0.05$ versus the rats treated with ACEi, high ACEi, or HRP. Scale bars: 30 μ m.

4.3. Tissue angiotensin II content and TGF β mRNA expression in the kidneys

As shown in Figure 4, the renal angiotensin II content of the 8-week-old diabetic SHRsp treated with the vehicle averaged 347 ± 75 fmol/g and was significantly and similarly reduced by the 4-week treatments with ACEi, high ACEi, HRP, or ACEi+HRP. The renal angiotensin II contents of the 8-week-old diabetic SHRsp treated with ACEi, high ACEi, HRP, and ACEi+HRP averaged 180 ± 69 , 179 ± 74 , 108 ± 96 , and 208 ± 53 fmol/g, respectively. The ratio of the renal TGF β mRNA level to that of the GAPDH mRNA level in the 8-week-old diabetic SHRsp treated with the vehicle averaged 1.00 ± 0.23 and was significantly and similarly reduced by the 4-week treatments with ACEi, high ACEi, HRP, or ACEi+HRP. The renal TGF β mRNA levels (relative to the GAPDH mRNA level) of the 8-week-old diabetic SHRsp treated with ACEi, high ACEi, HRP, and ACEi+HRP averaged 0.58 ± 0.06 , 0.48 ± 0.12 , 0.75 ± 0.13 , and 0.49 ± 0.19 , respectively.

4.4. Heart weight and cardiac fibrosis

As shown in Figure 5, the ratio of the heart weight to the body weight of the 8-week-old diabetic SHRsp treated with the vehicle averaged $0.50 \pm 0.03\%$ and was significantly reduced by the 4-week treatment with ACEi+HRP but was not influenced by the other treatments. The ratios of the heart weight to the body weight of the 8-week-old diabetic SHRsp treated with ACEi, high ACEi, HRP, and ACEi+HRP averaged $0.54 \pm 0.05\%$, $0.49 \pm 0.05\%$, $0.50 \pm 0.05\%$, and $0.42 \pm 0.05\%$, respectively. Mild cardiac fibrosis was observed in the hearts of 8-week-old diabetic SHRsp treated with the vehicle and was unaffected by the 4-week treatments with ACEi or HRP but tended to be reduced by the combined therapy with ACEi and HRP.

4.5. Tissue angiotensin II content and collagen I mRNA expression in the heart

As shown in Figure 6, the cardiac angiotensin II content of the 8-week-old diabetic SHRsp treated with the vehicle averaged 512 ± 160 fmol/g and was significantly and similarly reduced by the 4-week treatments with ACEi, high ACEi, HRP, or ACEi+HRP. The cardiac angiotensin II contents of the 8-week-old diabetic SHRsp treated with ACEi, high ACEi, HRP, and ACEi+HRP averaged 310 ± 150 , 274 ± 144 , 307 ± 18 , and 253 ± 50 fmol/g, respectively. The ratio of the cardiac collagen I mRNA level to the GAPDH mRNA level in the 8-week-old diabetic SHRsp treated with the vehicle averaged 15.11 ± 1.67 and was significantly and similarly reduced by the 4-week treatments with ACEi, high ACEi, HRP, or ACEi+HRP. The cardiac collagen I mRNA levels (relative to the GAPDH mRNA level) of the 8-week-old diabetic SHRsp treated with ACEi, high ACEi, HRP, and ACEi+HRP averaged 2.56 ± 1.25 , 5.20 ± 0.50 , 4.54 ± 1.52 , and 4.53 ± 2.32 , respectively.

5. DISCUSSION

The urinary protein excretion level and heart weight of the rats treated with ACEi+HRP were

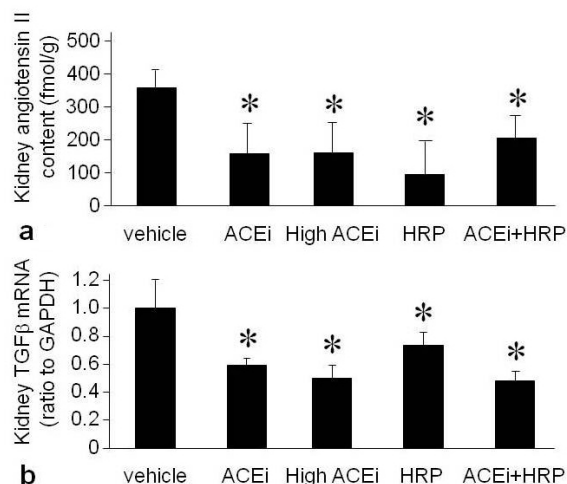


Figure 4. Kidney angiotensin II content (a) and transforming-growth-factor beta (TGFβ) mRNA expression levels (b) in the kidneys of 8-week-old diabetic SHRsp treated with the vehicle (n=4), 15 mg/kg/day of the angiotensin-converting enzyme inhibitor imidapril (ACEi, n=3), 60 mg/kg/day of imidapril (high ACEi, n=4), 0.1 mg/kg/day of handle region peptide (HRP, n=5), or combined therapy with ACEi and HRP (ACEi+HRP, n=5). * $P < 0.05$ versus the rats treated with the vehicle.

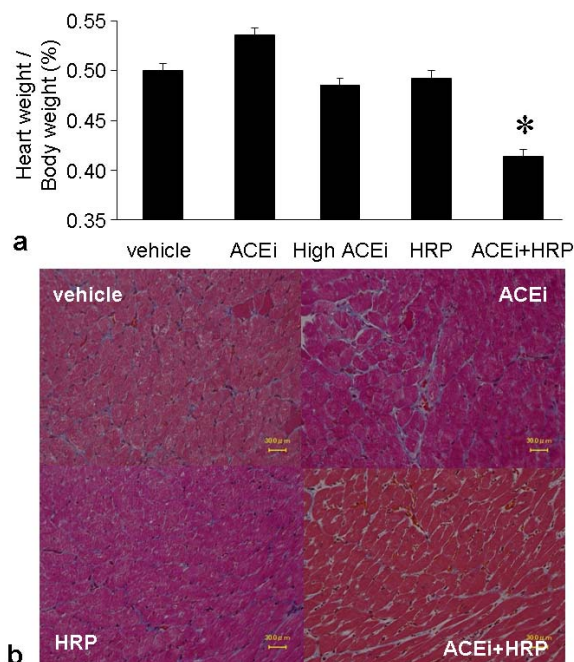


Figure 5. Ratio of heart weight to body weight (a) and representative heart fibrosis (b) in 8-week-old diabetic SHRsp treated with the vehicle (n=8), 15 mg/kg/day of the angiotensin-converting enzyme inhibitor imidapril (ACEi, n=8), 60 mg/kg/day of imidapril (high ACEi, n=11), 0.1 mg/kg/day of handle region peptide (HRP, n=8), or combined therapy with ACEi and HRP (ACEi+HRP, n=9). * $P < 0.05$ versus the rats treated with the vehicle. Scale bars: 30 μm.

significantly lower than those of the rats treated with ACEi or HRP. Since the angiotensin II contents in the heart and kidneys were similar in the rats treated with ACEi and high ACEi, the dose of ACEi used in the present study was sufficient to inhibit tissue angiotensin II synthesis. In addition, the levels of angiotensin II contents and the mRNA levels of downstream factors such as collagen I and TGFβ, which contribute to cardiac hypertrophy and nephropathy, respectively, were similar among the rats treated with ACEi, HRP, or ACEi+HRP. In addition, average heart rates of the rats treated with vehicle, ACEi, high ACEi, HRP, and ACEi+HRP were 339 ± 16 , 303 ± 15 , 340 ± 21 , 318 ± 18 , and 293 ± 25 , respectively, and similar among the groups. This result suggested that the decreased heart weight of the rats treated with ACEi+HRP was not due to its possible effects on nervous system as suggested previously (15). Thus, the further reduction in proteinuria and heart weight observed in the rats treated with ACEi+HRP likely results from an angiotensin II-independent mechanism. Treatment with ACEi significantly up-regulates the plasma levels of renin and prorenin via a negative feedback loop in the renin-angiotensin system. The increased plasma levels of renin and prorenin reportedly down-regulate the mRNA levels of the (pro)renin receptor in mesangial cells (16) and diabetic rat kidneys (17). Thus, ACEi treatment may result in a decrease in the number of (pro)renin receptors in the tissues. On the other hand, long-term treatment with HRP not only inhibited angiotensin II generation in the heart and kidneys (4, 6, 7), but also suppressed the activation of angiotensin II-independent, (pro)renin receptor-dependent mitogen-activated protein kinase (MAPK) signals in the kidneys of diabetic animals (18). Therefore, combined therapy with ACEi and HRP might have additional benefits in the heart and kidneys by reducing the numbers and activity levels of the (pro)renin receptor.

Susic *et al.* recently showed that the *in vivo* benefits of HRP were greater in hypertensive rats fed a high-salt diet than in rats fed a normal-salt diet (8). In SHR fed a high-salt diet, treatment with HRP significantly decreased the serum creatinine level, cardiac mass, and extent of cardiac fibrosis and improved cardiac function, whereas it only decreased the cardiac mass in rats fed a normal-salt diet. Similar results were observed in the present study and in hypertensive rats with high plasma aldosterone levels (19, 20). Thus, a high-salt diet was needed for the development of end-organ damage in the presence of increased plasma aldosterone levels, and eplerenone reportedly benefits rats fed a high-salt diet only (20-22), similar to the results for HRP. Since (pro)renin receptor transgenic rats have elevated plasma aldosterone levels (23), aldosterone may be involved in (pro)renin receptor-induced end-organ damage. Further studies are needed to confirm this hypothesis.

While Müller *et al.* reported that short-term treatment with HRP had no effect on the heart and kidneys of two-kidneys, one-clip (2K1C) hypertensive rats (24) or human renin- and human angiotensinogen-double transgenic rats (dTGR) (25), several investigators have shown that long-term treatment with HRP benefits the heart

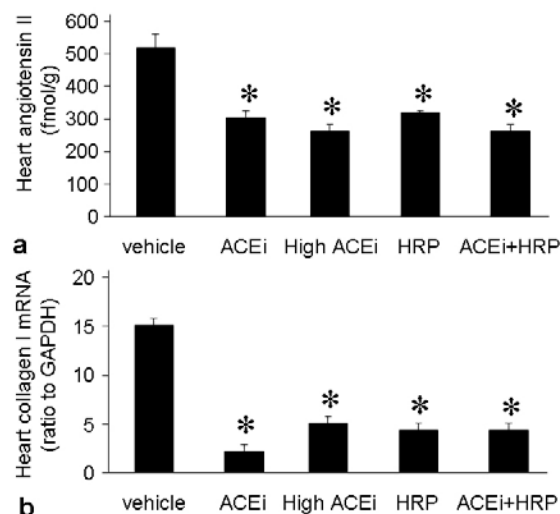


Figure 6. Angiotensin II content (a) and collagen I mRNA expression (b) in the hearts of 8-week-old diabetic SHRsp treated with the vehicle (n=5), 15 mg/kg/day of the angiotensin-converting enzyme inhibitor imidapril (ACEi, n=5), 60 mg/kg/day of imidapril (high ACEi, n=5), 0.1 mg/kg/day of handle region peptide (HRP, n=5), or combined therapy with ACEi and HRP (ACEi+HRP, n=5). * $P < 0.05$ versus the rats treated with the vehicle.

and kidneys of diabetic rats (4), SHR (8), and SHRsp (6, 7). This discrepancy in the *in vivo* effectiveness of HRP observed between Müller's group and the other groups may be explained by the unique characteristics of the (pro)renin receptor. The (pro)renin receptor can bind to both renin and prorenin (9). While renin remains an active enzyme in the presence or absence of the (pro)renin receptor, prorenin is an inactive enzyme in the absence of the (pro)renin receptor and exerts its enzymatic activity only when it binds to the (pro)renin receptor (26). In addition, renin and prorenin competitively bind to the (pro)renin receptor, and their binding affinities are similar (26), suggesting that renin is a natural competitive inhibitor of the binding of prorenin to the (pro)renin receptor. Thus, under conditions with higher plasma levels of renin than prorenin, such as the situations in 2K1C and dTGR rats, the amount of prorenin that binds to the (pro)renin receptor may be relatively small, since the (pro)renin receptor is likely to be occupied by renin; consequently, the HRP would lose its object. In the present study, however, immunostaining for activated prorenin was enhanced in the kidneys of diabetic SHRsp, and this enhanced staining pattern was inhibited by HRP, but not by ACEi (Figure 2). These results suggest that the amount of prorenin bound to the (pro)renin receptor is not negligible and may contribute to the pathogenesis that occurs in the kidneys of diabetic SHRsp. Alternatively, the intracellular shedding of the (pro)renin receptor may influence the *in vivo* and *in vitro* effectiveness of HRP. Recently, the full-length (pro)renin receptor was reportedly ligated at its hydrophilic region by furin, which is present in the trans Golgi network, thereby dividing the receptor into soluble and insoluble forms (27). Since the

soluble (pro)renin receptor includes the sites necessary for the binding of the receptor to renin and prorenin, HRP is no longer capable of inhibiting the intracellular signals of the (pro)renin receptor shed by furin. Although furin is a constitutive enzyme, the ratio of soluble (pro)renin receptor to full length (pro)renin receptor reportedly differs among cell types (27). Accordingly, the *in vivo* and *in vitro* effectiveness of HRP may depend on the activity and expression of furin in individual cells. Müller *et al.* showed that HRP has no *in vitro* effect in vascular smooth muscle cells (28) or monocyte cells (29), whereas other investigators have observed *in vitro* effects of HRP in neuronal cells (15) and mesangial cells (30). Thus, determining the ratio of soluble (pro)renin receptor to full-length (pro)renin receptor in patients and animal models with various diseases may be necessary to determine whether the (pro)renin receptor contributes to pathogenesis.

In conclusion, combined therapy with HRP and ACEi further reduced proteinuria and cardiac hypertrophy, compared with monotherapy, in diabetic SHRsp. Since the tissue angiotensin II levels were similar in the rats treated with either monotherapy or the combined therapy, angiotensin II-independent, (pro)renin receptor-dependent mechanisms may contribute to these additional benefits of combined therapy with HRP and ACEi. Thus, the (pro)renin receptor may be involved in the mechanisms attributable to the acceleration of end-organ damage that occurs in patients with diabetes associated with hypertension.

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