Hypertension Research (2011) 34, 599–605

www.nature.com/hr

ORIGINAL ARTICLE

# The (pro)renin receptor is cleaved by ADAM19 in the Golgi leading to its secretion into extracellular space

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**The (pro)renin receptor ((P)RR), which is a recently discovered molecule of the renin–angiotensin system, plays an important role in the development of cardiovascular diseases. However, the molecular properties and the subcellular distribution of (P)RR remain controversial. In this study, (P)RR-Venus in Chinese hamster ovary (CHO) cells ((P)RR-Venus-CHO) or endogenous (P)RR in human vascular smooth muscle cells (VSMC) were constitutively cleaved without any stimulation, and secretion of the amino-terminal fragment (NTF-(P)RR) into the media was determined using western blot analysis. Immunofluorescent analysis showed robust expression of (P)RR in the endoplasmic reticulum (ER) or the Golgi but not in the plasma membrane. Moreover, we identified ADAM19, which is expressed in the Golgi, as one of cleaving proteases of (P)RR. Transfected ADAM19 evoked the shedding of (P)RR, whereas transfected dominant negative ADAM19 suppressed it. Although (P)RR contains a furin cleavage site, neither the furin-deficient LoVo cells nor furin inhibitor-treated VSMC lost NTF-(P)RR in the media. The secreted NTF-(P)RR induced the renin activity of prorenin in the extracellular space. We describe that (P)RR is mainly localized in the subcellular organelles, such as the ER and Golgi, and (P)RR is cleaved by ADAM19 in the Golgi resulting in two fragments, NTF-(P)RR and CTF-(P)RR. These results may suggest that (P)RR is predominantly secreted into the extracellular space.**
*Hypertension Research* (2011) **34,** 599–605; doi:10.1038/hr.2010.284; published online 27 January 2011

**Keywords:** ADAM19; ectodomain shedding; (pro)renin receptor; renin–angiotensin system

## INTRODUCTION

The (pro)renin receptor ((P)RR) is a recently discovered molecule of the renin–angiotensin system (RAS), which plays pivotal roles in the regulation of the cardiovascular system under normal and pathological conditions. (P)RR binds both renin and prorenin, which is the precursor form of renin.[1] Although the binding of renin to (P)RR may increase its catalytic activity, the binding affinity between (P)RR and renin is lower than that of (P)RR and prorenin. These results indicate that it may be necessary to focus on the interaction between (P)RR and prorenin. Prorenin does not display protease activity in the plasma because the enzymatic cleft is covered by the prosegment.[2] However, the binding of prorenin to (P)RR evokes the renin activity without removal of its prosegment. This nonproteolytic activation of prorenin contributes to the activation of the local RAS. In addition to the enzymatic activity, renin/prorenin has been shown to provide other (P)RR-mediated effects.[3] The binding of renin/prorenin to (P)RR induces the activation of intracellular signaling, including the p38 MAP kinase-HSP27 cascade, the PI3K pathway and the ERK 1/2 pathway; these effects occur independently of angiotensin II, which is a final product of RAS.[2,4,5] Although this evidence strongly supports (P)RR localization in the plasma membrane, the subcellular localization of (P)RR remains unknown. (P)RR is found on the cell surface of adipose stromal cells and in mesangial cells that overexpress (P)RR, whereas (P)RR is expressed in the intracellular compartments in vascular smooth muscle cells (VSMC) and is overexpressed in HeLa-S3 cells.[2,5,6,7] Furthermore, Batenburg *et al.* demonstrated that (P)RR is recycled between the cell surface and the intracellular compartment in VSMC that were harvested from human (P)RR transgenic rats.[8] Recently, Cousin *et al.* reported that there are soluble and receptor forms of (P)RR; in addition, a 8.9 kDa protein, M8–9, which corresponds to the carboxyl-terminal amino acid sequences of (P)RR, associates with the vacuolar $H^+$-ATPase in the chromaffin cells of the adrenal medulla.[9,10] Thus, we evaluated the subcellular localization and molecular properties of (P)RR.

In this study, we investigated the cleavage of (P)RR, the secretion of the (P)RR fragment and its subcellular localization. The majority of the amino-terminal fragment of (P)RR is secreted into the extracellular space leading to an increase in the renin activity of prorenin.

## METHODS

### Reagents

Brefeldin A (BFA) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St Louis, MO, USA). Monensin and *N*-ethyl-maleimide (NEM) were purchased from Nacalai Tesque (Kyoto, Japan). GM6001, TAPI-1 and

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Received 9 August 2010; revised 19 November 2010; accepted 24 November 2010; published online 27 January 2011

Furin Inhibitor I (Decanoyl-RVKR-CMK) were purchased from Calbiochem (San Diego, CA, USA).

### Antibodies

The cDNA fragment of the extracellular domain of human (P)RR, (P)RRex, was amplified by PCR and subcloned into the pET-31b vector (Novagen, Madison, WI, USA). The expression vector (pET-(P)RRex-his) was transformed into the BL21 (DE3) pLysS strain of *Escherichia coli* (Stratagene, La Jolla, CA, USA). The recombinant (P)RRex was purified using TALON Polyhistidine-Tag Purification Resins (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer's protocol for denaturing conditions. Rabbits were immunized with the human (P)RRex and antisera were collected. Anti-(P)RRex antibodies (N-(P)RR) were affinity-purified using a nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK), which was blotted with the extracellular domain of human (P)RR. The following monoclonal (McAb) and polyclonal (PcAb) antibodies were used: mouse anti-green fluorescent protein (GFP) McAb (mFX73; Wako, Osaka, Japan), mouse anti-GFP McAb (GF200, Nacalai Tesque), mouse anti-V5 McAb (V5005, Nacalai Tesque), mouse anti-syntaxin 6 McAb (3D10), rabbit anti-calnexin PcAb (Stressgen Biotech, Victoria, BC, Canada), Alexa 488-conjugated goat anti-rabbit PcAb (Molecular Probes, Eugene, OR, USA), Alexa 488-conjugated goat anti-mouse PcAb (Molecular Probes), Cy3-conjugated goat anti-rabbit PcAb (Molecular Probes) and Cy3-conjugated goat anti-mouse PcAb (Molecular Probes).

### Cell culture, stable cell lines and transfection of small interfering RNAs

The human adenocarcinoma cell lines, HeLa and HeLaS3, the human embryonic kidney cell lines, HEK293 and HEK293T, and the human epithelial carcinoma cell line, A431, were obtained from Riken Cell Bank (Tsukuba, Japan) and were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U ml$^{-1}$ penicillin G, and 100 μg ml$^{-1}$ streptomycin. U937 cells were obtained from Riken Cell Bank and were cultured using RPMI medium supplemented with 10% FBS, 100 U ml$^{-1}$ penicillin G, and 100 μg ml$^{-1}$ streptomycin. Human metastatic mammary carcinoma MCF-7 cells were obtained from JCRB (Japanese Collection of Research Bioresources) Cell Bank (Osaka, Japan) and were cultured in DMEM supplemented with 10% FBS, 0.1 mM NEAA, 1 mM sodium pyruvate, 100 U ml$^{-1}$ penicillin G, and 100 μg ml$^{-1}$ streptomycin. LoVo cells and Chinese hamster ovary (CHO)-K1 cells were obtained from Riken Cell Bank, and Flp-In-CHO cells were obtained from Invitrogen (Carlsbad, CA, USA), and were cultured in HamF-12 supplemented with 10% FBS. Human umbilical vein endothelial cells were obtained from Cell Applications, Inc. (San Diego, CA, USA) and were cultured using Endothelial Cell Growth Medium (Cell Applications, Inc.) with Endothelial Cell Growth Supplement (Cell Applications Inc.). Human umbilical artery smooth muscle cells were obtained from Cell Applications Inc. and were cultured using Smooth Muscle Cell Growth Medium (Cell Applications Inc.) with Smooth Muscle Cell Growth Supplement (Cell Applications Inc.). Normal human aortic VSMC were obtained from Loza (Walkersville, MD, USA), Cells were grown in SmGM2 medium (Loza) and were used between passages 4 and 8.

The Flp-In System (Invitrogen) was used for the establishment of stably expressing cell lines. The cDNAs of human (P)RR plus Venus or that of human (P)RR alone was inserted into the pcDNA5/FRT vector and transfected with pOG44 (Flp recombinase expression vector) into Flp-In-CHO cells using Fugene 6 (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. These stably expressed cell lines were maintained in media containing 500 mg l$^{-1}$ Hygromycin B. Venus was kindly provided by A. Miyawaki (RIKEN, Saitama, Japan).[11] Venus protein is a modified GFP, and an anti-GFP antibody can recognize Venus-fused proteins.

The small interfering RNAs (siRNAs) duplexes against human (P)RR were obtained from Invitrogen (hPRR #1; ATP6AP2-HSS115475, hPRR #2; ATP6AP2-HSS115476). HEK293 cells were cultured in a 12-well plate for 24 h and were transfected with 12 pmol of siRNA duplexes using Lipofectamine RNAiMax Reagent (Invitrogen) according to the manufacturer's protocol. Cells were used 48 h after transfection.

### Immunoblotting

Cells were washed with cold phosphate-buffered saline and were solubilized in 1×SDS–PAGE sample buffer (62.5 mmol l$^{-1}$ Tris-HCl, 2% SDS, pH 6.8), RIPA buffer (50 mmol l$^{-1}$ Tris-HCl, pH 8.0, 150 mmol l$^{-1}$ NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate and 5 mmol l$^{-1}$ EDTA) containing a protease inhibitor cocktail (Nacalai Tesque) or HNE buffer (20 mmol l$^{-1}$ HEPES-NaOH, pH 7.5, 150 mmol l$^{-1}$ NaCl, 1% Triton X-100, and 5 mmol l$^{-1}$ EDTA) containing a protease inhibitor cocktail. To detect the fragment of (P)RR, cells were incubated in serum-free medium for 24 h. The medium was concentrated 20-fold using Amicon Ultra-4 (Millipore, Billerica, MA, USA). The protein determination was performed using the BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were subjected to SDS–PAGE followed by western blot analysis. The reactive bands were visualized with enhanced chemiluminescence or advance enhanced chemiluminescence (GE Healthcare) substrates.

### Immunocytochemistry

VSMCs were fixed with phosphate-buffered saline containing 2% paraformaldehyde at room temperature for 20 min and were then permeabilized with phosphate-buffered saline containing 0.05% Triton X-100 at room temperature for 5 min. They were blocked with 5% bovine serum albumin in phosphate-buffered saline for 30 min and were treated with appropriately diluted primary antibodies at 4 °C overnight; they were then washed and incubated with Alexa 488-conjugated or Cy3-conjugated secondary antibodies for 1 h at room temperature. For confocal microscopic analysis, fluorescence imaging was performed using a TE300/Radiance2000 confocal microscope (Bio-Rad Laboratories, Hercules, CA, USA).

### Assay of renin activity

Renin activity was measured using modified SensoLyte 520 Renin Assay Kit protocol (Anaspec, San Jose, CA, USA). This is an assay using a 5-FAM/QXL 520 fluorescence resonance energy transfer peptide as the renin substrate. In this peptide, the fluorescence of 5-FAM is quenched by QXL 520. Upon cleavage into two fragments by renin, the fluorescence of 5-FAM is recovered. After concentration of the conditioned medium using Amicon Ultra-4 (Millipore), recombinant human prorenin (17.7 nmol l$^{-1}$; Cayman Chemical, Ann Arbor, MI) and the renin substrate peptide (3.3 μmol l$^{-1}$) were added. During incubation at 37 °C for 30 min, fluorescence signals were measured at excitation/emission wavelengths of 490/520 nm using a Flex Station imaging plate reader (Molecular Devices, Sunnyvale, CA, USA). The data showed relative fluorescent units per second (RFU per sec).

### Statistical analysis

All values are expressed as mean ± s.e.m. Statistical significance was determined by a one-way analysis of variance. If a significant difference was found, the group means were compared using the Dunnett's multiple comparison of means test.

## RESULTS

### The cleavage of (P)RR

Stable CHO cell lines, which express either human (P)RR tagged with Venus at the carboxyl-terminus, (P)RR-Venus-CHO or untagged human (P)RR, (P)RR-CHO, were established. Western blot analysis using the anti-GFP antibody showed two bands at 74 and 35 kDa in the cell lysate of (P)RR-Venus-CHO cells without any stimulation; these bands were detected in the pellet of the nondetergent homogenate of the cell lysate, but not in the supernatant fraction. In contrast, these bands were detected in the supernatant fraction of the detergent (RIPA (+) buffer) homogenate of the cell lysate, but not in the pellet (Figure 1a). This result indicated that both bands contain hydrophobic regions (Figure 1b). Taken together, the 74 kDa band represented full-length (P)RR tagged with Venus (FL-(P)RR-Venus), whereas the 35 kDa band represented a carboxyl-terminal fragment of (P)RR tagged with Venus (CTF-(P)RR-Venus) including its transmembrane region and its cytoplasmic domain (Figure 1b).

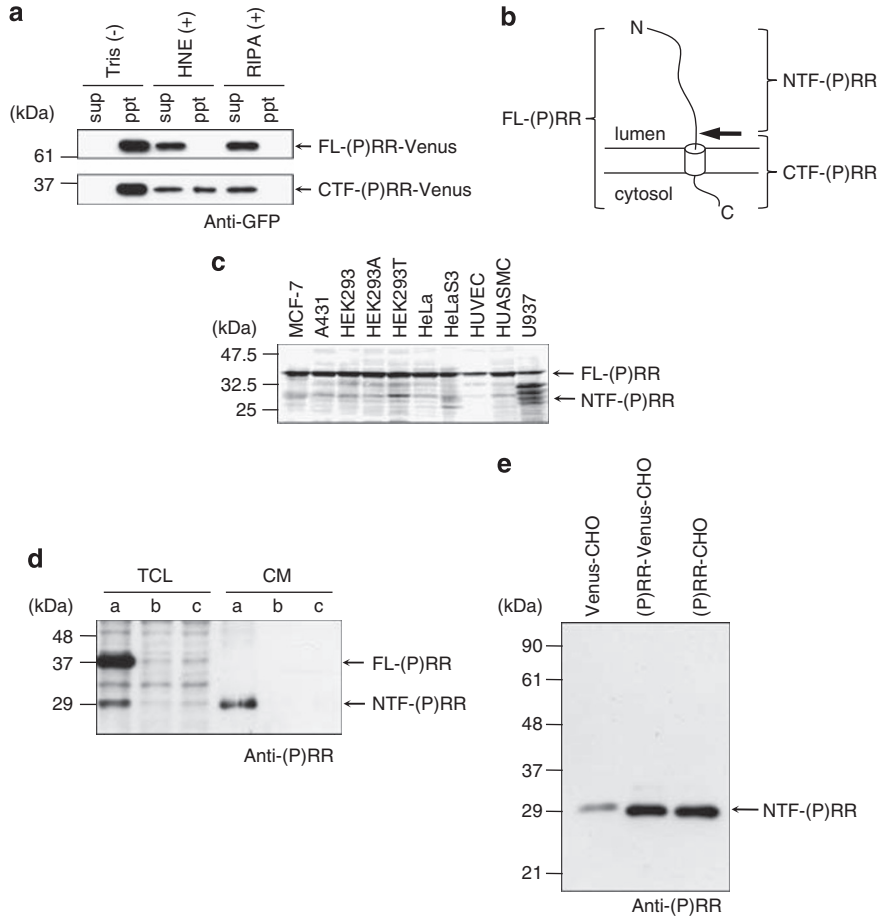


**Figure 1** Cleavage of (P)RR and secretion of NTF-(P)RR. (**a**) (P)RR-Venus-CHO cells were harvested and disrupted in Tris (−) buffer containing nondetergent, HNE (+) buffer containing 1% Triton X-100, or RIPA (+) buffer containing 1% NP-40, 0.1% SDS and 0.5% DOC. Each extract was centrifuged at 100 000×*g* for 1 h to separate the supernatant fraction (sup) from the pellet fraction (ppt). (**b**) The cleavage site of (P)RR is located in its juxtamembrane region (arrow). (P)RR, (pro)renin receptor; FL-(P)RR, full-length (P)RR; NTF-(P)RR, amino-terminal fragment of (P)RR; CTF-(P)RR, carboxyl-terminal fragment of (P)RR. (**c**) Total cell lysates (100 μg) of human cells were immunoblotted using the ProX antibody. (P)RR was endogenously expressed by all of the cell types examined. MCF-7, human breast adenocarcinoma; A431, human epithelial carcinoma; HEK293/HEK293A/HEK293T, human embryonic kidney; HeLa/HeLaS3 human cervical carcinoma; HUVECs, human umbilical vein endothelial cells; HUASMCs, human umbilical artery smooth muscle cells; U937, human leukemic monocyte lymphoma. (**d**) HEK293 cells were transfected with a negative control or a human (P)RR siRNA duplex for 2 days. Total cell lysate (TCL) (5 μg proteins) and the conditioned medium (CM) were immunoblotted using the N2-(P)RR antibody. a: A siRNA duplex of negative control, b: a siRNA duplex of human (P)RR (hPRR#1), c: a siRNA duplex of human (P)RR (hPRR#2). (**e**) After serum starvation for 24 h, the conditioned media from the indicated stable CHO cell lines were collected and concentrated. Western blotting was performed using the N2-(P)RR antibody.

To investigate whether (P)RR truncated-forms are produced endogenously, an antibody against the extracellular domain of (P)RR, the N-(P)RR antibody, was generated. For the reliability of the N-(P)RR antibody in the detection of endogenous (P)RR, (P)RR was detected at 39 and 29 kDa in MCF-7, A431, HEK293, HEK293T, human umbilical artery smooth muscle cells, HeLa, HeLaS3, human umbilical vein endothelial cells, and U937 cells using western blot analysis with the N-(P)RR antibody (Figure 1c). These bands at 39 and 29 kDa indicated endogenous FL-(P)RR and an amino-terminal fragment of endogenous (P)RR (NTF-(P)RR), respectively (Figure 1b). To confirm the reliability of the N-(P)RR antibody, western blot analysis revealed two bands at 39 and 29 kDa in the lysate of HEK293 cells, and these bands were diminished by siRNA against human (P)RR (Figure 1d). The endogenous NTF-(P)RR was also detected in the conditioned media of HEK293 cells (Figure 1d). In VSMC, the same results were obtained (data not shown). The N-(P)RR antibody was extremely reliable and sufficiently credible for the detection of the (P)RR protein.

In the conditioned medium of Venus-CHO cells, NTF-(P)RR at 29 kDa was also detected by the N-(P)RR antibody (Figure 1e). The overexpression of (P)RR caused an increase in the levels of NTF-(P)RR in the medium (Figure 1e). These results indicated that (P)RR was cleaved constitutively and the majority of the extracellular domain of (P)RR was released into the extracellular space.

### The subcellular localization of (P)RR

In VSMC, the distribution of strong staining with the N-(P)RR antibody was colocalized with GM130, which is a Golgi marker protein (Figure 2a). Moreover, the distribution of moderate staining was colocalized with PDI, which is an endoplasmic reticulum (ER) marker protein (Figure 2a). However, the endogenous (P)RR in VSMC did not show strong staining in the plasma membrane. In the (P)RR-Venus-CHO cells, the distribution of immunofluorescent staining with the anti-GFP antibody was similar to that of the VSMC (data not shown).

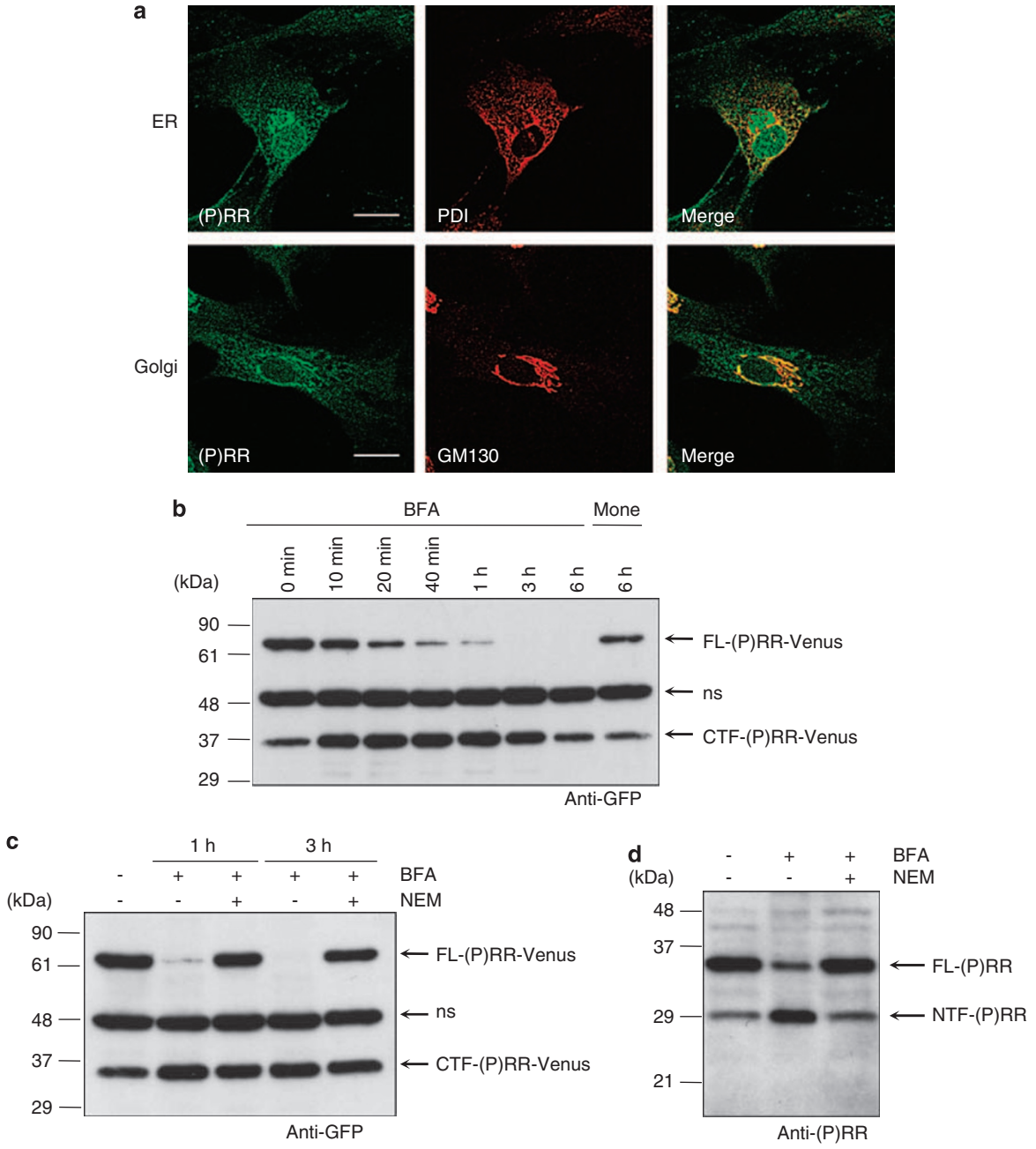


**Figure 2** Subcellular localization of (P)RR in VSMC and the effect of the treatment with BFA. (**a**) Double immunofluorescence staining was performed using antibodies against (P)RR(N2-(P)RR) and PDI (upper panels), or GM130 (lower panels). Intracellular localizations were analyzed by confocal fluorescence microscopy. Scale bar, 20 μm. (**b**) (P)RR-Venus-CHO cells were stimulated with BFA (10 mg l$^{-1}$) or monensin (Mone) (4 μmol l$^{-1}$) for the indicated period of time. After stimulation, western blotting was performed using anti-GFP antibody. (**c**) (P)RR-Venus-CHO cells were stimulated with BFA (10 mg l$^{-1}$) or BFA and NEM (200 μmol l$^{-1}$) for 1 or 3 h. After stimulation, western blotting was performed using anti-GFP antibody. (**d**) HEK293 cells were stimulated with BFA (10 mg l$^{-1}$) or BFA and NEM (200 μmol l$^{-1}$) for 3 h. After stimulation, western blotting was performed using the N2-(P)RR antibody. NS, nonspecific protein band in Figure 2b, c.

To investigate the cellular location of (P)RR cleavage, BFA was used. After treatment with BFA for 3 h, the FL-(P)RR-Venus in the cell lysate was nearly abolished, and the CTF-(P)RR-Venus was increased (Figure 2b). In contrast, treatment with monensin, which blocks vesicular transport at the Golgi, had no effect on both the amount of FL-(P)RR-Venus and CTF-(P)RR-Venus (Figure 2b). Treatment with NEM in addition to BFA reversed the BFA-mediated abolishment of FL-(P)RR-Venus levels (Figure 2c). In HEK293 cells, the treatment with BFA significantly decreased the levels of endogenous FL-(P)RR and increased the levels of NTF-(P)RR; treatment with NEM in addition to BFA reversed the BFA effects (Figure 2d).

### The effect of proteases on the cleavage of (P)RR

We investigated the effect of various protease inhibitors on the levels of NTF-(P)RR. The tumor necrosis factor α-converting enzyme inhibitor TAPI-1, the MMP inhibitor GM6001 and the furin inhibitor did not

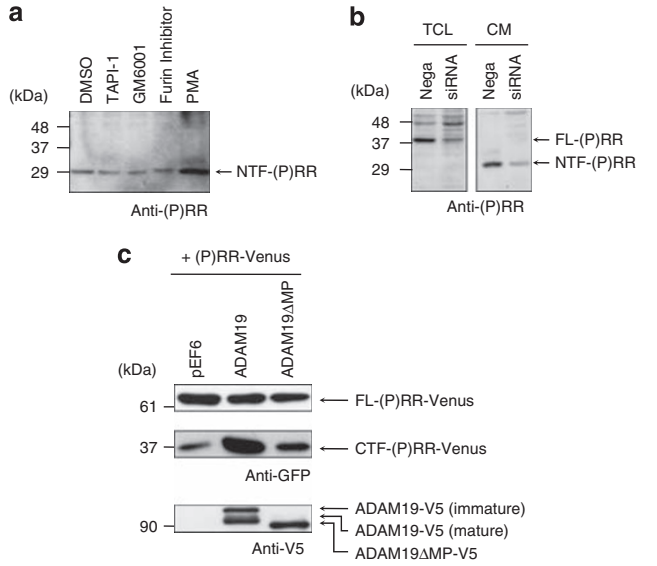


**Figure 3** Proteases to mediate the cleavage of (P)RR. (**a**) VSMC were incubated for 24 h in the presence of tumor necrosis factor α-converting enzyme (TACE, ADAM17) inhibitor TAPI-1 (20 μmol l$^{-1}$), matrix metalloprotease inhibitor GM6001 (25 μmol l$^{-1}$), furin Inhibitor (50 μmol l$^{-1}$), or PMA (30 nmol l$^{-1}$). The conditioned media were collected and concentrated by ultrafiltration. Western blotting was performed using the N2-(P)RR antibody. (**b**) LoVo cells were transfected with a negative control (Nega) or a human (P)RR siRNA duplex (hPRR#2) for 2 days. Total cell lysate (TCL) (10 μg proteins) and the conditioned medium (CM) were immunoblotted using the N2-(P)RR antibody. (**c**) CHO cells were transiently transfected with (P)RR-Venus and V5-tagged ADAM19 or mutated ADAM19 (ADAM19ΔMP) and were collected after 72 h. Western blotting was performed using the anti-GFP antibody ((P)RR) and the anti-V5 antibody (ADAM19).

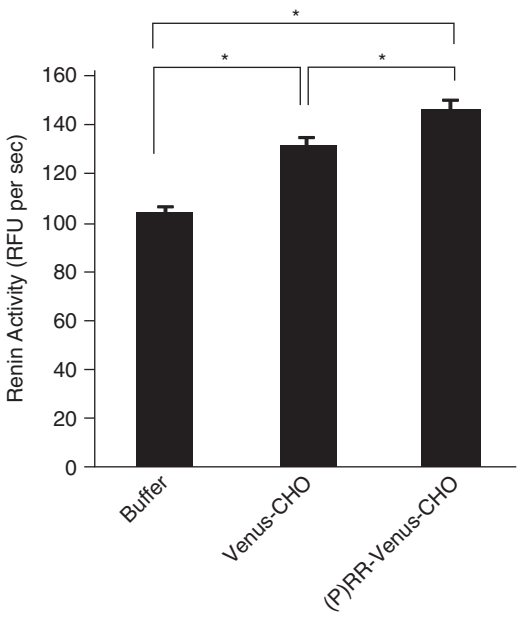


**Figure 4** The NTF-(P)RR induced the renin activity of prorenin. After serum starvation for 24 h, the conditioned media of the indicated cell lines were collected and concentrated. Using these concentrated media, the renin activity of prorenin was measured. Bars show the means ± s.e.m. *$P<0.01$ ($n=3$).

suppress the production of the NTF-(P)RR in VSMC (Figure 3a). The release of a wide variety of membrane proteins from the cell surface has been shown to be stimulated by phorbol esters, such as PMA, through the activation of protein kinase C. Interestingly, treatment with PMA considerably increased the amount of the NTF-(P)RR in the medium of VSMC (Figure 3a). Although (P)RR possesses a putative furin cleavage site in its juxtamembrane region, the furin inhibitor did not suppress the production of the NTF-(P)RR in VSMC (Figure 3a). To confirm this finding, we performed western blot analysis using the LoVo cells, which express (P)RR without functional furin.[12,13] Using the N-(P)RR antibody, LoVo cell lysates showed FL-(P)RR at 39 kDa, and conditioned medium from the LoVo cells showed NTF-(P)RR at 29 kDa. Both bands were significantly suppressed by transfection with siRNA against (P)RR (Figure 3b).

In CHO cells that were dual-transfected with (P)RR-Venus and ADAM19-V5, the ratio of the CTF-(P)RR considerably increased because of its association with matured ADAM19, which is an active form of ADAM19; however, an increase in the ratio of the CTF-(P)RR was not observed in CHO cells that were dual-transfected with (P)RR-Venus and ADAM19ΔMP, in which the metalloprotease domain is deleted (Figure 3c).

### The effect of the NTF-(P)RR on renin activity

The generation of angiotensin I by renin is increased by its binding to the extracellular domain of (P)RR, which contains 292 amino acids.[12] The cleavage site of (P)RR was located near to the transmembrane domain, and the NTF-(P)RR contained a large part of the extracellular domain (Figure 1b). The NTF-(P)RR was abundantly found in the medium of (P)RR-Venus-CHO cells. To validate whether the NTF-(P)RR increased renin activity, we performed a renin activity assay to detect the renin activity of prorenin using the medium from (P)RR-Venus-CHO cells. The concentrated medium from (P)RR-Venus-CHO cells significantly induced renin activity (146.8 ± 3.7 RFU per sec), whereas the renin activity detected in the concentrated medium from Venus-CHO cells was 132.4 ± 2.7 RFU per sec; the renin activity detected in a negative control buffer was 104.8 ± 1.9 RFU per sec (Figure 4).

## DISCUSSION

This study demonstrates the following: (1) The (P)RR is mainly located in the subcellular organelles, such as the ER and Golgi but not in the plasma membrane, (2) (P)RR is cleaved to the NTF-(P)RR and CTF-(P)RR by ADAM19 in the Golgi, and (3) The NTF-(P)RR induces the renin activity of prorenin (Figure 5).

### The secretion of the NTF-(P)RR

The NTF-(P)RR was secreted into the culture media of HEK293 cells and VSMC without any stimulation. Generally, secretion has two distinct pathways: the regulated secretory pathway and the constitutive secretory pathway.[14] In regulated secretion, secretory molecules persist for many hours in vesicles near the plasma membrane. Stimulations induce fusion of the vesicle to the plasma membrane, which results in exocytosis. In contrast, in constitutive secretion, secreted proteins are transported from the Golgi to the cell surface over a short period of time (< 10 min) without storage in post-Golgi vesicles. The constitutive secretory pathway is a more rapid process than the regulated pathway. In our study, the exogenous NTF-(P)RR was released into the medium of transfected CHO cells, and the endogenous NTF-

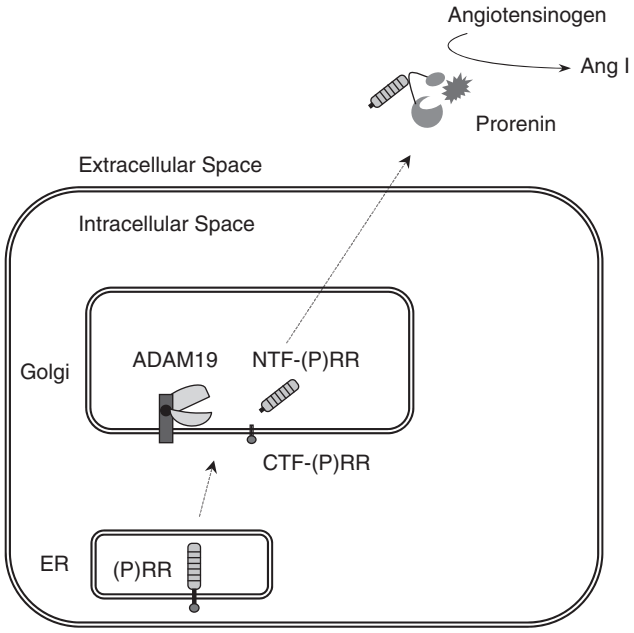


**Figure 5** Scheme of (P)RR fragments. The FL-(P)RR is located in the ER. (P)RR is cleaved by proteases in the Golgi, such as ADAM19. The NTF-(P)RR is secreted into the extracellular space, where it increases the renin activity of prorenin.

(P)RR was also in the medium of HEK293 cells or VSMC that did not undergo any stimulation. Therefore, our results support the hypothesis that the NTF-(P)RR may be secreted through the constitutive secretory pathway.

**(P)RR is cleaved in the Golgi**

(P)RR mainly localizes in the Golgi and the ER. BFA induces the formation of tubules from the Golgi and the fusion to the ER membrane, which results in the complete redistribution of the Golgi enzymes into the ER.[15] Our results showed that the treatment with BFA for 3 h reduced the levels of FL-(P)RR and concurrent treatment with NEM, which inhibits the fusion, recovered it (Figures 2b and c). In addition, after the treatment with BFA, HEK293 cells had similar results to the (P)RR-Venus-CHO cells (Figure 2d). These results represent clear evidence to support the shedding of (P)RR in the Golgi.

**ADAM19 cleaves (P)RR**

PMA increased the NTF-(P)RR in the medium of VSMC. The ADAM family consists of cleaving proteases that cleave (P)RR because it is activated by PMA through protein kinase C activation.[16,17] The multiple functions of the ADAM proteins include the proteolytic processing of transmembrane proteins, cell adhesion and cell signaling events. In the ADAM family, ADAM19 is localized in the Golgi.[18,19] Dual transfection with (P)RR-Venus and ADAM19-V5 significantly increased the ratio of the CTF-(P)RR and the dominant negative ADAM19 reversed this CTF-(P)RR increase. Yokozeki *et al.* reported that the shedding of Neuregulin β1 is mediated by ADAM19 in the Golgi.[19] Taken together, these results suggest that ADAM19 may be a protease that is involved in the shedding of (P)RR in the Golgi. Cousin *et al.* reported that (P)RR has soluble and receptor forms, which is consistent with our results.[10] On the other hand, they also reported that furin is the protease that is involved in the shedding of (P)RR in the Golgi because (P)RR is processed at RXXR, which is the furin cleavage site near the transmembrane region.[10] Our experiment revealed that the N-(P)RR antibody detected the NTF-(P)RR at 29 kDa in the conditioned medium from LoVo cells, which express endogenous (P)RR without furin expression. Furthermore, the furin inhibitor did not suppress the protein levels of NTF-(P)RR in the medium from VSMC. Remacle *et al.* reported that RXKR or RXRR, but not RKXR, in (P)RR strongly contributes to the furin cleaving sites.[20] Hence, furin may not be pivotal in the processing of (P)RR. Furthermore, M8–9, which is associated with the V-ATPase, corresponds to carboxyl-terminal amino acid sequences of (P)RR.[9] However, the furin cleavage site is not compatible with the amino-terminal end of M8–9. Two possibilities may explain these observations: either another protease cleaves (P)RR or further processing occurs after the processing by furin.

Melenhorst *et al.* reported that under various pathologic conditions in the human kidney, the expression levels of ADAM19 significantly increases in the distal tubular cells and the glomerular visceral epithelial cells, and the glomerular mesangial and proximal tubular cells endogenously express ADAM19.[21] As Nguyen *et al.* reported that (P)RR is expressed in glomerular mesangial cells and distal tubular cells, the localization of (P)RR and ADAM19 may be influence the development of particular diseases.[1,22] The relevance of localization and participated diseases in (P)RR and ADAM19 may be compatible. In contrast, furin is expressed widely under normal conditions. Proteases that cleave (P)RR may be altered between physiological and pathological conditions.

**Significance of the NTF-(P)RR on the local RAS**

Local RAS at the tissue site is widely accepted. Prorenin is expressed in various tissues, such as the retina, adrenal glands, ovary, testis and placenta.[23] Prorenin is not converted to active renin in various tissues. However, the local activation of prorenin after binding to (P)RR contributes to the local generation angiotensin I. In fact, Nishiyama *et al.* reported a very high concentration of angiotensin II in the renal intercellular space.[24] In this study, we were able to demonstrate that the NTF-(P)RR induced the renin activity of prorenin after secretion into the extracellular space. Therefore, the secreted NTF-(P)RR into the extracellular fluid may generate local angiotensin I, especially in the kidneys, heart and brain.

**Perspectives**

Various studies have suggested that (P)RR induces its intracellular signaling pathways independent of angiotensin II. From our results, it is difficult to interpret the trigger of intracellular signaling because (P)RR may be mainly located in the ER and Golgi, and in the process of intracellular protein transport, the (P)RR is shed by ADAM19 in the Golgi, which leads to secretion of the NTF-(P)RR into the extracellular space. Otherwise, the secretion of the NTF-(P)RR may participate in the activation of intracellular signaling. Cruciat *et al.* reported that the full-length (P)RR in the plasma membrane binds to V-ATPase and the Wnt receptor, which leads to activation of intracellular Wnt signaling.[25] The detailed mechanisms that determine the proportion of the NTF-(P)RR/the receptor form are not completely understood. Based on our results, we speculate that (P)RR may be modified to the NTF-(P)RR, although in HEK293 cells that were pretreated with BFA, the levels of the endogenous full-length (P)RR were not completely abolished (Figure 2d). The (P)RR shedding may be a new therapeutic target for cardiovascular and renal diseases.

## ACKNOWLEDGEMENTS

We wish to thank Dr Makoto Endo and Dr Kei Maruyama for their valuable suggestions and their critical reading of this manuscript. This study was supported in part by the Uehara Memorial Foundation, the Takeda Science Foundation, the Maruki Memorial Foundation and the Saitama Medical University Internal Grant 21-2-2-04.

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