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Expression of (Pro)renin Receptor During Rapamycin-Induced Erythropoiesis in K562 Erythroleukemia Cells and Its Possible Dual Actions on Erythropoiesis

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(Pro)renin receptor ((P)RR), a specific receptor for renin and prorenin, is expressed in erythroblastic cells. (P)RR has multiple biological actions: prorenin activation, stimulation of the intracellular signaling including extracellular signal-regulated kinases, and functional complex formation with vacuolar H^+-ATPase (v-ATPase). However, the functional implication of (P)RR in erythroblast cells has not been clarified. The aim of the present study was to clarify changes of (P)RR expression during erythropoiesis and a role of (P)RR in the heme synthesis. (P)RR expression was studied during rapamycin-induced erythropoiesis in a human erythroleukemia cell line, K562. Treatment with rapamycin (100 nM) for 48 hours significantly increased %number of hemoglobin-producing cells, γ-globin mRNA levels, erythroid specific 5-aminolevulinate synthase (ALAS2) mRNA levels, and heme content in K562 cells. Both (P)RR protein and mRNA levels increased about 1.4-fold during rapamycin-induced erythropoiesis. Suppression of (P)RR expression by (P)RR-specific small interference RNA increased ALAS2 mRNA levels about 1.6-fold in K562 cells, compared to control using scramble RNA, suggesting that (P)RR may down-regulate ALAS2 expression. By contrast, treatment with bafilomycin A1, an inhibitor of v-ATPase, decreased greatly % number of hemoglobin-producing cells and heme content in K562 cells, indicating that the v-ATPase function is essential for hemoglobinization and erythropoiesis. Treatment with bafilomycin A1 increased (P)RR protein and mRNA levels. In conclusion, we propose that (P)RR has dual actions on erythropoiesis: the promotion of erythropoiesis via v-ATPase function and the down-regulation of ALAS2 mRNA expression. Thus, (P)RR may contribute to the homeostatic control of erythropoiesis.

Keywords: erythropoiesis; heme; prorenin; (pro)renin receptor; rapamycin
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Introduction

(Pro)renin receptor ((P)RR), a specific receptor for renin and prorenin, is a 350-amino acid protein with a single transmembrane domain (Nguyen et al. 2002; Nguyen and Muller 2010; Nguyen 2011). The enzymatic activity of prorenin in converting angiotensinogen to angiotensin I is activated when it binds to (P)RR. Moreover, the binding of prorenin or renin to (P)RR directly stimulates the intracellular signaling pathways, including extracellular signal-regulated kinase (ERK 1/2) and Akt, resulting in cell proliferation and/or cell dysfunction (Liu et al. 2011; Nguyen 2011). Soluble (P)RR comprising of the extracellular domain of (P)RR, and a truncated form of (P)RR at the C-terminal region (truncated (P)RR) are generated from full length (P)RR by furin (Cousin et al. 2009; Nguyen 2011).

The truncated (P)RR is associated with vacuolar H^+-ATPase (v-ATPase), an ATP-dependent multi-subunit proton pump (Ludwig et al. 1998). v-ATPase plays a physiological role in maintaining the acidic environment of intracellular components and the extracellular space. Cardiomyocyte-specific ablation of the (P)RR gene caused loss-of-function of v-ATPase and resulted in lethal heart failure (Kinouchi et al. 2010). Moreover, podocyte-specific (P)RR-knockout mice died of kidney failure with severe proteinuria within 2 to 4 weeks of birth (Oshima et al. 2011,

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Riediger et al. 2011). The deficiency of (P)RR in podocytes resulted in v-ATPase dysfunction, impaired autophagy and podocyte death. These reports have suggested that (P)RR plays an essential role in the v-ATPase function and survival of cells, at least in cardiomyocytes and podocyes. Furthermore, the complex of (P)RR and v-ATPase is also involved Wnt/β-catenin pathway, which is essential for embryonic development, and various diseases including cancers (Cruciat et al. 2010).

(P)RR is expressed in various types of cells, including neurons, endocrine cells, lymphocytes, cardiomyocytes, and renal tubular cells (Hirose et al. 2009; Takahashi et al. 2010a, 2010b, 2013; Narumi et al. 2015). We have previously reported expression of (P)RR in a human erythroblastic cell line, YN-1 and its clonal variant cell line, YN-1-0-A (Kaneko et al. 2012). Erythropoietin increased (P)RR mRNA expression levels, and interferon-γ increased protein accumulation of (P)RR in YN-1-0-A cells. By contrast, treatment of transforming growth factor-β (TGF-β) had no significant effects on expression levels of (P)RR mRNA and protein in YN-1-0-A cells, whereas it induced expression of erythroid specific 5-aminolevulinate synthase (ALAS2) mRNA. Because (P)RR is a multi-functional molecule, (P)RR may affect erythropoiesis via either the renin-angiotensin system, the intracellular signaling such as ERK1/2, or v-ATPase. The functional role of (P)RR has not been studied, however, in erythropoiesis.

Rapamycin is a potent inducer of erythroid differentiation of human erythroleukemic K562 cells (Yamamoto-Yamaguchi et al. 2001; Mischiati et al. 2004). Moreover, rapamycin induced accumulation of α-, β- and γ-globin mRNAs in erythroid precursor cells obtained from β-thalassemia patients (Fibach et al. 2006). Rapamycin is an inhibitor of the mammalian target of rapamycin (mTOR) activity, and possesses immunosuppressive, anti-fungal and anti-tumor properties (Price et al. 1992; Hay and Sonenberg 2004). The aim of the present study was to clarify changes of expression of (P)RR during rapamycin-induced erythropoiesis, and effects of (P)RR on erythropoiesis in K562 cells.

Materials and Methods

Cell culture

A human erythroleukemia cell line, K562, was cultured in RPMI 1640 medium (Sigma-Aldrich, Tokyo, Japan), containing 10% heat-inactivated fetal bovine serum (FBS) (Biomeda, CA, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere. The K562 cell line was used in the present study, because rapamycin-induced erythropoiesis was well established in this cell line (Yamamoto-Yamaguchi et al. 2001; Mischiati et al. 2004), and the efficiency of siRNA transfection was low in YN-1-0-A cells, which were used in our previous study (Kaneko et al. 2012).

Erythropoiesis of K562 cells was induced by culturing for 48 hours in the medium containing rapamycin (Wako Pure Chemicals Industries Ltd., Osaka, Japan), and then harvested for the analysis of hemoglobin production, cellular heme content, and expression of γ-globin, ALAS2 and (P)RR. In another experiment, effects of (P)RR on the expression levels of ALAS2 mRNA and cellular heme content were studied in K562 cells by using (P)RR-specific small interference RNA (siRNA). Finally, we wished to clarify whether or not the action of (P)RR-specific siRNA on heme synthesis was reproduced by bafilomycin A1, an inhibitor of v-ATPase, and whether the change in (P)RR expression by rapamycin was caused by alteration of the v-ATPase function. Cells were incubated with bafilomycin A1 (Santa Cruz Biotechnology Inc., Dallas, Texas) at indicated concentrations for 48 hours, and then harvested for the analysis of hemoglobin production, cellular heme content, and expression of γ-globin, ALAS2, and (P)RR.

Hemoglobin production and heme content

Hemoglobin production of K562 cells was determined by *o*-dianisidine staining (Minegishi et al. 1994). Heme contents in cells were determined as described (Sassa 1976).

Western blot analysis

Cells were washed twice with cold phosphate-buffered saline on cultured dishes. Cells were scraped into 1.5 ml microtubes, and centrifuged at 4°C for 10 minutes by 2,500 × g. Cells were harvested, and were then lysed in M-PER™ Mammalian Protein Extraction Reagent (Thermo scientific, Rockford, IL, USA), which contained Halt® Protease and Phosphatase Inhibitor Cocktail (Thermo scientific). The mixture was shaken gently for 10 minutes, and centrifuged at 4°C for 10 minutes by 14,000 × g. The supernatant was then transferred to another tube. Protein was quantified by Bradford protein assay (Bio-rad, Hercules, CA, USA). Cell lysates were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA), as previously described (Udono-Fujimori et al. 2004; Kaneko et al. 2012).

The membranes were blocked for 1 hour at 4°C by immersion into a Tris buffered saline with Tween 20 (TBST) containing 5% skimmed milk. The membrane was incubated with the primary antibody at 4°C overnight. Dilutions of primary antibodies used in this study were as follows: (P)RR (Hirose et al. 2009, 2010), 1:10,000 and α-tubulin (Santa Cruz), 1:20,000. Antibodies against (P)RR were diluted in Can Get Signal® Immunoreaction Enhancer Solution (TOYOBO, Tokyo, Japan). The membrane was washed several times with TBST buffer and then incubated with horseradish peroxidase-coupled anti-rabbit IgG secondary antibodies (1:20,000; NA934, GE Healthcare, Buckinghamshire, UK) for 1 hour at 4°C. The membrane was washed with TBST buffer, and then developed using Immobilon Western (Millipore) and LAS 4000 (Fuji Film, Tokyo, Japan). The relative intensities of the bands were determined using NIH Image software for quantification of protein expression.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Quantitative real-time RT-PCR of (P)RR, γ-globin, ALAS2 and 18S ribosomal RNA was performed using a 7500 Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA), according to the manufacturer's instruction, as previously reported (Kaneko et al. 2009; Kaneko et al. 2012). The SYBR PrimeScript RT-PCR Kit II (Takara Bio Inc., Shiga, Japan) was used for all reactions. The copy number of (P)RR mRNA, γ-globin mRNA, ALAS2 mRNA or 18S ribosomal RNA was calculated with 7500 system sequence detection

software (Applied Biosystems Inc.), using each cDNA as a standard. Relative expression levels of (P)RR mRNA, γ-globin mRNA and ALAS2 mRNA were determined after normalization with 18S ribosomal RNA expression levels.

Transient transfection and small interference RNA

(P)RR-specific siRNA was purchased from Invitrogen; ID HSS115474, (siB) sense, 5-AGUUGACCUGCUCUUUCUUUCUGAA-3; anti-sense, 5-UUCAGAAAGA AAGAGCAGGCUAACU-3. RNAi Negative Control Duplexes (Invitrogen) (scramble RNA) was used as a negative control. K562 cells were transfected with 20 nM (P)RR-specific siRNA using SF Cell Line 4D-Nucleofector™ X Kit (Lonza, Belgium), following the manufacturer's instruction. Cells were harvested 48 hours after the transfection, and subjected to real-time RT-PCR of (P)RR mRNA and ALAS2 mRNA, and measurement of cellular heme content.

Statistics

Data are shown as mean ± SEM. Data between two groups were compared by unpaired Student's t-test. Multiple comparison was performed by one-way analysis of variance, followed by Tukey-Kramer method.

Results

Increased hemoglobinization and (P)RR expression by rapamycin

Treatment with rapamycin for 48 hours increased % number of hemoglobin-producing cells in K562 cells significantly at concentrations of 100 nM and 1,000 nM (Fig. 1A). We therefore used 100 nM rapamycin in the following experiments. Treatment with rapamycin (100 nM) for 48 hours increased γ-globin mRNA levels (Fig. 1B), ALAS2 mRNA levels (Fig. 1C), and heme content (Fig. 1D) in K562 cells significantly. Thus, the effect of rapamycin on erythropoiesis was confirmed in K562 cells.

Western blot analysis showed that the intensity of both the 35 kDa band (representing full length (P)RR) and the 28 kDa band (representing soluble (P)RR) was more intense in rapamycin-treated K562 cells than in control (Fig. 2A, upper panel). Relative expression levels of full-length (P)RR and 'full-length + soluble (P)RR' were significantly greater (about 1.4-fold) in rapamycin-treated K562 cells than in control, respectively, whereas those of soluble (P)RR were not significantly changed by rapamycin (Fig. 2A,

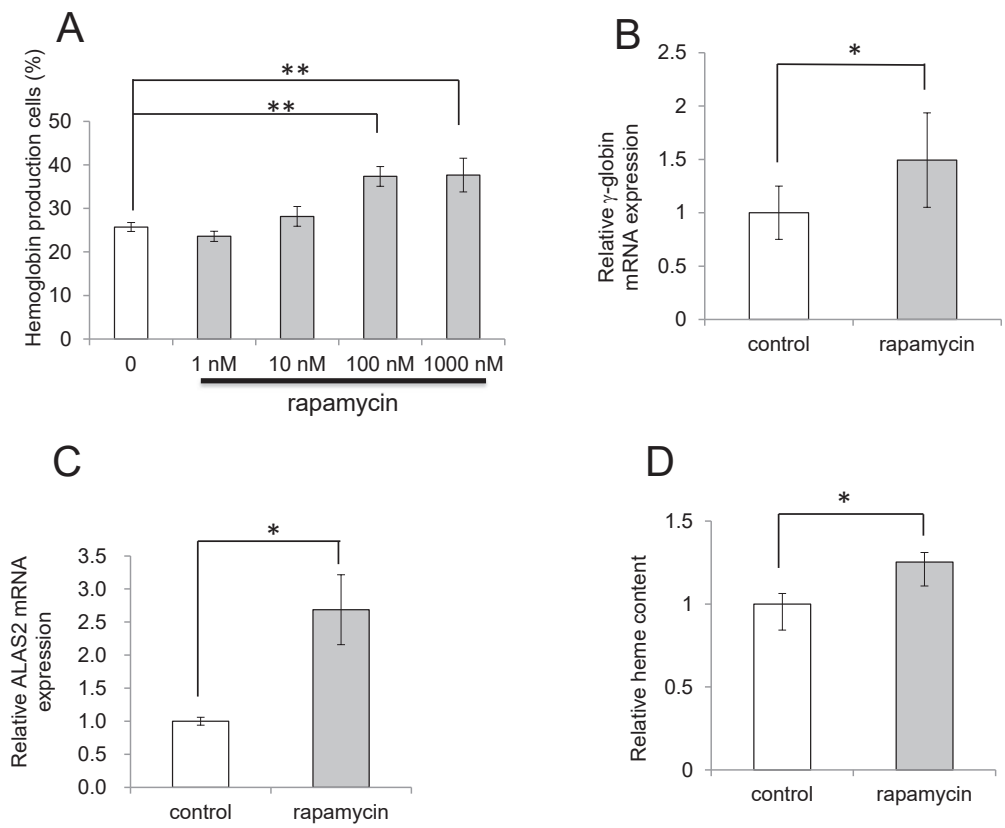

Fig. 1. Effects of rapamycin on erythropoiesis in K562 cells.
Shown are % of hemoglobin-producing cells (A), relative expression levels of γ-globin mRNA (B), relative expression levels of erythroid specific 5-aminolevulinate synthase (ALAS2) mRNA (C), and relative heme content (D). K562 cells were incubated with rapamycin at concentrations of 1 nM-1,000 nM (A) or 100 nM (B, C, D) for 48 hours. Hemoglobin-producing cells were determined using *o*-dianisidine staining. Expression levels of of γ-globin mRNA and ALAS2 mRNA were normalized by those of 18S ribosomal RNA. The relative levels in B, C, and D were determined by the ratio of each level to the mean level of control. Bar graphs represent mean ± SEM ($n = 3$ in A; $n = 6$ in B, C and D). *$p < 0.05$; **$p < 0.01$.

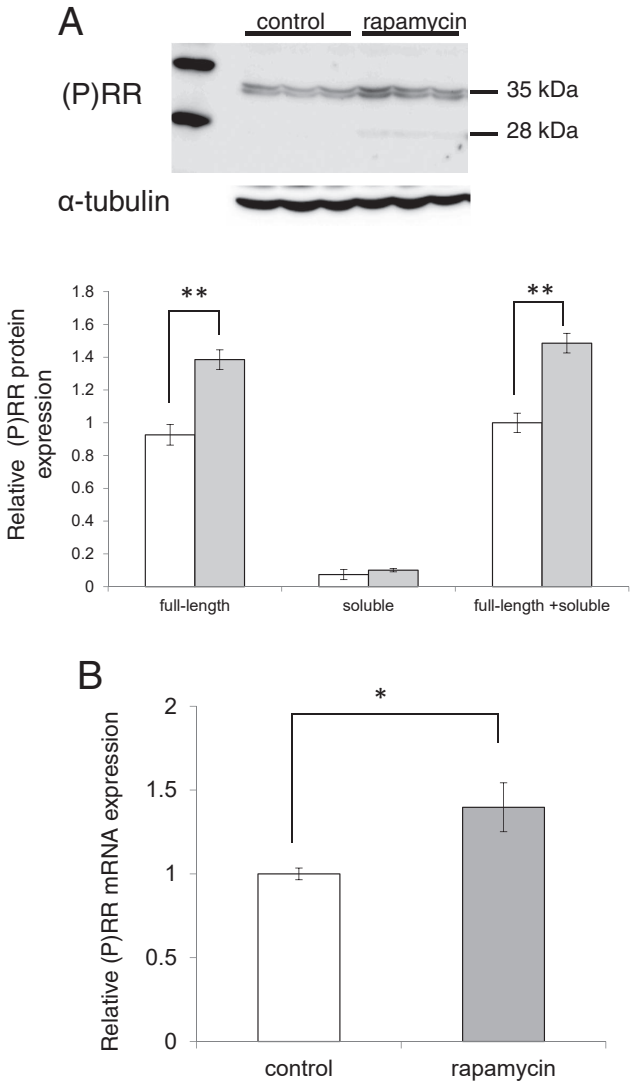

Fig. 2. Effects of rapamycin on the expression of (pro)renin receptor [(P)RR] protein and mRNA in K562 cells.
Cells were incubated with 100 nM rapamycin for 48 hours. Expression of (P)RR protein and mRNA was studied by western blot analysis (A) and real-time reverse-transcriptase polymerase chain reaction (B), respectively. (A) Western blot analysis of (P)RR protein (upper panel) and relative expression levels of full-length (P)RR protein (full length, *left*), soluble (P)RR protein (soluble, *middle*), and the total of full-length and soluble (P)RR protein (full-lenghth + soluble, *right*) (mean ± SEM, $n = 6$) (lower panel). White and dark columns indicate the control and rapamycin-treated cells, respectively. The intensity of the bands in western blot analysis was quantified using NIH Image software. The intensity of the bands representing full-length (P)RR at 35 kDa and soluble (P)RR at 28 kDa was normalized by that of the α-tubulin band. Expression of α-tubulin was studied as an internal control. The ratio of the normalized value to that of the mean value of the control is shown as the relative (P)RR protein expression. (B) Real-time reverse-transcriptase polymerase chain reaction of (P)RR mRNA. Expression levels of (P)RR mRNA were normalized by those of 18S ribosomal RNA. The ratio of the normalized value to that of the mean value of the control is shown as relative (P)RR mRNA expression (mean ± SEM, $n = 9$). $*p < 0.05$; $**p < 0.01$.

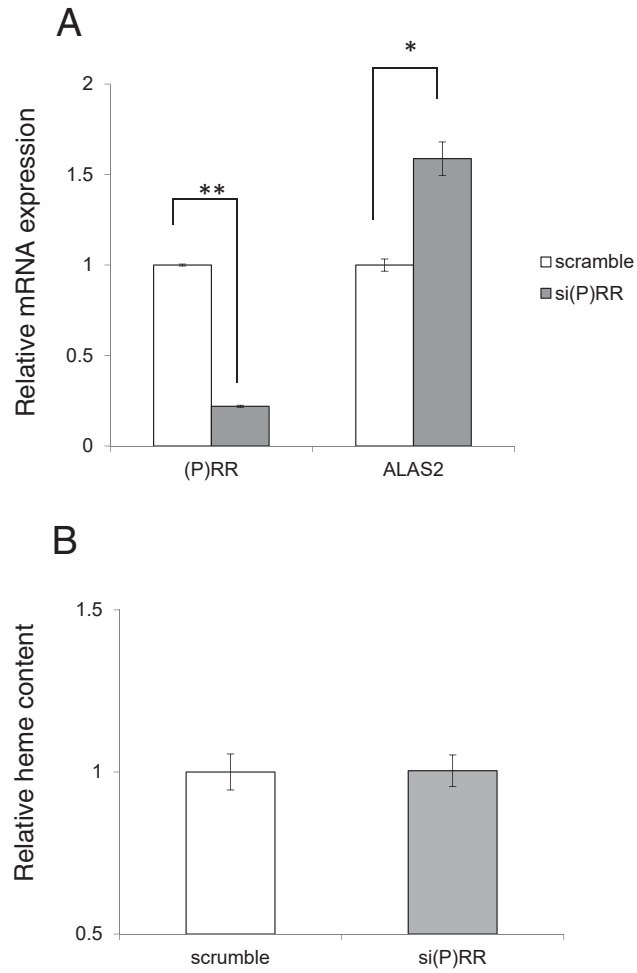

Fig. 3. Effect of (P)RR-specific small interference RNA [(P)RR siRNA] on relative expression levels of erythroid specific 5-aminolevulinate synthase (ALAS2) mRNA and relative heme conten in K562 cells.
Cells were transfected with (P)RR siRNA following the manufacturer's instruction. Cells were harvested 48 hours after the transfection for real-time reverse-transcriptase polymerase chain reaction of (P)RR mRNA and ALAS2 mRNA (A), and measurement of cellular heme content (B). White and gray columns show scramble siRNA as a control and (P)RR siRNA, respectively. Expression levels of (P)RR mRNA and ALAS2 mRNA were normalized by those of 18S ribosomal RNA. The ratio of the normalized value to that of the mean value of the control (scramble siRNA) is shown as relative mRNA expression (A) (mean ± SEM, $n = 3$). $*p < 0.05$; $**p < 0.01$.

lower panel). Real-time RT-PCR showed that (P)RR mRNA levels were increased 1.4-fold in rapamycin-treated K562 cells compared to control (Fig. 2B).

Increased ALAS2 mRNA expression levels by suppression of (P)RR expression

The effect of (P)RR on erythropoiesis was then studied in K562 cells by using (P)RR-specific siRNA. Treatment with (P)RR-specific siRNA suppressed (P)RR mRNA levels to about 20%, and increased ALAS2 mRNA levels about

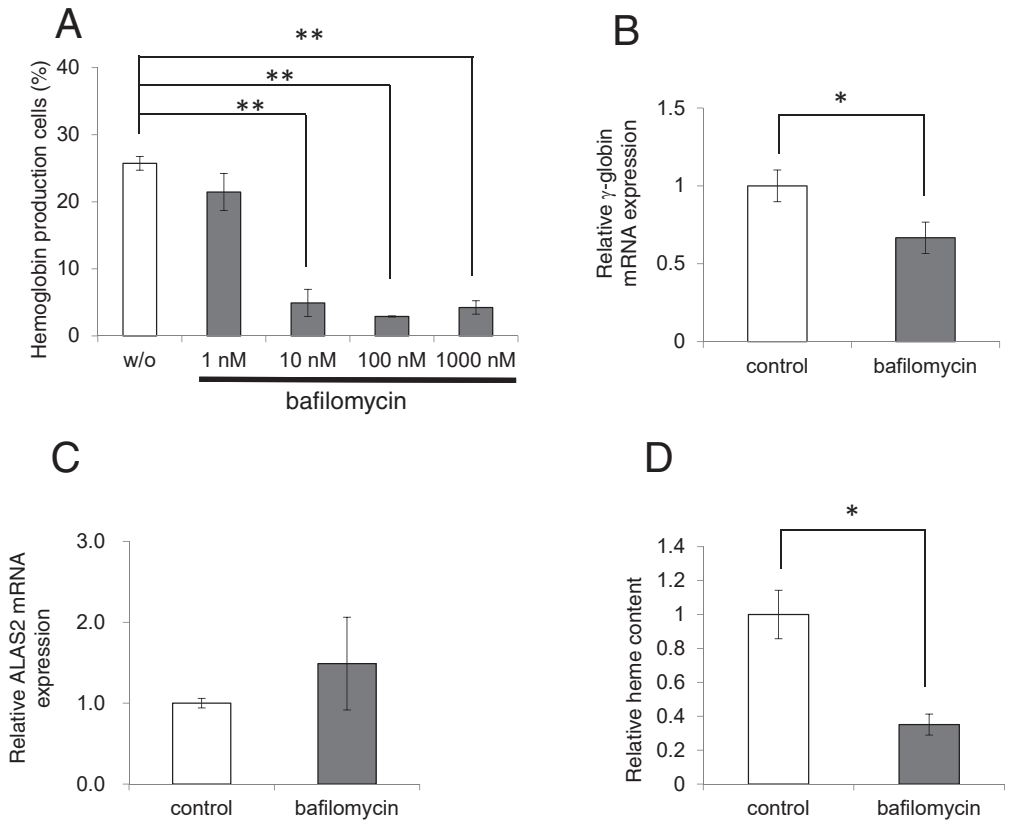

Fig. 4. Effects of bafilomycin A1 on erythropoiesis in K562 cells.
Shown are % of hemoglobin-producing cells (A), relative expression levels of γ-globin mRNA (B), relative expression levels of erythroid specific 5-aminolevulinate synthase (ALAS2) mRNA (C), and relative heme content (D). Cells were incubated with bafilomycin A1 at concentrations of 1 nM-1,000 nM (A) or 100 nM (B, C, D) for 48 hours. Hemoglobin-producing cells were determined using *o*-dianisidine staining. Expression levels of of γ-globin mRNA and ALAS2 mRNA were normalized by those of 18S ribosomal RNA. The relative levels in B, C, and D were determined by the ratio of each level to the mean level of control. Bar graphs represent mean ± SEM ($n = 3$ in A; $n = 6$ in B, C and D). $^{*}p < 0.05$; $^{**}p < 0.01$.

1.6-fold in K562 cells, compared to control using scramble RNA, indicating that (P)RR negatively regulate ALAS2 mRNA expression (Fig. 3A). By contrast, there was no significant increase in cellular heme content by the transfection of (P)RR-specific siRNA (Fig. 3B).

Suppression of hemoglobinization and increase in (P)RR expression levels by bafilomycin A1

Effects of bafilomycin A1, an inhibitor of v-ATPase, on hemoglobin production (Fig. 4A), expression of γ-globin mRNA (Fig. 4B), expression levels of ALAS2 mRNA (Fig. 4C) and cellular heme content (Fig. 4D) were studied. In contrast to rapamycin or (P)RR-specific siRNA, treatment with bafilomycin A1 for 48 hours decreased greatly %number of hemoglobin-producing cells at concentrations of 10 nM, 100 nM and 1,000 nM (Fig. 4A), γ-globin mRNA levels (Fig. 4B) and cellular heme content in K562 cells (Fig. 4D) at a concentration of 100 nM, but had no significant effects on ALAS2 mRNA levels (Fig. 4C). Finally, effects of bafilomycin A1 on (P)RR expression were studied in order to clarify whether the increase in (P)RR expression by rapamycin was caused by alteration in the v-ATPase function or not. Western blot analysis showed that relative expression levels of soluble (P)RR and 'full-length + soluble (P)RR' were significantly greater in bafilomycin A1-treated K562 cells than in control, but those of full-length (P)RR were not (Fig. 5A). Real-time RT-PCR showed that (P)RR mRNA levels were increased 1.6-fold in bafilomycin A1-treated K562 cells compared to control (Fig. 5B).

Discussion

The present study has shown for the first time that expression levels of (P)RR were elevated during rapamycin-induced erythropoiesis, and (P)RR may have dual actions on erythropoiesis in K562 erythroleukemic cells; the promotion of erythropoiesis via the functional complex formation with v-ATPase and the negative regulation on ALAS2 mRNA expression. In our previous study (Kaneko et al. 2012), no significant change was observed in the (P)RR expression levels during TGF-β-induced erythropoiesis in YN-1-0-A cells. Finotti et al. (2015) reported that the inhibition of mammalian TOR complex 1 (mTORC1) functions by mithramycin caused an erythroid induction of

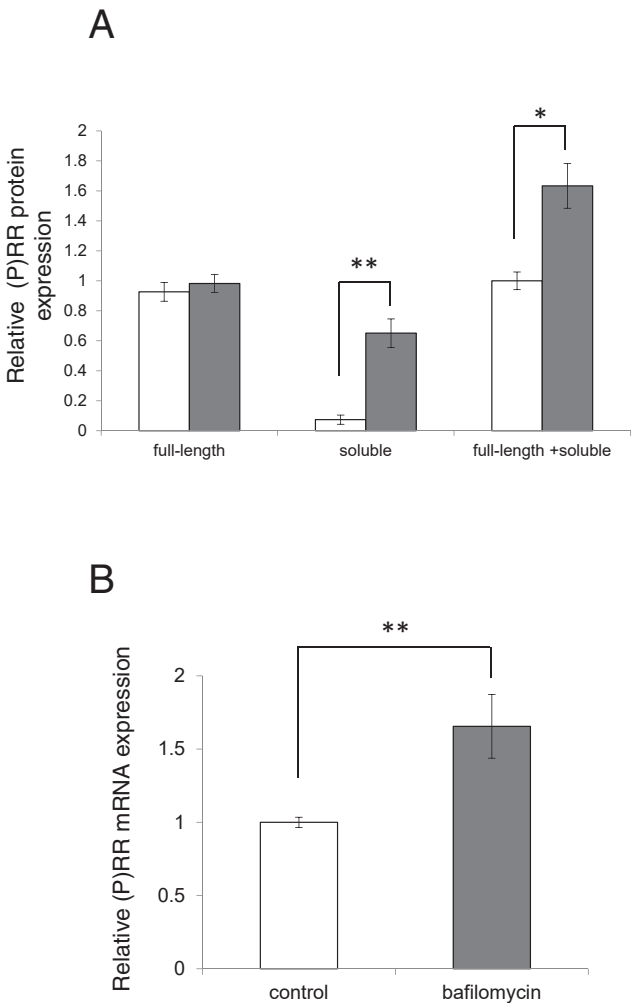

Fig. 5. Effects of bafilomycin A1 on the expression of (pro) renin receptor [(P)RR] protein and mRNA in K562 cells. Cells were incubated with 100 nM bafilomycin A1 for 48 hours. Expression of (P)RR protein and mRNA was studied by western blot analysis (A) and real-time reverse-transcriptase polymerase chain reaction (B), respectively. (A) Relative expression levels of full-length (P)RR protein (full length, *left*), soluble (P)RR protein (soluble, *middle*), and the total of full-length and soluble (P)RR protein (full-lenghth + soluble, *right*) (mean ± SEM, $n = 6$). White and dark columns indicate the control and bafilomycin A1-treated cells, respectively. The intensity of the bands in western blot analysis was quantified using NIH Image software. The intensity of the bands representing full-length (P)RR at 35 kDa and soluble (P)RR at 28 kDa was normalized by that of the α-tubulin band. Expression of α-tubulin was studied as an internal control. The ratio of the normalized value to that of the mean value of the control is shown as the relative (P)RR protein expression. (B) Real-time reverse-transcriptase polymerase chain reaction of (P)RR mRNA. Expression levels of (P)RR mRNA were normalized by those of 18S ribosomal RNA. The ratio of the normalized value to that of the mean value of the control is shown as relative (P)RR mRNA expression (mean ± SEM, $n = 9$). $^{**}p < 0.01$.

K562 cells. Elevated expression levels of (P)RR during rapamycin-induced erythropoiesis in the present study may, therefore, be mediated by the inhibitory action of rapamycin on mTORC1.

mTORC1 is a pivotal regulator of cell growth and proliferation, and a critical player in the etiology of cancer and metabolic diseases, including diabetes mellitus (Hay and Sonenberg 2004). mTORC1 is composed of the atypical serine/threonine kinase mTOR, the regulatory associated protein of mTOR (raptor), mammalian LST8/G-protein β-subunit like protein, PRAS40 and DEPTOR. mTORC1 senses and integrates diverse extra-and intracellular signals to anabolic and to inhibit catabolic cellular processes. Inhibition of the mTORC1 activity represses cell cycle and exosome complex component, and induces erythroid differentiation. mTOR and a transcriptional factor, FOXO3, form a metabolism-mediated regulatory network and control the balanced production and maturation of erythroid cells (Zhang et al. 2014).

Moreover, mTORC1 regulates the expression of v-ATPase (Peña-Llopis et al. 2011). It was therefore plausible that the inhibition of mTORC1 by rapamycin resulted in the reduced activity of v-ATPase in the present study. Indeed, the inhibition of v-ATPase by bafilomycin A1 elevated expression levels of (P)RR mRNA and protein, as shown in Fig. 5. Elevation in (P)RR expression levels during rapamycin-induced erythropoiesis is therefore likely to be induced by the reduced v-ATPase activity. (P)RR forms a functional complex with v-ATPase (Ludwig et al. 1998), but (P)RR expression is not under the direct control by mTORC1. The reduced v-ATPase activity may therefore stimulate the expression of (P)RR to compensate for its activity by a certain unknown feedback mechanism. V-ATPase activity helps to protect cells from endogenous oxidative stress, and loss of v-ATPase activity results in chronic oxidative stress (Milgrom et al. 2007). Oxidative stress may therefore be one of the candidates that induce (P)RR expression. It remains to be clarified why rapamycin treatment increased full-length (P)RR protein whereas bafilomycin A1 increased soluble (P)RR protein.

In contrast to rapamycin-induced erythropoiesis, bafilomycin A1 decreased hemoglobin production and heme content, suggesting that the v-ATPase function is essential for hemoglobinization. This finding is consistent with previous report by Zhang et al. (2005), who showed that bafilomycin A1 prevented endosomal Fe incorporation into heme. Elevated (P)RR during rapamycin-induced erythropoiesis may act to maintain the v-ATPase activity via the complex formation with v-ATPase. The minimum activity of v-ATPase required for hemoglobinization may exist during rapamycin-induced erythropoiesis in contrast to the bafilomycin A1 treatment, which inhibited the v-ATPase activity nearly completely. In addition, the inhibition of mTORC1 by rapamycin may more strongly induce hemoglobinization in K562 cells, when compared with the inhibitory action of (P)RR on ALAS2 mRNA expression. Thus,

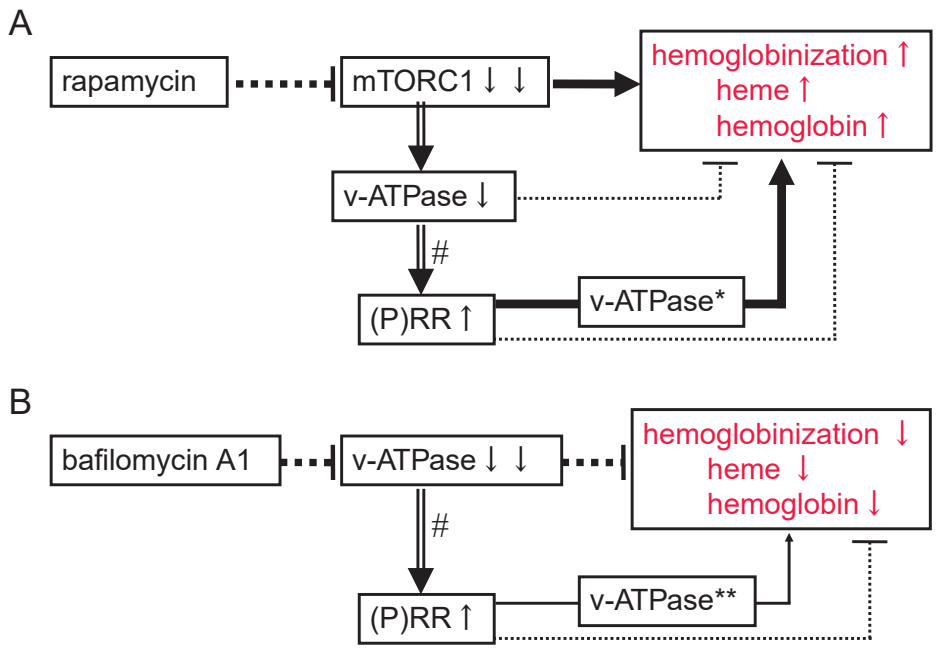

Fig. 6. Possible relationship among mammalian target of rapamycin complex 1 (mTORC1), vacuolar H^+-ATPase (v-ATPase) and (pro)renin receptor [(P)RR] in erythropoiesis.
(A) Erythropoiesis is induced by the inhibition of mTORC1 by rapamycin. The decreased activity of mTORC1 may result in a reduced activity of v-ATPase. The reduced activity of v-ATPase may stimulate the (P)RR expression to compensate for its activity by a certain unknown feedback mechanism (indicated by the arrow ⇩ and #). Elevated (P)RR may promote erythropoiesis via the complex formation with v-ATPase (indicated by *), whereas it may suppress ALAS2 mRNA expression. Although the v-ATP activity was reduced, its minimum activity required for hemoglobinization may be kept during rapamycin-induced erythropoiesis. In addition, the inhibition of mTORC1 by rapamycin may more strongly induce hemoglobinization in K562 cells, when compared with the inhibitory action of (P)RR on ALAS2 mRNA.
(B) Bafilomycin A1 inhibits the v-ATPase activity. The reduced activity of v-ATPase may stimulate the (P)RR expression to compensate for its activity by a certain unknown feedback mechanism (indicated by the arrow ⇩ and #). Elevated (P)RR may act to maintain the v-ATPase activity via the complex formation with v-ATPase (indicated by **). By contrast, both the reduced v-ATPase activity and elevated (P)RR expression resulted in decreased hemoglobin production and heme content. Thus, the v-ATPase function may be essential for hemoglobinization and erythropoiesis. Solid lines with arrows indicate positive signals. Dotted lines indicate negative signals.

there appears to be a functional interaction among mTORC1, v-ATPase and (P)RR in erythropoiesis, as shown in Fig. 6.

(P)RR is essential for function and survival of cardiomyocytes and podocytes by maintaining autophagy and protein-turnover machinery via the v-ATPase function (Kinouchi et al. 2010; Oshima et al. 2011; Riediger et al. 2011). The inhibition of the v-ATPase function by bafilomycin A1 reproduced the phenotype observed for the (P)RR-deficient cardiomyocytes or podocytes in these previous reports. In this regard, it is intriguing that the treatment with (P)RR-specific siRNA elevated ALAS2 mRNA expression, but did not affect survival of K562 cells. Moreover, bafilomycin A1 treatment did not reproduce the phenotype observed in K562 cells treated with (P)RR-specific siRNA in the present study. This discrepant response may be due to the difference between non-neoplastic cells such as cardiomyocytes and podocytes, and neoplastic cells such as erythroleukemia cells.

The suppression of endogenous (P)RR expression by (P)RR siRNA may result in the reduced activity of v-ATPase / (P)RR complex, and is likely to decrease heme synthesis. Thus, elevated ALAS2 mRNA expression under this condition may play a compensatory role for the heme synthesis. Bafilomycin A1 treatment had no significant effects on ALAS2 mRNA levels, but suppressed hemoglobin production in K562 cells. The effect of (P)RR on the ALAS2 mRNA expression was therefore unlikely to be mediated by the (P)RR/v-ATPase complex. Binding of prorenin/renin to (P)RR stimulates the intracellular signaling including ERK1/2 (Liu et al. 2011; Nguyen 2011). Mardini et al. (2010) showed that the ERK pathway inhibitor U0126 increased intracellular heme and hemoglobin levels, as well as ALAS2 mRNA expression levels, in Friend-virus-infected MEL (mouse erythroleukemia) cells. The ERK1/2 activation by the (P)RR-mediated signaling may be silenced by (P)RR-specific siRNA, resulting in elevated expression levels of ALAS2 mRNA in the present study. In contrast, the v-ATPase function may be related mostly to the post-transcriptional events of hemoglobin production, such as Fe incorporation into heme molecule (Zhang et al. 2005).

Cruciat et al. (2010) reported that (P)RR functions in a renin-independent manner as an adaptor between Wnt receptors and v-ATPase complex. (P)RR and v-ATPase were shown to be required to mediate Wnt signaling during antero-posterior patterning of Xenopus early central nervous system development. There has been accumulating evidence which shows the close relationship among iron, the Wnt signaling and erythropoiesis (Brookes et al. 2008; Tarafdar et al. 2013). Canonical Wnt/β-catenin signaling plays important roles in mesodermal specification, primitive erythropoiesis and early hematopoietic progenitor formation during hematopoietic induction (Tarafdar et al. 2013). Brookes et al. (2008) speculated that iron-medicated Wnt signaling and c-myc induced expression of transferrin receptor 1. We therefore could not deny the possibility that the Wnt/β-catenin signaling acts in cooperation with (P)RR, v-ATPase and mTORC1 in erythropoiesis.

Previous studies have suggested that (P)RR is related to the pathophysiology of various diseases, such as hypertension, diabetes mellitus, kidney disease (Ichihara et al. 2004, 2006), dehydration (Tamura et al. 2016), and obstructive sleep apnea syndrome (Nishijima et al. 2016). The present study has shown that expression levels of (P)RR are elevated during rapamycin-induced erythropoiesis, and (P)RR may have dual actions on erythropoiesis in K562 erythroleukemic cells; the promotion of erythropoiesis via the functional complex formation with v-ATPase, and the negative regulation on ALAS2 mRNA expression. Reduced v-ATPase activity may induce (P)RR expression. These findings have raised the possibility that (P)RR, together with mTORC1 and v-ATPase, is related to physiology of erythropoiesis, and possibly to pathophysiology of certain types of anemia.

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Conflict of Interest

The authors declare no conflict of interest.

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