Non-proteolytic activation of prorenin: activation by (pro)renin receptor and its inhibition by a prorenin prosegment, “decoy peptide”

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1. ABSTRACT

Prorenin is the enzymatically inactive precursor of renin. Recent interest has focused on the nonproteolytic activation of prorenin by antibodies and (pro)renin receptors since markedly increased levels of circulating prorenin have been associated with both physiological and pathological changes. Prorenin has been considered to be activated in vivo proteolytically and/or non-proteolytically. It has been demonstrated in vitro the "gate" and "handle" regions in the prorenin molecule is crucial for its non-proteolytic activation by a protein-protein interaction. Prorenin was also activated by the renin/prorenin receptors. Decapeptides (10P-19P) known as “decoy” peptide and pentapeptides (11P-15P) named as “handle” region peptide, were observed to inhibit the binding of both prorenins to receptors. The “handle” region plays an important role in prorenin binding to the receptor and its enzymatic activity by non-proteolytic activation. Prorenin receptors so far revealed by animal experiments have indicated that the decoy peptide prevented diabetes nephropathy and retinopathy. It was postulated the existence of novel regulatory system that stimulated signal transduction as well as that of renin-angiotensin system inhibitors.

2. INTRODUCTION

2.1. Renin-angiotensin (RA) system

Renin is the rate-limiting enzyme for the generation of angiotensin II (Ang II) in the renin-angiotensin-aldosterone cascade. This cascade is known to regulate blood pressure and electrolyte balance, but it also plays important roles in growth, angiogenesis, reproduction, aldosterone production, and catecholamine release from nerve endings. Whereas renin of renal origin appears to be predominantly responsible for the generation of Ang II in the circulation, some of the effects mentioned above may be mediated by local renin-angiotensin systems (1-4).

2.2. Structure and function of prorenin

Prorenin is the inactive precursor of renin (EC 3.4.23.15). Prorenin has a prosegment with 43 residue extension from the N terminus of mature renin with 339–341 residues (5-8). The prosegment, which is folded in the active site cleft of renin, prevents its interaction with angiotensinogen (5, 7-10).

In certain body fluids such as that in the ovarian follicle or amniotic fluid, vitreous fluid of diabetics with proliferative retinopathy, prorenin concentrations can approach 100 times the levels found in plasma (11-14). Since the initial identification of human inactive renin by Lumbers (13) in 1971, much progress has been made in
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understanding the relation between prorenin and renin. This progress is largely the result of purification of native human renin with generation of antibodies against renin (15-18). These events made it possible to produce recombinant prorenin and renin and to provide knowledge of their complete amino acid sequences (19-22).

Evidences confirming the identity of human inactive renin as prorenin are that 1) antibodies developed against peptide sequences of the prosegment of prorenin cross-react with human inactive renin extracted from plasma, tumor, kidney, and amniotic fluid (23-26). 2) inactive renin and human recombinant prorenin share multiple biochemical similarities including molecular weight, kinetics of enzyme action on angiotensinogen.

Some investigators have recently proposed that prorenin is a useful marker of diabetic microvascular complications, Wilms' tumor (27-29) and retinopathy of prematurity (30).

Prorenin concentration has not been considered a significant parameter in the pathophysiological roles of the renin angiotensin system until recently. However, recent discovery of prorenin/renin receptor (pro)renin receptor and a series of investigations on prorenin and (pro)renin receptor (31-34) finding (35). A regulatory system, “receptor associated prorenin system” has been also proposed during those studies (32).

In this article we will review studies on (1) proteolytic and nonproteolytic 2) the three-dimensional structure of prorenin with assessment of the mechanisms by which the prosegment inactivates the enzyme; 3) pathophysiological effects of prorenin activation and clinical implication., 4) the structure and function of prorenin that is the source of investigations of the “decoy peptide.” and 5) interaction of prorenin with PRR in vitro and on the cell membrane.

2.3. Circulating prorenin

The cardiac renin levels are too high to be explained on the basis of its content in blood plasma in the heart (36, 37). Thus, circulating renin and prorenin either diffuse into the interstitial space (38) and or bind to prorenin receptors. Perfusion studies with modified version of the isolated perfused rat Langendorff heart, renin was found to diffuse slowly into the interstitial space (39).

In addition, studies in rat and porcine hearts have shown that part of cardiac renin is membrane-associated (40, 41). Moreover, isolated perfused hearts of rats expressing transgenic human angiotensinogen release Ang I during renin (but not prorenin) perfusion and this release continues after stopping the renin perfusion (42, 43). These findings support the idea that circulating renin binds to a cardiac renin-binding protein/receptor, and that bound renin is catalytically active.

The idea of renin binding is not new. In fact, evidence for renin binding was already obtained 20 years ago, when it was observed that vascular renin disappeared more slowly than circulating renin following a bilateral nephrectomy (44).

3. ACTIVATION OF PRORENNIN

3.1. Proteolytic activation of prorenin by cleavage of the prosegment

Activation of prorenin requires either proteolytic or non-proteolytic removal of this prosegment from the active site cleft. The proteolytic activation involves hydrolysis of a specific scissile peptide bond e.g., Arg<sup>43</sup>-Leu<sup>44</sup> of human prorenin, by any of the known renin-prorenin processing enzyme e.g., cathepsin (45), kallikrein (46), and prohormone convertases (47), thereby, irreversibly removing the prosegment. The proteolytic activation was considered to be a physiological activation mechanism.

Amino terminal sequencing of pure human renal renin indicates that the kidney cleaves the prosegment after two dibasic residues (Lys<sup>2</sup>-Arg<sup>3</sup>) to yield a Leu at the one position of active renin. This prosegment cleavage site is similar to those in other polypeptide hormone systems, which occur after a pair of basic amino acids, particularly Lys-Arg (48).

However, since there are 10 pairs of dibasic amino acids in prorenin, it remains to be clarified why this particular site conserved to the others is recognized. It is likely that a specific enzyme is involved in prorenin processing. Current view suggests that requirements for the renal prorenin processing enzyme include 1) activation of prorenin, 2) correct cleavage of the prosegment, and 3) lack of activity to degrade renin. Shinagawa et al. identified an enzyme in the kidney that satisfied the first three of these criteria and showed it as a thiol protease by isolating it from human renal cortical homogenates. It activated human recombinant prorenin and correctly cleaved the complete 43-amino-acid prosegment (49).

3.2. Proteolytic Activation by Various Proteases.

Cleavage of prorenin at sites other than the conventional one can also generate active renin. Heinriksen et al (50) found that cleavage of prorenin between residues -34 and -30 of the prosegment yields an active enzyme. Plasmin cleaves prorenin between Lys<sup>2</sup>-Arg<sup>3</sup> to generate active renin and trypsin cleaves prorenin at Arg<sup>1</sup>-Leu<sup>2</sup> as well as between Arg<sup>32</sup>-Leu<sup>33</sup> to generate active renin (50). Shinagawa et al purified recombinant prorenin by a procedure involving acid precipitation and found a substantial activation of prorenin by removing 32 amino acids of the prosegment (51). It is plausible that cleavage at these alternative sites could occur in vivo to activate prorenin, though there is currently no evidence that this occurs.

3.3. Nonproteolytic activation of prorenin under low pH or low temperature

The non-proteolytic activation such as treatment under acidic pH or low temperature (cryo-activation) reversibly altered the stereo structure of intact prorenin molecule into a catalytically active form (10, 52,53).
and anti-27/41 antibodies at neutral pH. Their dissociation constants were 13, 40, 8.6, 3.6, and 14 nM, respectively. The acid-activated prorenin had also been known to be re-inactivated at a neutral pH. Anti-07/10 and anti-11/26 antibodies were found to inhibit such re-inactivation by more than 90% and 50%, respectively, whereas other antibodies directed to other segments did not prevent the re-inactivation. The region of T13PFLKR10P must have an essential role for inactivation of prorenin.

Based on these observations, we have proposed that there are two key regions, "gate" (T13PFLKR10P) and "handle" (I11PFLKR15P) which play important roles in nonproteolytic activation of human prorenin (Fig 1.)

3.5. Nonproteolytic activation of prorenin by (pro)renin receptor

A renin binding protein (RnBP) and the mannose 6-phosphate/IGF2 receptor (M6P/IGF2R) have been reported as an intracellular renin inhibitor and a renin/prorenin receptor, respectively as reviewed previously (33, 34). The RnBP localized in the cytosolic fraction of cells does not contribute to plasma renin activity, nor does it play a role in blood pressure control. The M6P/IGF2R bound renin and prorenin are internalized with the receptor, but the binding per se did not activate prorenin. The M6P/IGF2R probably is a clearance receptor for renin and prorenin and other mannose-6-P containing protein (33). Recently, a novel receptor, (pro)renin receptor, was found in human mesangial cells (35). This receptor consists of 350 amino acid residues with a single transmembrane domain. (pro)renin receptor binds renin as well as prorenin to activate them. Its C-terminal region were shown to be associated with PLZF (54). (pro)renin receptor is highly homologous in human, mouse and rat, as well as in chicken, fish, xenopus and C. elegans (55). We also cloned rat (pro)renin receptor cDNA (AB188298 in DDJB) to investigate nonproteolytic activation of rat prorenin due to binding to rat (pro)renin receptor in cultured cells and on the isolated membrane fractions. The rat recombinant (pro)renin receptor was expressed in a baculovirus expression system (56). The recombinant receptor, prepared from the cytoplasmic fraction of the insect cells bound prorenin as well as renin with different binding affinities. Their $K_d$ values were estimated at around 8.0 and 20 nM, respectively. Prorenin had higher affinity to the receptor than renin. Receptor-bond rat prorenin developed renin enzyme activity. On the other hand, the enzyme activity of receptor bound renin remained more or less unchanged. The $K_m$ of their complexes was comparable at 3.3 $\mu M$ for sheep angiotensinogen used as the substrate. Their $V_{max}$ values were 1.7 and 10 nM·h$^{-1}$, respectively. These results indicate that rat prorenin as well as renin is enzymatically active in their recombinant receptor-bound states.

The rat (pro)renin receptor was also expressed on the membranes of COS-7 cells and we determined its time and dose dependence. The highest expression of (pro)renin receptor was attained 18h following transfection of the (pro)renin receptor cDNA vector (57).
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Figure 2. Time dependency for binding of rat and human prorenin to the receptors expressed on COS-7 cells. Recombinant rat and human prorenin (2.0 nM) were incubated at 37°C for 0, 6, 12, 18 and 24 h. The amount of the total prorenin and unbound prorenin were determined as the renin activity after trypsinization of the prorenin media during the incubation. The binding percentages [rat prorenin (-△-) and human prorenin (-■-)] were estimated by subtracting the percentages of receptor bound prorenin from the non-specific binding. For control, rat (-△-) and human (-■-) prorenin preparations at 2.0 nM were incubated with the cells transfected only with the expression vector lacking the receptor cDNA. Each data represents mean ± S.D. (n = 6).

Figure 3. Receptor-Associated Prorenin System (RAPS).

We observed (57) that rat, as well as human prorenin, bound to receptors of their respective species, underwent enzymic activation on the cell membrane and the binding properties. However, we noted a difference in binding properties between the two species (Figure 2).

Nguyen et al (35) cloned a functional human prorenin/renin receptor. It was found to convert inactive prorenin to an enzymatically active form by a non-enzymatic mechanism. Since prorenin activation without proteolytic removal of the prosegment requires lifting of the prosegment it may involve receptor binding to the prosegment (Figure 3). Thus, prorenin and renin have dual functional potentials as activatable enzyme precursor or active enzyme and as receptor ligand. Furthermore, these authors found that prorenin or renin binding activates the receptor as a receptor ligand. The activated receptor in the mesangial cells and vascular smooth muscle cells mediated transmission of the signal to activate a mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (ERK) 1/2. Using immunohistochemical techniques the receptor was localized in mesangial cells and distal tubular cells in the macula densa region. Ichihara et al identified podocytes as another (pro)renin receptor expressing cell type by fluorescence merge in reference to nephrin, a podocyte marker protein (58).

3.6. (Pro) renin, its receptor and handle region decoy peptide

We prepared a peptide that is capable of competing with the handle region of prorenin for binding to receptor and inhibiting the nonproteolytic activation of
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Figure 4. The amino acid sequence of the prorenin segment is given. Ten amino acids were elected the Handle Region Peptide (HRP). The constructed HRP peptide is shown.

prorenin (31). We constructed a decapeptide (NH2-RILLKKMPSV-COOH) that contains the Handle Region sequence and is called either “decoy peptide” or “handle region peptide, (HRP)” (Figure 4).

To study the effectiveness of the decoy, we generated a rabbit antibody directed against rat HRP. This antibody bound prorenin, however, the HRP sequence (NH2-RILLKKMPSV-COOH) effectively blocked all binding, showing that the decoy sequence is effective to prevent binding. We (31) then expressed the prorenin/renin receptor on COS7 cells. Recombinant prorenin was added to the cells and bound to the prorenin/renin receptor. The prorenin was then nonproteolytically activated, permitting the cleavage of angiotensinogen and Ang I leading to the generation of Ang II. However prorenin activation by the prorenin/renin receptor on COS7 cells. Recombinant prorenin was added to the system. Another prorenin peptide sequence, located outside of the handle region, did not block prorenin activation. The rabbit anti-rat HRP antibody directed against the NH2-RILLKKMPSV-COOH sequence bound to prorenin and was also able to activate prorenin. This activation was effectively blocked by HRP.

We developed diabetic rats with streptozotocin and produced four groups: controls plus saline, controls plus HRP, diabetics plus saline and diabetics plus HRP. Blood sugar values were elevated to a similar degree in the two diabetic groups. Blood pressure levels were the same in all four groups. Urinary protein excretion, glomerulosclerosis index, and collagen deposition all indicated severe nephropathy in the saline-treated diabetic group. The diabetes plus HRP group was markedly improved by the HRP treatment. Plasma renin activity was lower in the diabetic groups, compared to the nondiabetic groups. HRP did not influence plasma renin activity. Plasma prorenin was elevated in the diabetic groups. Circulating Ang II was lower in the diabetic groups compared to controls and HRP did not lower circulating Ang II further. On the other hand, kidney Ang I and Ang II were higher in the diabetic rats, compared to controls. HRP lowered Ang I and Ang II in the kidney to control values. We concluded that nonproteolytic prorenin activation in the kidney is a probable mechanism contributing to diabetic nephropathy.

We investigated endotoxin-induced uveitis. In this study, Long-Evans rats were given a single intraperitoneal injection of 100 µg lipopolysaccharide (LPS). Prorenin, activated prorenin, and the prorenin/renin receptor were studied in the eye. HRP was given intraperitoneally before LPS administration and again afterwards. Retinal vessels in rats receiving LPS and vehicle were positively stained for prorenin, prorenin receptor, and activated prorenin. In addition, leukocyte adhesion, infiltration, and capillary leakage were apparent. Adhesion molecules and cytokines were expressed. HRP treatments inhibited prorenin activation and greatly ameliorated all of the LPS-induced inflammatory effects (59).

To study the efficacy of HRP in preventing or ameliorating end organ damage in hypertension, we (60) selected the stroke-prone spontaneously hypertensive rat (SHRSP), with the normotensive the Wistar-Kyoto control rat (WKY). In this model, SHRSPs develop severe hypertension and stroke when given a high-salt diet. When salt was given, blood pressure increased similarly in SHRSP treated with or without HRP. Thus, HRP did not lower blood pressure. Plasma prorenin, renin, Ang I and Ang II were not influenced by HRP treatment. SHRSPs had higher values for all these parameters than WKY. However, HRP provided marked cardiac protection. Cardiac perivascular fibrosis and hypertrophy were attenuated by HRP treatment. Cardiac prorenin/renin receptor mRNA was markedly increased in SHRSPs. Cardiac total renin content was elevated in both SHRSP groups, compared to WKY and was not influenced by HRP. However, cardiac Ang II levels were lowered to the levels of WKY by HRP. While total positive prorenin-staining cardiac cells were elevated in both SHRSP groups, activated prorenin-positive cells were reduced in SHRSPs receiving HRP.

We concluded that whether prorenin and renin could signal via the prorenin/renin receptor and thereby cause target organ injury independent of Ang II-related effects. We (61) investigated this possibility in Ang
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Figure 5. Interferences of the binding of rat and human prorenin to their corresponding receptors on COS-7 cells with the respective synthetic pentapeptides. In Figure A, the 4.0 nM of the synthetic peptide 11P-15P (■) inhibited 2 nM of rat prorenin binding to the receptor by almost 40% of the total binding (○), whereas the 4.0 nM of the synthetic peptide 11P-15P (□) inhibited 2 nM of human prorenin binding to the receptor by almost 30% of the total binding (△) as shown in Figure B. In both the cases, the synthetic pentapeptides inhibited binding of their corresponding prorenin molecules as efficiently as their respective decoy peptides, 10P-19P (■). For controls, rat and human prorenin preparations (2.0 nM) were incubated with the cells transfected only with the expression vector lacking the receptor cDNA (■). Each data represents mean ± S.D. (n = 4).

II type 1a (AT1a) receptor gene-deficient mice. AT1a gene-deficient and wild-type mice were made diabetic with streptozotocin; appropriate controls were included. Both wild-type and AT1a gene-deficient diabetic mice developed proteinuria by week 8 and substantial glomerulosclerosis by week 16. To examine whether renal end organ damage is elicited by and Ang II receptor mediated mechanism, or prorenin receptor mediated mechanism. Mice were given ACE inhibition, HRP, or the combination of both from the beginning of the experiment. AT1a gene-deficient mice had high renal Ang I and Ang II levels because of the absence of negative feedback control of plasma renin. In wild-type diabetic mice, ACE inhibition and HRP both ameliorated proteinuria and glomerulosclerosis index, but HRP was more effective than ACE inhibitor treatment. Both ACE inhibition and HRP lowered renal Ang II levels. In AT1a gene-deficient mice, ACE inhibition afforded no benefit, while HRP was effective to the same degree as in wild-type mice. In diabetic wild-type mice, phospho-ERK staining in the kidney was markedly increased, but was diminished by both HRP and ACE inhibition, although again HRP was more effective than ACE inhibition. In diabetic AT1a gene-deficient mice, phospho-ERK staining in the kidney was again markedly increased. This increase was abolished by HRP, but not by ACE inhibition. These experiments show that diabetic mice can develop proteinuria and nephrosclerosis even when the AT1a receptor is absent, although under those circumstances they no longer benefited from an ACE inhibitor. We also showed that ERK activation can occur in diabetic AT1a gene-deficient mice. Blockade of prorenin/renin receptor activation is protective in this model, strongly suggesting that the prorenin/renin receptor can elicit signaling leading to target-organ damage. The prorenin/renin molecules thus can act independently of Ang II-related effects.

We recently (62) developed transgenic (TG) rat strains overexpression the human (pro)renin receptor to define the pathogenic effects of the (pro)renin receptor. We showed that human prorenin receptor elicits slowly progressive nephropathy by angiotensin II-independent MAPK activation in rats. This study clearly provided in vivo evidence for the angiotensin II-independent MAPK activation by human (pro)renin receptor and induction of glomerulosclerosis with increased TGF-β1 expression (62).

3.7. Interference of prorenin binding to the receptor by handle region decoy peptide

We (57) observed that binding of rat and human prorenin to the receptor were inhibited by their respective Handle Region peptides (11P-15P) as shown in Figure 5. The pentapeptide regions within the prosegment sequences of prorenin molecules termed as the “handle” peptides (64). Such inhibition was not observed by using the “gate” region (7P-10P) and 30P-36P peptides, which were also parts of the prosegment sequences of prorenin molecules. Though the binding affinity for the synthetic peptide inhibitor (11P-15P) was lower (Ki = 6.6 nM) than that of the prorenin, its capability as inhibitor was clearly determined. Thus, the present study demonstrates that the “handle” region plays an important role in the paradigm of prorenin binding to the (pro)renin receptor. The rat decoy as well as handle region peptide inhibited the rat prorenin binding to the rat (pro)renin receptor on the plasma membrane of COS-7. Their Ki values were similar in the range around 7 nM. Therefore, the rat “handle” region probably also plays a crucial role in prorenin-(pro)renin receptor binding in vivo.

4. CONCLUSION AND PERSPECTIVES

Several renin receptors or binding protein have been described (33-35). These receptors also bind prorenin, and
such binding results in prorenin activation, either proteolytically or nonproteolytically. Thus, for the first time, a physiological role for prorenin might be established.

Prorenin gains Ang-I generating activity by binding to its receptor, without undergoing proteolytic cleavage. Receptor binding activation of protein, like low pH and low temperature, induces conformational changes in the prorenin molecule leading to the unfolding of the prosegment from the enzymatic cleft. This change is reversible and receptor binding prorenin activation can be blocked by a (pro)renin receptor blocker (63).

This is important in view of earlier observations that high prorenin levels in diabetic subjects are an indication of microvascular complications. Unexpectedly, renin and prorenin binding to their receptors not only facilitated angiotensin generation but also led to activation of the (pro)renin receptor signal pathways distinct from Ang II receptor signals. These results clearly indicate that renin and prorenin have dual functions, as receptor ligands and as enzyme or its precursor.

All of the experimental data on (pro)renin receptor obtained by animal experiments have indicated that the decay peptide prevented diabetes nephropathy and retinopathy, and indicates the existence of a novel regulatory system that stimulates novel signal system in diabetes and hypertension which conventional renin-angiotensin system inhibitors which did not provide a complete solution.

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