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New perspectives on secretion of (pro)renin receptor into extracellular space

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1. ABSTRACT

(Pro)renin receptor is a new molecule of the renin-angiotensin system. The (pro)renin receptor binds both renin and prorenin leading to protease activity. Furthermore, the binding of renin/prorenin to (pro)renin receptor activates intracellular signaling. Although these studies show the classical function of the (pro)renin receptor on the plasma membrane as a receptor, subcellular distribution and extracellular secretion of (pro)renin receptor remained controversial until recently when Cousin *et al.* reported possible existence of the soluble form of (pro)renin receptor. Chinese hamster ovary (CHO) cells transfected with human (pro)renin tagged with Venus showed bands at 74kDa and 35kDa without any stimulation in Western blot analysis. Moreover, these cells secreted a 29kDa form, which was the amino-terminal fragment of the (pro)renin receptor. In immunofluorescent staining, (pro)renin receptor tagged with Venus was mainly stained on the endoplasmic reticulum and in vesicle-like structures, but not on the plasma membrane. These data suggest that the (pro)renin receptor may be cleaved in the intracellular compartments of cells and secreted into the extracellular space.

2. INTRODUCTION

(Pro)renin receptor ((P)RR) is a new molecule in renin-angiotensin system (RAS), which is a 39 kDa type I transmembrane protein consisting of a large unglycosylated amino-terminal domain, a single transmembrane, and a short cytoplasmic tail (1). (P)RR binds not only to renin but also to prorenin. The binding of renin to (P)RR leads to an increased catalytic activity by unknown mechanisms, whereas the binding of prorenin to (P)RR opens the active center covered by the prodomain resulting in enzymatic activity (2). Although little is known of the relevance between prorenin and renin bound to (P)RR, prorenin does bind to (P)RR with higher affinity than renin (3,4). Furthermore, (P)RR induces the activation of intracellular signaling, including p38 mitogen-activated protein kinase (MAPK), HSP27 cascade, phosphoinositide 3-kinase (PI3K) pathway and extracellular signal-regulated kinase (ERK) 1/2 pathway by the extracellular administration of renin or prorenin (1, 5, 6). Although these studies are based upon (P)RR on the plasma membrane as a receptor, accumulating evidence of (P)RR localization show that (P)RR is mainly localized in the intracellular compartments, but not on the plasma membrane despite the

receptor (1, 7). (P)RR is localized in the intracellular compartments in vascular smooth muscle cells (VSMC) and in HeLa-S3 cells overexpressing (P)RR (5, 8, 9). Cousin *et al.* reported the possible occurrence of the soluble form of (pro)renin receptor (10). Moreover, a 8.9 kDa protein, M8-9, associated with the vacuolar H^+-ATPase in the chromaffin cells of the adrenal medulla corresponds to carboxyl-terminal amino acid sequences of (P)RR (11). Thus, the intracellular distribution of (P)RR may contribute to its cleaving properties.

Using Chinese hamster ovary (CHO) cell lines stably expressing human (P)RR tagged with Venus at the carboxyl terminus, we evaluated the subcellular distribution of (P)RR (12).

3. BACKGROUND

RAS, a pivotal circulating system with a history of over 100 years of generating of angiotensin II (Ang II), plays a key role in the regulation of blood pressure, cardiac function, and senescence through its specific receptors under physiological and pathological conditions (13). It has been thought that there are a circulating and several tissue-located forms of RAS (13). Particularly, the tissue-located RAS may participate in the development of pathologically cardiovascular diseases such as atherosclerosis, hypertrophy, remodeling, inflammation, and fibrosis. Most evidence based medicines (EBMs) concerning RAS inhibitors such as angiotensin converting enzyme inhibitor (ACEI), Ang II receptor blocker (ARB) and direct renin inhibitor (DRI) prove that the RAS inhibitors have strong benefits for patients who have cardiovascular disease, despite a low level of plasma renin activity in most patients (14, 15). Furthermore, the research suggests that the nephrectomy induces the depletion of renin, but not Ang II (16). This evidence may support the consensus that the tissue-located RAS is the most important system for the development of cardiovascular diseases. However, there still remains an enigma about RAS. How is renin produced, besides being produced in the kidneys?

Renin belongs to the aspartyl-protease family and has a high substrate specificity, which means that renin only cleaves angiotensinogen between Leu10-Val11 leading to the conversion of Ang I (17). There are two forms: the proenzyme prorenin and the mature renin. The circulating renin is secreted from the juxtaglomerular cells in the kidneys, where processing of prorenin to renin cleaves from the prodomain by several proteases such as trypsin, kallikrein or cathepsin B, whereas prorenin is synthesized by the adrenal gland, eyes, placenta, in addition to the kidneys (1). Interestingly, despite the much higher plasma concentration of prorenin than that of renin in normal subjects, the mechanisms of activation of prorenin in the plasma has been covered (18). This aspect has suggested the hypothesis that a modification of renin activity, or a functional role of prorenin has resulted in the discovery of several renin- or prorenin-binding proteins.

4. RENIN-BINDING PROTEIN

The N-acyl-D-glucosamine 2-epimerase was discovered by several groups as an intracellular renin-binding protein (RnBP) (19-23). However, the binding of this RnBP to renin reduces renin activity resulting in decreased Ang I synthesis besides RnBP and renin do not co-localize (24). The RnBP knockout mice showed normal blood pressure and renin activity (25). This accumulating evidence seems that RnBP and renin do not participate in the tissue-located RAS.

Subsequently, the mannose 6-phosphate (M6P), an insulin-like growth factor II receptor, was identified as the second renin/prorenin-binding protein (26, 27). The M6P equally binds renin and prorenin (28). After binding, the binary then was internalized leading to the conversion from prorenin to renin by proteolysis. However, since the intracellular renin was degraded, Ang I generation was not evoked. So it appears that M6P does not participate in the tissue-located RAS.

In 2002, Nguyen *et al.* discovered the third renin-binding protein, which contains 350-amino acids with the signal peptide at the amino terminus and a single transmembrane domain in the carboxyl terminus (1). Since this renin binding protein also binds to the prodomain of prorenin resulting in renin enzymatic activity of prorenin without maturing, this molecule was named (pro)renin receptor. This evidence indicates that the renin-binding protein may strongly contribute to the tissue-located RAS as the renin supplier (29). Since direct renin inhibitor aliskiren is a competitive inhibitor against the active center of renin/prorenin, the enzymatic activity through prorenin bound to (P)RR is suppressed by aliskiren (30). In addition to the enzymatic activity of prorenin, the binding of renin/prorenin to (P)RR induces the activation of intracellular signaling (2). This signaling of (P)RR is never suppressed by aliskiren because renin/prorenin binds to (P)RR as a trigger for the activation of intracellular signaling of (P)RR, and this trigger happens prior to opening the active center of prorenin (31). On the other hand, there is a handle region peptide (HRP) as a (P)RR blocker. Although the potency of HRP still remains controversial, HRP was designed on the handle region of the prodomain of prorenin and theoretically blocked the binding of prorenin to (P)RR resulting in suppression of both enzymatic activity of prorenin and intracellular signaling of (P)RR (32). Ichihara et. al. reported that HRP may have more renoprotective effects than those of other RAS inhibitors in diabetes mellitus (DM) rat model treated with streptozotocin (STZ) (33). These results show that the intracellular signaling of (P)RR may play a major role for the development of nephropathy based upon (P)RR on the plasma membrane as a receptor. However, accumulating evidence of (P)RR localization has shown that (P)RR may be localized in the intracellular compartments, but not on the plasma membrane despite the receptor (5, 8, 9). Thus, we evaluated that the intracellular distribution of (P)RR may be an important aspect in uncovering the precise functions of (P)RR.

5. NEW CONCEPT OF (PRO)RENIN RECEPTOR

Human (P)RR tagged with Venus at the carboxyl terminus was permanently transfected into CHO cell lines

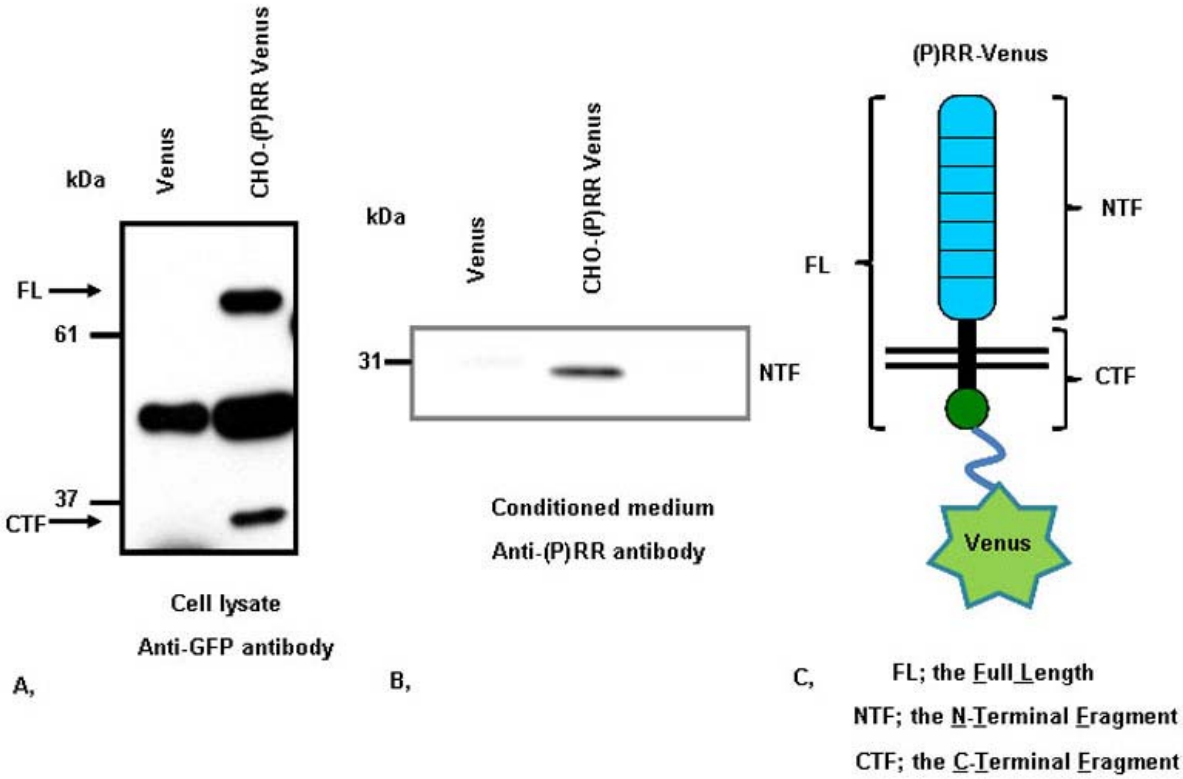

Figure 1. Cleavage of (P)RR and secretion of the NTF-(P)RR. A, CHO cell lines stably expressed Venus and (P)RR-Venus were established. Western blotting of the extracts from these cell lines was performed using anti-GFP antibody. B, After serum starvation for 24 h, the conditioned media of indicated stable CHO cell lines were collected and then concentrated. Western blotting was performed using the (P)RR antibody, is against the extracellular domain of (P)RR. C, Schema of (P)RR-Venus.

((P)RR-Venus-CHO) (Figure 1C). Venus protein is a modified green fluorescent protein (GFP) and anti-GFP antibody can recognize Venus-fused proteins. Without any stimulation, the cell lysate of (P)RR-Venus-CHO showed dual bands at 74kDa and 35kDa using anti-GFP antibody in Western blot analysis (Figure 1A, 1C) (12). This result indicates that the 74kDa band represented a full-length (P)RR tagged with Venus (FL-(P)RR-Venus), and the 35kDa band represented a carboxyl-terminal fragment of (P)RR tagged with Venus (CTF-(P)RR-Venus) containing its transmembrane region. An antibody against the extracellular domain in (P)RR, detected an amino-terminal fragment (NTF-(P)RR) at 29kDa in the conditioned media of (P)RR-Venus-CHO cells (Figure 1B, 1C). The distribution of immunofluorescent staining of (P)RR-Venus-CHO cells using anti-GFP antibody revealed combined endoplasmic reticulum (ER) characteristic pattern and Golgi characteristic pattern (Figure 2). However, (P)RR-Venus was not strongly stained on the plasma membrane. Prorenin never activated ERK 1/2 in (P)RR-Venus-CHO cells despite functional ERK 1/2. These results suggest that (P)RR-Venus may be localized in the ER or Golgi but not on the plasma membrane, and some of (P)RR-Venus may be cleaved constitutively and then secreted to the extracellular space (Figure 3). Sakoda *et al*. reported that (P)RR is localized in the intracellular compartments in VSMC and Schefe *et al*. also reported using HeLa-S3 cells overexpressing (P)RR (5, 8). Recently Cousin *et al*. reported the possible occurrence of the soluble form of (P)RR (10). (P)RR is cleaved by furin at the furin cleaving site of (P)RR. These facts strongly support that (P)RR is localized in the intracellular compartments and cleaved, and the NTF-(P)RR is secreted to the extracellular space. However, the detail mechanisms of relevance between secreted and receptor form of (P)RR postulate further investigations for future research.

If (P)RR is not localized on the plasma membrane, then there is no triggering of intracellular signaling, and hence no expected biological effects. The ratio of secreted and receptor form (P)RR may contribute to the activation of intracellular signaling, meanwhile the secreted NTF-(P)RR may participate in the activation of intracellular signaling, but its exact functions remain elucidated. Although we performed (P)RR-Venus but not endogenous (P)RR, Cousin *et al.* exhibits the soluble form of (P)RR using the endogenous (P)RR (10). The direct renin inhibitor aliskiren, in the Evaluation of Proteinuria in Diabetes (AVOID), Aliskiren Observation of Heart Failure Treatment (ALOFT) and Aliskiren in Left Ventricular Hypertrophy (ALLAY) trials, shows advantageous

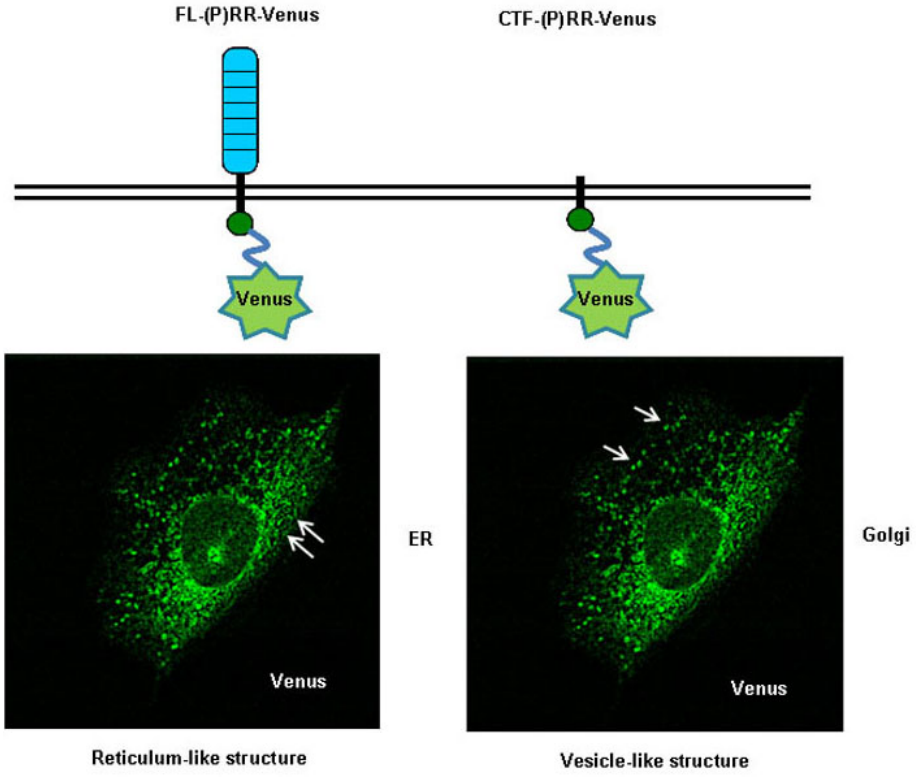

Figure 2. The immunofluorescent staining of (P)RR-Venus. (P)RR-Venus staining combined ER characteristic pattern and Golgi characteristic pattern.

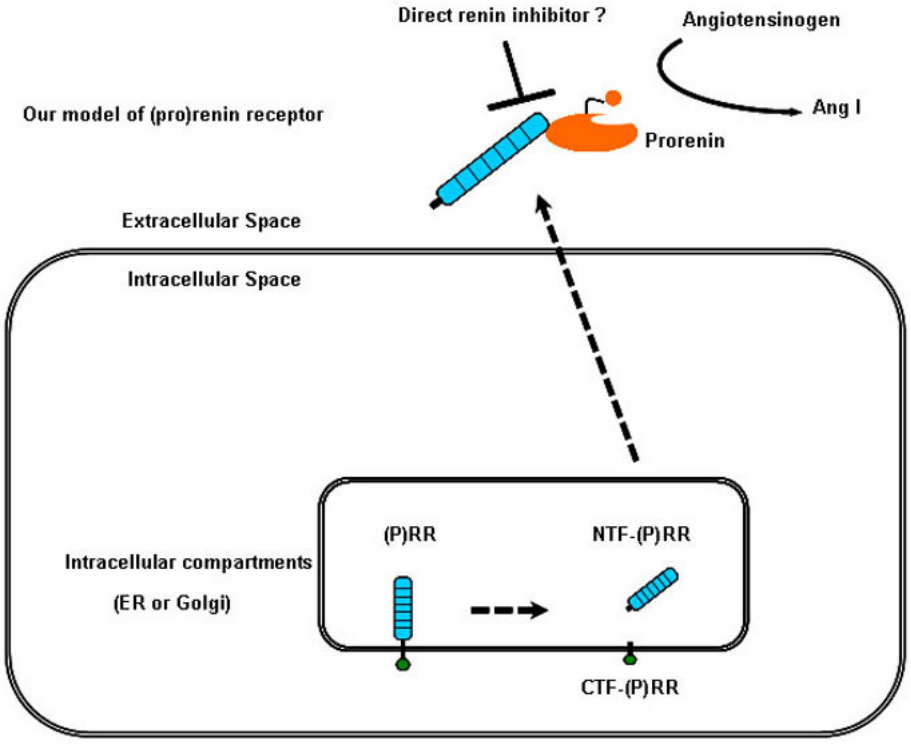

Figure 3. Our model of (P)RR behavior. Some of (P)RR may be cleaved and secreted to the extracellular space.

benefits as cardiovascular protective effects compared to those of other RAS inhibitors despite the same target of all RAS inhibitors (14, 34, 35). Aliskiren, which is the competitively direct renin inhibitor, suppresses renin activity through renin and prorenin evoked by (P)RR but not intracellular signaling by the binding of prorenin to (P)RR. The advantageous benefits of aliskiren may contribute as an interpretation in the new concept of (P)RR.

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