Nonproteolytic Activation of ProreninContributes to Development of Cardiac Fibrosis in Genetic Hypertension

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Abstract—In contrast to proteolytic activation of inactive prorenin by cleavage of the N-terminal 43 residue peptide, we found that prorenin is activated without proteolysis by binding of the prorenin receptor to the pentameric “handle region” I\textsuperscript{119}LLKK\textsuperscript{150}. We hypothesized that such activation occurs in hypertensive rats and causes cardiac renin–angiotensin system (RAS) activation and end-organ damage. To test this hypothesis, we devised methods of specifically inhibiting nonproteolytic activation by decapptide spanning the pentameric handle region peptide as a decoy. In stroke-prone spontaneously hypertensive rats (SHRsp) fed a high-salt diet, arterial pressure started to rise significantly with a marked increase in the cardiac prorenin receptor mRNA level at 8 weeks of age, and cardiac fibrosis had developed by 12 weeks of age. By immunohistochemistry using antibodies to the active site of the renin molecule, we demonstrated increased proteolytic or nonproteolytic activation of prorenin in the heart but not in plasma of SHRsp. Continuous subcutaneous administration of the handle region peptide completely inhibited the increased staining by antibodies to the active site of the renin molecule, indicating the increased nonproteolytic but not proteolytic activation of prorenin in the heart of SHRsp. Administration of the handle region peptide also inactivated tissue RAS without affecting circulating RAS or arterial pressure and significantly attenuated the development and progression of cardiac fibrosis. These results clearly demonstrate the significant role of nonproteolytically activated tissue prorenin in tissue RAS activation leading to cardiac fibrosis and significant inhibition of the cardiac damage produced by chronic infusion of the handle region peptide. (Hypertension. 2006;47:894-900.)

Key Words: angiotensin ■ antibodies ■ renin

Progressive end-organ damage of the heart is a hallmark of hypertensive diseases, which results in an irreversible morbid outcome leading to death. Direct mechanical stress by high perfusion pressure and an activated renin-angiotensin system (RAS) are considered to play decisive roles in the development and progression of hypertensive cardiac damage. However, because in many types of essential hypertension plasma RAS is not elevated and circulating RAS is even subnormal, we cannot explain the morbidity by elevation of circulating RAS alone. Alternatively, we postulated elevated tissue renin activity in critical organs, such as the heart, as a possible mechanism. We, and others, have found that a prorenin binding protein exists on the plasma membrane surface in humans\textsuperscript{1-5} and now in rat tissues.\textsuperscript{6} The present study focused on the prorenin receptor described by Nguyen et al,\textsuperscript{4} because its binding to prorenin activates the proenzyme via a conformational change without proteolytically cleaving the 43 amino acid prosegment off the main body of active (mature) renin.\textsuperscript{6} Because the prorenin receptor localizes to sensitive organs like the heart,\textsuperscript{4} we hypothesized that the prorenin receptor will sequester prorenin and activate it on the cell surfaces of critical organs susceptible to end-organ damage. The activated prorenin will generate angiotensin I and II locally, thereby exerting local actions leading to tissue damage. Because the exact in vivo mechanism of this process has not been demonstrated, we devised an intervention technique to inhibit prorenin binding to the receptor in vivo. A short pentapeptide sequence in the prosegment of prorenin was identified as the binding region to the receptor, and the peptide was infused into hypertensive rats to compete for prorenin binding to the receptor or binding antibody, thus preventing the nonproteolytic activation of prorenin by the prorenin receptor.\textsuperscript{6} It is noteworthy that such prorenin activation does not occur in blood by a nonproteolytic mechanism, because the prorenin receptor is not present in plasma.\textsuperscript{4}

We demonstrate herein that the elevated mRNA of prorenin receptor, nonproteolytically activated prorenin, elevated tissue angiotensin I and II concentrations, and marked fibrosis occur in the hypertensive heart. Furthermore, the short peptide competitive inhibitor almost completely blocked the nonpro-
teolytic activation of prorenin, resulting in the normalization of angiotensin I and II in the hearts of hypertensive animals to the levels of normotensive animals without lowering the plasma concentrations of angiotensin I and II. These results may be taken as evidence of the important roles of nonproteolytically activated prorenin in tissues showing hypertensive cardiac damage.

**Methods**

**Animals**
We maintained male stroke-prone spontaneously hypertensive rats (SHRsp) and normotensive control Wyster Kyoto rats (WKY; Charles River Labs, Yokohama, Japan) in a temperature-controlled room, 23°C, and on a 12:12-hour light-dark cycle. Rats had free access to 1% NaCl water and a normal-salt–diet rat chow (0.4% NaCl; CE-2, Nihon Clea). The Keio University Animal Care and Use Committee approved all of the experimental protocols including the implantation of both minipumps and radiotelemetry transmitters in rats at 4 weeks of age. At 4 weeks of age, we subcutaneously implanted osmotic minipumps (model 2004 for 28-day use, Alzet, containing saline or a decapetide, NH2-RLLKKMPSV-COOH, as the “handle region” of rat prorenin (HRP, 0.1 mg/kg) under sodium pentobarbital anesthesia (50 mg/kg IP) and divided the rats (100- to 150-g body weight) into 4 groups: SHRsp, SHRsp+HRP, WKY, and WKY+HRP. At 8 weeks of age, we replaced the minipump with another pump filled with the same solution, and 4 weeks later (12 weeks of age) decapitated 6 to 8 rats at 12 weeks of age to collect their blood and hearts. In our preliminary study, an osmotic minipump with an NH2-SFGR-COOH (0.1 mg/kg, n=3) or NH2-MTRISAE-COOH (0.1 mg/kg, n=3) was also implanted into the SHRsp group. However, these peptides did not inhibit the development of glomerulosclerosis or increase in angiotensin II levels in the kidneys of SHRsp.

**Telemetry Probe Implantation**
At 4 weeks of age, we implanted a telemetry transmitter probe (model TA11PA-C40, Data Sciences International) into rats under sodium pentobarbital anesthesia (50 mg/kg IP), and 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 then returned to their home cages and allowed to recover for 6 days before starting measurements. We monitored conscious mean arterial pressure, heart rate, and activity in unrestricted and untethered animals with the Dataquest IV system (Data Sciences International), which consisted 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 pressure, heart rate, and activity waveforms were sampled every 5 minutes throughout the course of the study, and daily averages and SDs were then calculated.

**Morphological and Immunohistochemical Evaluation**
Part of the heart removed from each animal was fixed in 10% formalin in phosphate buffer (pH 7.4), and paraffin-embedded sections of the heart were stained by the Masson trichrome method. For immunohistochemical staining, deparaffinized sections were immersed in 3% H2O2 in methanol to buffer with microwaving to unmask antigenic sites, and endogenous pretreated with proteinase K and after boiling the sections in citrate for immunohistochemical staining, deparaffinized sections were collected into a tube containing 30 µL of EDTA (500 mmol/L), 15 µL of enalaprilat (1 mmol/L), and 30 µL of o-phenanthroline (24.8 mg/mL) and pepstatin (0.2 mmol/L), and plasma samples were obtained by centrifugation. Plasma levels of components of the circulating RAS were determined as described previously. For the measurement of total cardiac renin, a part of the removed cardiac ventricle was weighed, placed in 5 mL of buffer containing 2.6 mmol/L EDTA, 1.6 mmol/L dimeracrol, 3.4 mmol/L 8-hydroxyquinoline sulfate, 0.2 mmol/L PMSF, and 5 mmol/L ammonium acetate; homogenized with a chilled glass homogenizer; and centrifuged. The homogenate was frozen and thawed 4 times, spun at 5000 rpm for 30 minutes at 4°C, and the supernatant was removed. Then, 500 µL of plasma obtained from nephrectomized male rats were added to an equal volume of the supernatant as a substrate for the enzymatic reaction. The renin activity was determined as described previously. Angiotensin I and II levels in the heart were determined as reported previously.

**Real-Time Quantitative RT-PCR Analysis**
We extracted total RNA from the heart removed from each animal with an Rneasy Mini kit (Qiagen) and performed a real-time quantitative RT-PCR with the TaqMan One-Step RT-PCR Master Mix Reagents kit, an ABI Prism 7700 HT Detection System (Applied Biosystems), and probes and primers for the rat genes encoding renin, angiotensinogen, angiotensin-converting enzyme (ACE), collagen I, and GAPDH, as described previously. We used the commercially available probes and primers for the rat genes encoding collagen III (Applied Biosystems) and designed the probe and primers for the rat prorenin receptor (forward, 5'-CATTCCGAGAATCTCTGTTG-3'; reverse, 5'-AAGGTTGTAGGGCTTTGGGTG-3'; and probe, 5'-FAM-AAGTCAAGGACCATCCTTGAGACGAAACAA-TAMRA-3'), based on its cDNA sequence reported in the Genbank database (Accession No. AB188298 in DNA Databank of Japan), which showed a high sequence homology with the human renin/prorenin receptor mRNA described by Nguyen et al.

**Statistical Analyses**
Within-group statistical comparisons were made by 1-way ANOVA for repeated measures followed by the Newman-Keuls post hoc test. Differences between 2 groups were evaluated by 2-way ANOVA for repeated measures combined with the Newman-Keuls post hoc test. P<0.05 was considered significant. Data are reported as mean±SEM.

**Results**

**Arterial Pressure and Prorenin Receptor mRNA Expression**
We investigated changes in arterial pressure by implanting minipumps containing an HRP or saline for 8 weeks in SHRsp and WKY, in the treatment and control groups, respectively (Figure 1). Mean arterial pressure in the SHRsp group started to increase significantly at 8 weeks of age and further increased for the following 4 weeks as compared with arterial pressure in the WKY rats, whereas HRP did not affect mean arterial pressure in either the SHRsp or the WKY group. At 8 weeks of age, when no organ damage was present in either the SHRsp or the WKY rats, cardiac prorenin receptor mRNA levels were significantly
increased in the SHRsp group as compared with the WKY group. However, HRP did not affect cardiac prorenin receptor mRNA levels in either the SHRsp or the WKY group, as shown in Figure 1. At 12 weeks of age, when significant organ damage was seen in the SHRsp group, cardiac prorenin receptor mRNA levels in this group were decreased, having fallen to levels similar to those in the WKY group.

Heart Damage
We also investigated cardiac morphology, heart weight, and fibrosis in the SHRsp, SHRsp+HRP, WKY, and WKY+HRP groups at 12 weeks of age after 8 weeks of treatment (Figure 2). The areas of fibrosis stained blue by Masson trichrome were increased in perivascular areas (Figure 2a) and the myocardium (Figure 2b) in the SHRsp group as compared with the WKY and WKY+HRP groups, whereas in the SHRsp+HRP group, fibrosis remained only slightly higher than in the control. The ventricular size (Figure 2c) and heart weight (Figure 2d) were also greater in the 12-week-old SHRsp group than in the WKY and WKY+HRP groups. HRP mitigated the increases in ventricular size and heart weight in the SHRsp group.

Circulating RAS
At 12 weeks of age, after 8 weeks of treatment with HRP or saline, plasma renin activity was significantly higher in the SHRsp group (3.6±1.0 ng/mL per hour) than in the WKY group (1.7±0.6 ng/mL per hour) and HRP had no effect in either group (Figure 3a). At this point, the plasma prorenin was significantly higher in the SHRsp group (6.7±0.4 ng/mL
per hour) than in the WKY group (3.9 ± 0.8 ng/mL per hour), and HRP had no effect in either group (Figure 3b). At 12 weeks of age, plasma angiotensin I and II levels were also higher in the SHRsp group (249 ± 74 and 169 ± 10 fmol/L, respectively) than in the WKY group (132 ± 26 and 38 ± 8 fmol/L, respectively), and HRP had no influence on these levels in either group (Figure 3c and 3d). Thus, the ratio of angiotensin II to angiotensin I in plasma appeared to be lower in the WKY group than in the SHRsp group. The plasma ACE activity may be lower in the WKY group than in the SHRsp group.

Cardiac RAS and Collagen I and III mRNA Levels
At 12 weeks of age, cardiac renin mRNA levels were similar in the SHRsp, SHRsp + HRP, WKY, and WKY + HRP groups, but the cardiac total renin content in the 12-week-old SHRsp group was significantly higher than in the 12-week-old WKY group, and HRP had no effect on total cardiac renin content (Figure 4a and 4b). Cardiac angiotensin I and II contents were significantly higher in the SHRsp group than in the similarly low levels in the SHRsp + HRP, WKY, and WKY + HRP groups (Figure 4c and 4d). At 12 weeks of age, the cardiac angiotensin I contents in the SHRsp, SHRsp + HRP, WKY, and WKY + HRP groups averaged 91 ± 3, 43 ± 6, 42 ± 8, and 44 ± 6 fmol/g, respectively, and their cardiac angiotensin II contents averaged 92 ± 3, 35 ± 4, 29 ± 6, and 31 ± 5 fmol/g, respectively. Thus, HRP completely inhibited the increases in the cardiac angiotensin I and II contents in the SHRsp group. At 12 weeks of age, the cardiac mRNA levels for angiotensinogen and ACE did not differ significantly among SHRsp, SHRsp + HRP, WKY, and WKY + HRP (Figure 4e and 4f). Cardiac collagen I and III mRNA levels were significantly higher in the SHRsp group than in the WKY group, and HRP completely inhibited the increases in cardiac collagen I and III mRNA levels in the SHRsp group, while not affecting the cardiac collagen I and III mRNA levels in the WKY group (Figure 4g and 4h).

Total and Nonproteolytically Activated Prorenin in the Heart
To estimate cardiac levels of total and nonproteolytically activated prorenin, we performed an immunohistochemical analysis of cardiac ventricles collected from rats at 12 weeks of age. There were significantly greater numbers of prorenin-positive cells stained with antibody to the prorenin prosegment in the perivascular area of the SHRsp group than in that of the WKY group. The increased prorenin immunoreactivity was unaffected by HRP (Figure 5a and 5b). The nonproteolytically activated prorenin-positive cells stained with antibody to the active site of the renin molecule were also increased in the perivascular area in the SHRsp group, but their numbers were significantly decreased by HRP. The cardiac level of nonproteolytically activated prorenin in the SHRsp + HRP group was similar to those in the WKY and WKY + HRP groups (Figure 5a and 5c). These results suggest that the SHRsp heart contains an increased level of nonproteolytically activated prorenin.

Discussion
At 8 weeks of age, when no organ damage was present in the hearts of either SHRsp or WKY rats, significant increases in arterial pressure and cardiac prorenin receptor mRNA levels were observed in the SHRsp group as compared with the WKY group. At 12 weeks of age, significant cardiac fibrosis with activated tissue RAS and high levels of activated

Figure 3. Changes in plasma renin activity, prorenin levels, and angiotensin peptides levels in the SHRsp group (n=8), SHRsp + HRP (n=6), WKY (n=6), and WKY + HRP (n=6) group. (a) Plasma renin activity. (b) Plasma prorenin concentration. (c) Plasma angiotensin I concentration. (d) Plasma angiotensin II concentration. *P<0.05 vs WKY and WKY + HRP.
prorenin was demonstrated in the SHRsp heart. Because prorenin receptor binding to prorenin activates the proenzyme via a conformational change without proteolytically cleaving the prosegment off the main body of active renin, increased expression of the prorenin receptor may contribute to cardiac end-organ damage and tissue RAS activation. In the present study, chronic subcutaneous administration of HRP completely inhibited both nonproteolytic activation of prorenin and activation of tissue RAS without affecting circulating RAS or arterial pressure. In addition, HRP significantly attenuated the development and progression of hypertensive end-organ damage, despite the activated circulating RAS and sustained high arterial pressure, a possible source of mechanical end-organ stress. These findings suggest a crucial role of nonproteolytic activation of prorenin in tissue RAS activation in hypertensives and that nonproteolytic activation of prorenin should be considered as an important target of strategies to prevent hypertensive heart damage. Because cardiac prorenin receptor mRNA levels in the SHRsp group decreased to levels similar to those in the WKY group at 12 weeks of age when significant organ damage developed in the SHRsp group only, the prorenin receptor may contribute to the development but not progression of cardiac damage in the SHRsp group.

Immunohistochemical studies of tissues with antibody to the prorenin prosegment showed a higher level of prorenin in the perivascular area of the heart in the hypertensive SHRsp than in the normotensive WKY group. This in vivo observation indicates the increased tissue prorenin levels in hypertensive animals and corroborates previous data showing that exposure to high pressure inhibits conversion of prorenin to renin in juxtaglomerular cells and subsequently increases intracellular prorenin levels. We previously presented in vitro evidence that binding of a prorenin-binding protein, such as a prorenin receptor, to the handle region of the prorenin prosegment activated prorenin without proteolytic cleavage of prorenin and obtained in vitro and in vivo evidence that HRP, used as a decoy, out-competes for handle region binding and thereby inhibits the nonproteolytic activation of prorenin. In the present study, chronic administration of the decoy peptide HRP did not alter the number of total prorenin-positive cells but significantly decreased the active renin immunoreactivity to a level similar to that in WKY rats. If the active renin immunoreactivity represents proteolytically activated renin, the HRP decoy peptide should be unable to decrease the active renin immunoreactivity, because proteolytically activated renin does not contain the prorenin prosegment, including the handle region. However, HRP significantly decreased the active renin immunoreactivity, suggesting that the active renin immunoreactivity represents nonproteolytically activated prorenin, which contains the prorenin prosegment and handle region. These results suggest that prorenin levels were elevated in the damaged hearts of hypertensive animals and that a greater amount of prorenin was nonproteolytically activated, probably via elevated expression of the prorenin receptor.

The tissue levels of angiotensin I and II were also higher in the SHRsp heart than in the WKY heart, and peptide levels were completely normalized by HRP treatment, presumably by preventing the binding of prorenin to the prorenin receptor. There were no changes in other RAS components, suggesting nonproteolytic activation of prorenin to play a key role in tissue RAS activation in hypertensive animals. In addition to the increased total renin content in the SHRsp heart, we observed a significant

Figure 4. Changes in components of the RAS, collagen I mRNA, and collagen III mRNA in the heart of the SHRsp (n=8), SHRsp+HRP (n=8), WKY (n=6), and WKY+HRP (n=6) groups. (a) Cardiac renin mRNA level. (b) Cardiac total renin content. (c) Cardiac angiotensin I level. (d) Cardiac angiotensin II level. (e) Cardiac angiotensinogen mRNA level. (f) Heart ACE (ACE) mRNA level. (g) Cardiac collagen I mRNA level. (h) Cardiac collagen III mRNA level. *P<0.05 vs WKY and WKY+HRP.
increase in prorenin receptor mRNA levels in the hearts of 8-week-old SHRsp. Therefore, it is likely that increases in both the prorenin receptor and tissue prorenin are the determining factors in enhancing nonproteolytic activation.

HRP interferes with prorenin binding to a prorenin receptor as a decoy peptide and thereby inhibits RAS activation by the nonproteolytic activation of prorenin. Chronic infusion of the decoy peptide HRP markedly lowered tissue angiotensin I and II levels of SHRsp to those of WKY, whereas HRP did not lower arterial pressure or plasma angiotensins. These results indicate that inhibition of nonproteolytic activation occurs only in tissues, that is, not in plasma. The difference can be explained by the prorenin receptor being present exclusively in tissue, whereas none is detectable in plasma. Our preliminary study showed that, as well as in the heart, HRP did not affect the total renin content in the kidneys of SHRsp, although the renin secreted from the kidneys did influence the circulating RAS. Thus, HRP significantly decreased cardiac angiotensin I and II by inhibiting the nonproteolytic activation of prorenin in the heart but did not affect plasma angiotensin I or II levels. Because HRP had no effect on proteolytically activated circulating RAS or increased arterial pressure in the SHRsp group, increased plasma renin activity but not the plasma prorenin concentration may account for the activated circulating RAS and increased arterial pressure in SHRsp.

There appears to be a difference in cardiac total renin expression between the 2 strains, SHRsp and WKY. However, no matter what the details of the underlying mechanisms may be, it is clear that the total renin content of the heart was higher in the SHRsp group than in the WKY group despite a similar level of renin mRNA in the 2 groups. Although Peters et al suggested that increased tissue renin may be because of internalization by cardiac tissue from plasma, we found higher plasma prorenin concentrations in the SHRsp group than in the WKY group and predominant immunostaining of prorenin in the perivascular area of the heart in the SHRsp group. Further studies will be needed to clarify the mechanism regulating the proteolytically activated circulating RAS and the increased cardiac total renin content in the SHRsp group.

Despite a similar level of cardiac prorenin receptors in the SHRsp and WKY rats at 12 weeks of age, the number of cardiac prorenin-positive cells was higher in the SHRsp than in the
WKY rats. In addition, the inhibition of prorenin binding to the prorenin receptor by HRP did not alter the number of cardiac prorenin-positive cells or the perivascular localization of prorenin in the heart of SHRsp. These results suggest that the majority of cardiac prorenin may exist intracellularly and is not bound to the prorenin receptor.

In conclusion, the present studies using SHRsp show that the novel nonproteolytic prorenin activation mechanism has a specific role in hypertensive end-organ damage in tissues where tissue prorenin is activated via its binding proteins, such as the prorenin receptor, rather than via traditional activation by proteolytic cleavage of the 43 amino acid prosegment.

Perspectives
Lowering of blood pressure, if possible, may be another essential strategy for the prevention of organ damage in hypertensives, as suggested by several clinical studies. However, achieving the target blood pressure recommended in guidelines is difficult for hypertensive patients; some are unable to sufficiently control their blood pressure and ultimately develop end-organ damage, despite the best efforts of their physicians. In the present study, HRP significantly attenuated hypertensive end-organ damage without reducing high arterial pressure. Thus, we propose that tissue prorenin activation by the nonproteolytic mechanism can be an important target of strategies for preventing hypertensive end-organ damage.

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