Original Article

(Pro)Renin Receptor–Mediated Activation of Mitogen-Activated Protein Kinases in Human Vascular Smooth Muscle Cells

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Blockade of (pro)renin receptor has benefits in diabetic angiotensin II type-1a-receptor–deficient mice, suggesting the importance of (pro)renin receptor–mediated intracellular signals. To determine the mechanism whereby the human (pro)renin receptor activates mitogen-activated protein kinases in human vascular smooth muscle cells (hVSMC), we treated the cells with recombinant human prorenin. Prorenin enhanced hVSMC proliferation and activated extracellular-signal–related protein kinase (ERK) in a dose- and time-dependent manner but did not influence activation of p38 or c-Jun NH_2-terminal kinase. The activated ERK level was reduced to the control level by the tyrosine kinase inhibitor genistein, and the MEK inhibitor U0126 markedly reduced the activated ERK level to the control level, whereas the level of activated ERK was unaffected by the angiotensin-converting enzyme inhibitor imidaprilat or the angiotensin II receptor blocker candesartan. A human (pro)renin receptor was present in hVSMCs, and its knockdown with small interfering RNA (siRNA) significantly inhibited the prorenin-induced ERK activation. These results suggest that prorenin stimulates ERK phosphorylation in hVSMCs through the receptor-mediated activation of tyrosine kinase and subsequently MEK, independently of the generation of angiotensin II or the activation of its receptor. The (pro)renin receptor–mediated ERK signal transduction is thus a possible new therapeutic target for preventing vascular complications. (*Hypertens Res* 2007; 30: 1139–1146)

***Key Words*: extracellular-signal–related protein kinase, prorenin, receptor, small interfering RNA, vascular smooth muscle cells**

Introduction

Binding of prorenin to an intrinsic prorenin-binding receptor plays an important role in the development of cardiovascular complications in hypertension and diabetes, such as cardiac fibrosis (*1*), nephrosclerosis (*2*), and microvascular complications (*3*, *4*). These effects have been considered to be mediated by the receptor-associated prorenin (RAP) system and consist of two major pathways: activation of the renin-angiotensin system (RAS) by conversion of prorenin to its active form by a conformational change instead of proteolytic cleavage of the prosegment of prorenin (*3*, *5*), and stimulation of the RAS-independent intracellular pathways *via* the

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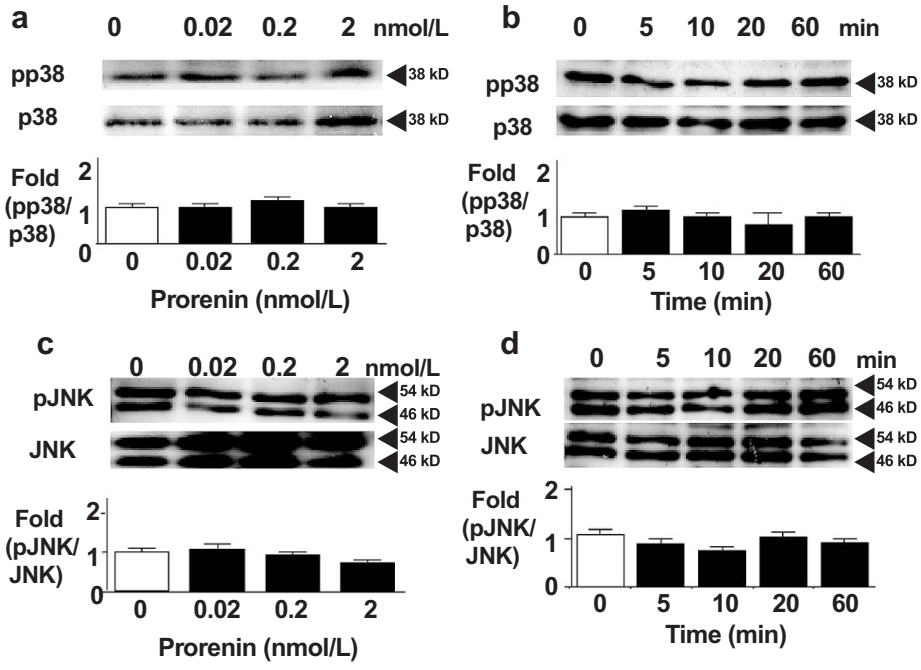

Fig. 1. *Dose response and time course of activation of p38 (a and b, respectively) and c-Jun NH_2-terminal kinase (JNK, c and d, respectively) after addition of human recombinant prorenin to human vascular smooth muscle cells (VSMCs; n = 5 in each). Representative Western blots are presented. The relative density of phosphorylated p38 (pp38) to p38 or phosphorylated JNK (pJNK) to JNK is shown in the bar graphs. Neither p38 nor JNK was stimulated by prorenin in human VSMCs.*

(pro)renin receptor (*5–8*).

Studies using cultured mesangial cells have provided evidence that stimulation of the (pro)renin receptor caused activation of extracellular-signal–related protein kinases (ERK) (*5*) and stimulation of transforming growth factor-β expression (*7*) through a RAS-independent mechanism. More recently, prorenin-treated cardiomyocytes showed stimulation of p38, but not ERK, through an angiotensin II–independent mechanism (*9*). Since RAP-dependent, RAS-independent activation of all three members of the mitogen-activated protein kinase (MAPK) family, including ERK, p38, and c-Jun NH_2-terminal kinase (JNK), was observed in the kidneys of diabetic mice (*8*), RAP-mediated intracellular signal transduction may depend on cell types. However, the signaling mechanisms of the (pro)renin receptor in vascular smooth muscle cells (VSMCs) have remained unclear despite the significant presence of this receptor in the small arteries (*5*).

The present study was designed to determine the involvement of the three members of the MAPK family in the (pro)renin receptor–mediated intracellular signal transduction in human VSMCs. Targeting of the (pro)renin receptor signal independent of angiotensin II receptor activation may provide a new therapeutic strategy for vascular complications in hypertension and diabetes.

Methods

Reagents

U0126, an MEK inhibitor, and genistain, a tyrosine kinase inhibitor, were purchased from A.G. Scientific Inc. (San Diego, USA) and Sigma (St. Louis, USA), respectively. Imidaprilat, an angiotensin-converting enzyme (ACE) inhibitor, and candesartan (CV11974), an angiotensin II type 1 receptor blocker (ARB), were kindly provided by Tanabe Seiyaku Co. (Osaka, Japan) and Takeda Chemical Industry (Osaka, Japan), respectively.

Prorenin Actions on Human VSMCs

Recombinant human prorenin was prepared from Chinese hamster ovary cell lines harboring human prorenin cDNA (*10*). Human VSMCs were obtained from Cell Systems (Kirkland, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% fetal calf serum according to the manufacturer's instructions. Experiments were performed with VSMCs maintained in culture for 4 to 10 passages. Before the experiments, cells were exposed to serum-free medium for 24 h. The cells were treated with human recombinant prorenin in a dose-dependent and time-dependent manner and then harvested for the measurement of

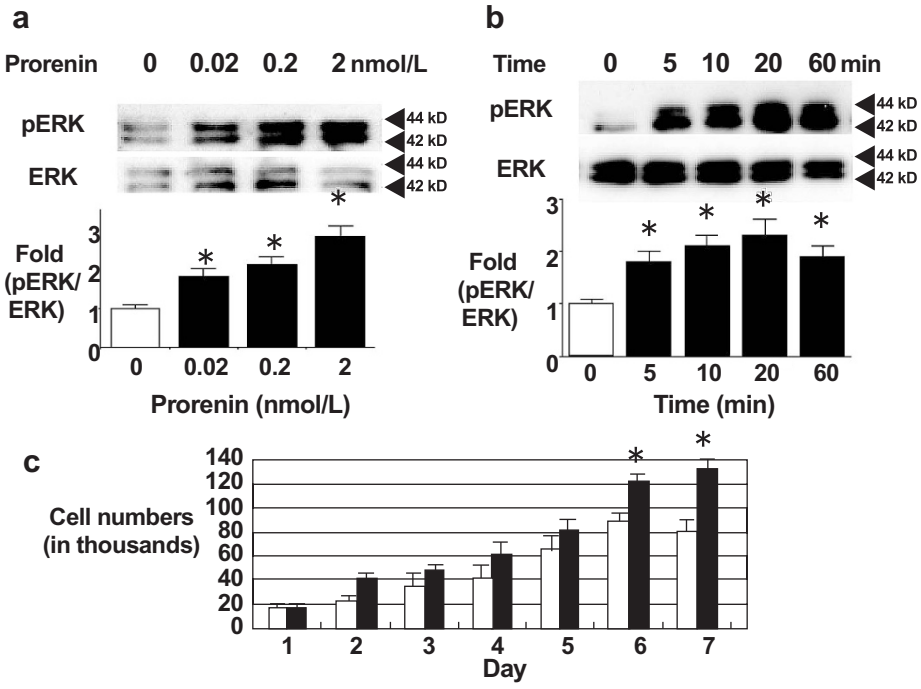

Fig. 2. *Extracellular-signal–related protein kinase (ERK) activation and growth by human recombinant prorenin in human vascular smooth muscle cells (VSMCs). a: The dose response was determined at 20 min after the addition of prorenin (n = 4), and b: the time course in response to 2 nmol/L prorenin was assessed (n = 4). Representative Western blots are presented. The relative density of phosphorylated ERK (pERK) to ERK is shown in the bar graphs. *p < 0.05 vs. the control. The stimulation of ERK (pERK) was dose-dependent and peaked at 20 min. c: Untreated human VSMCs (open bar; n = 3) and human VSMCs treated with 2 nmol/L human recombinant prorenin (closed bar; n = 3) were counted in triplicate once daily for 7 days by flow cytometry. *p < 0.05 vs. untreated VSMCs.*

(pro)renin receptor mRNA and ERK protein. Proliferation of VSMCs was determined by cell counts performed in triplicate once daily for 7 days with the use of a flow cytometer (Epics XL; Beckman Coulter, Fullerton, USA). Experiments were performed in the presence and absence of mannose-6-phosphate in the medium, but similar results were obtained.

MAPK Western Blot Analysis

Western blot analyses were performed as reported previously (*11*, *12*). Briefly, cultured human VSMCs were lysed in lysis buffer containing Tris (50 mmol/L), NaCl (100 mmol/L), NAF (50 mmol/L), EDTA (1 mmol/L), 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid sodium salt, 1% Triton X-100 and protease inhibitor (1 tab/9 mL buffer). Following centrifugation at 15,000 rpm for 15 min at 4°C, the supernatant was collected and subjected to SDS polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes, and after blocking the blots for 1 h with TBS-T containing 5% bovine serum albumin and 0.5% Tween 20, they were incubated for 24 h with mouse monoclonal anti-phosphorylated ERK antibody (1:1,000 dilution; Cell Signaling Technology, Beverly, USA), mouse monoclonal anti-ERK antibody (1:1,000 dilution; Cell Signaling Technology), mouse monoclonal anti-phosphorylated JNK antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, USA), rabbit polyclonal anti-JNK antibody (1:1,000 dilution; Santa Cruz Biotechnology), mouse monoclonal anti-phosphorylated p38 antibody (1:500 dilution; Santa Cruz Biotechnology), or rabbit polyclonal anti-p38 antibody (1:1,000 dilution; Santa Cruz Biotechnology). Immunoreactivity was detected by horseradish-peroxidase–conjugated donkey anti-mouse antibody and an enhanced chemiluminescence reaction, and the quantitative analyses were performed with Image 1D (Pharmacia, Peapack, USA).

RNA Preparation and Real-Time Reverse Transcription–Polymerase Chain Reaction of the (Pro)Renin Receptor

Total RNA was extracted from human VSMCs with an RNeasy Mini Kit (QIAGEN, Tokyo, Japan), and a real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed with the TaqMan One-Step RT-PCR Master Mix Reagents Kit, an ABI Prism 7700 HT Detection System (Applied Biosystems, Foster City, USA). We designed the probes and primers for the human (pro)renin receptor (forward, 5′-AGATGACATGTACAGTCTTTATG

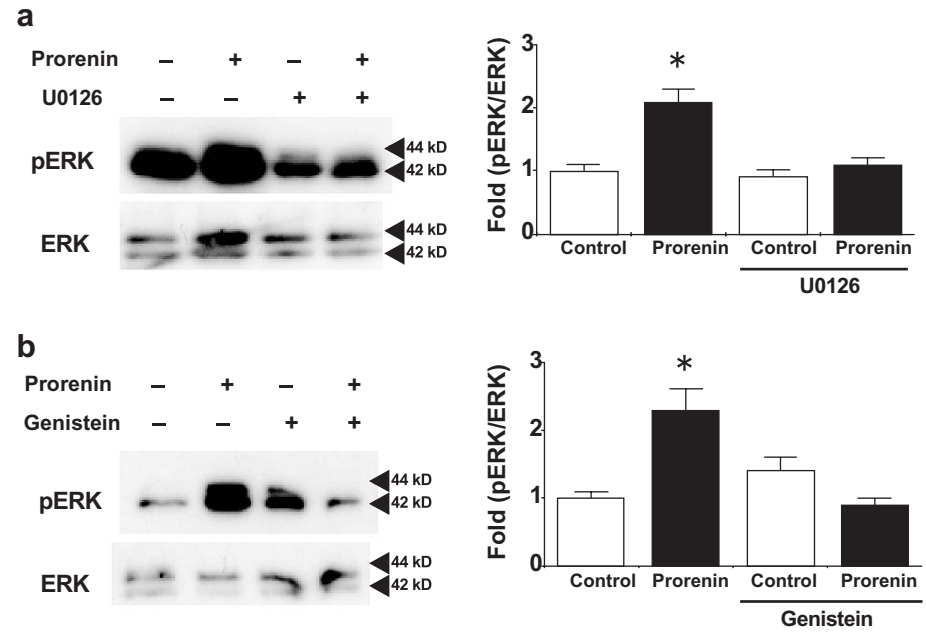

Fig. 3. *Extracellular-signal–related protein kinase (ERK) activation at 20 min after the addition of 2 nmol/L human prorenin in the presence and absence of 20 nmol/L U0126, an MEK inhibitor (n = 5; a) or 100 μmol/L genistain, a tyrosine kinase inhibitor (n = 5; b). Data are presented as a representative Western blots obtained from the experiments. The relative density of phosphorylated ERK (pERK) to ERK is presented in the bar graphs. ERK activation was completely blocked by U0126 or genistein. *p < 0.05 vs. the control.*

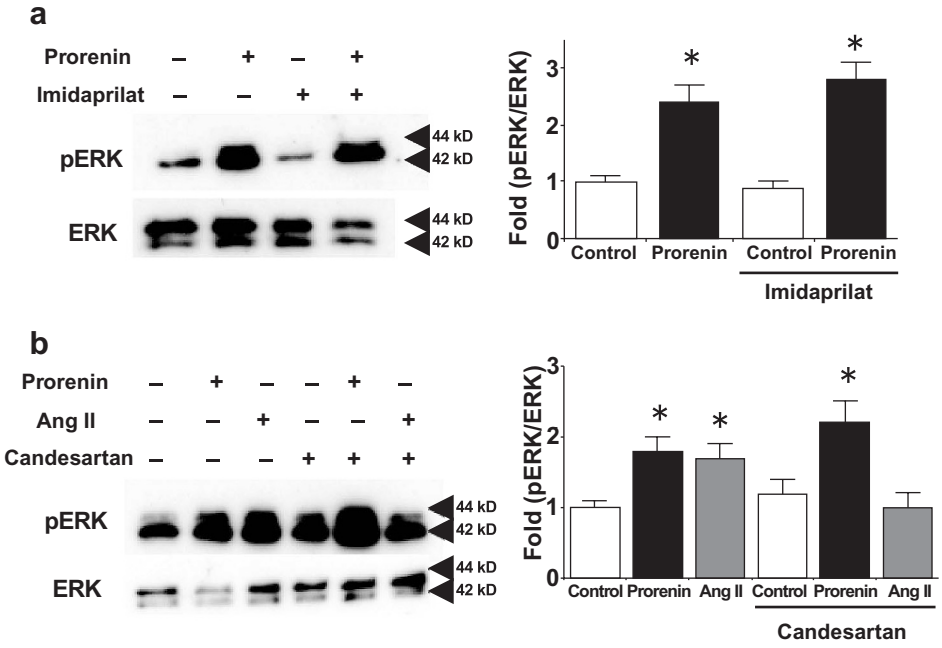

Fig. 4. *Extracellular-signal–related protein kinase (ERK) activation at 20 min after the addition of 2 nmol/L human prorenin or 1 μmol/L angiotensin (Ang) II in the presence and absence of 10 μmol/L imidaprilat, an ACE inhibitor (n = 5; a) or 10 μmol/L candesartan, an angiotensin II type 1 receptor blocker (n = 5; b). Data are presented as representative Western blots. The relative density of phosphorylated ERK (pERK) to ERK is given in the bar graphs. *p < 0.05 vs. the control. Neither imidapril nor candesartan inhibited prorenin stimulation of ERK.*

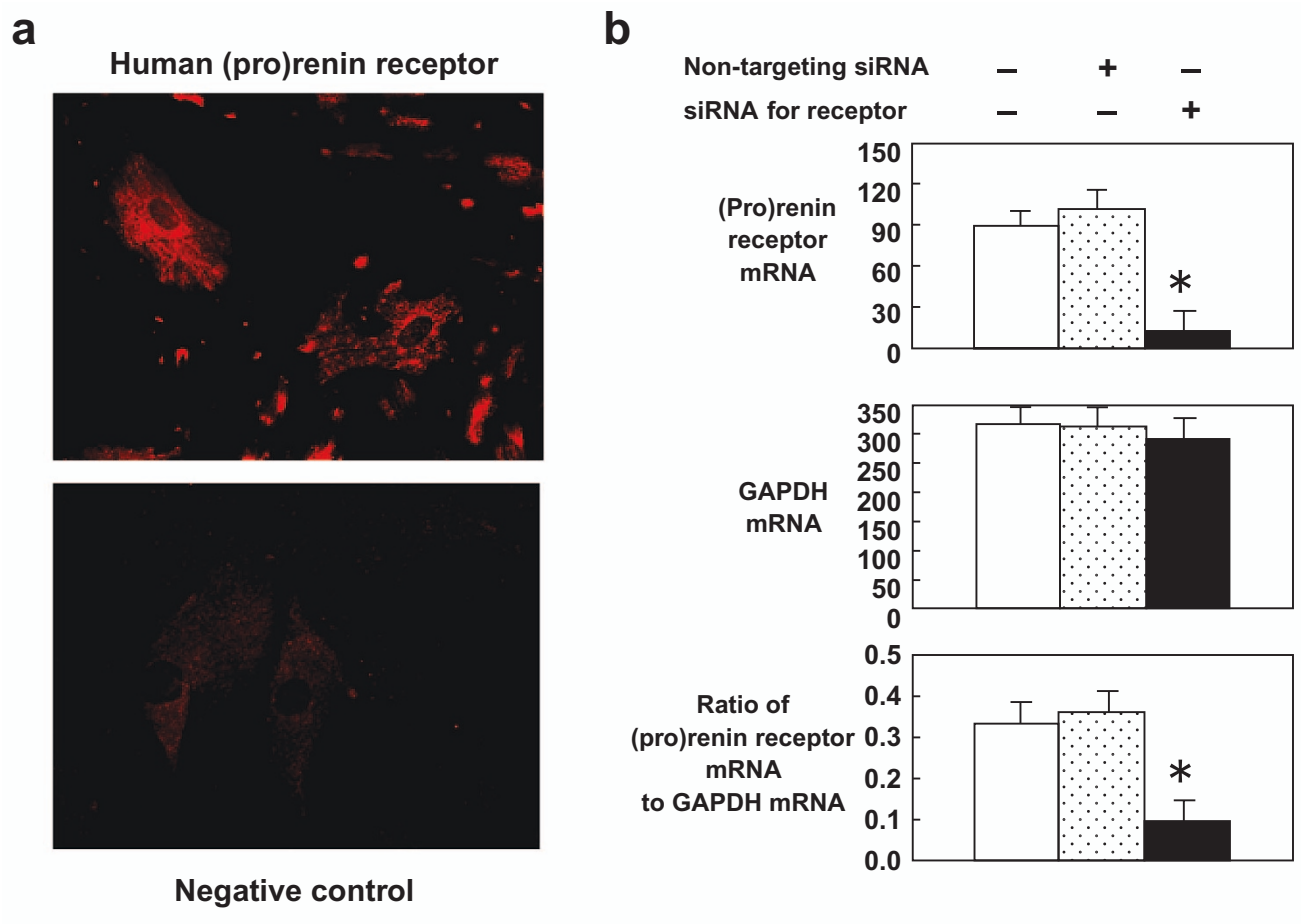

Fig. 5. *Photomicrograph of immunocytochemistry for human (pro)renin receptor (a) and effects of siRNA on human (pro)renin receptor mRNA (b) in human vascular smooth muscle cells. Targeting siRNA, but not control non-targeting siRNA, significantly decreased (pro)renin receptor mRNA expression as determined by real-time RT-PCR analysis for human (pro)renin receptor mRNA (n = 5). *p < 0.05 vs. untreated cells and cells treated with non-targeting siRNA.*

GTGG-3′; reverse, 5′-TGCTGGGTTCTTCGCTTGT-3′; probe, 5′-FAM-TTTGACACCTCCCTCATTAGGAAGAC AAGGACT-TAMRA-3′) and GAPDH (forward, 5′-TGACA ACTCCCTCAAGATTGTCA-3′; reverse, 5′-GGCATGGAC TGTGGTCATGA-3′; probe, 5′-FAM-TGCATCCTGCACC ACCAACTGCTTAG-TAMRA-3′), based on its previously reported cDNA sequence (*5*).

Silencing the Human (Pro)Renin Receptor

Human (pro)renin receptor mRNA was specifically knocked down using commercially available small interfering RNA (siRNA) oligonucleotides for ATP6AP2 (Dharmacon, Lafayette, USA). Before transfection, human VSMCs were maintained in DMEM with 10% fetal calf serum and then transfected with Dharmafect™ (Dharmacon), and the human (pro)renin receptor siRNA according to the manufacturer's instructions. Scrambled non-targeting siRNA was used as control.

Immunofluorescence Labeling for the (Pro)Renin Receptor

Cells were fixed with 4% paraformaldehyde and incubated in 100% methanol at −20°C for 10 min. After washing three times in PBS, the cells were incubated with the goat polyclonal anti-ATP6IP2 antibody (1:200 dilution; Abcam Inc., Cambridge, USA). After washing in PBS, the sections were incubated with TRITC-conjugated donkey anti-goat IgG (1:200 dilution; Molecular Probes Inc., Eugene, USA). Slides were examined with a Zeiss confocal microscope.

Statistical Analyses

Statistical comparisons were made by using two-way ANOVA and the Fisher's exact test. Values of $p<0.05$ were considered to indicate statistical significance. Data are reported as the means±SEM. Duplicate wells were analyzed for each experiment, and each experiment was performed independently at least five times.

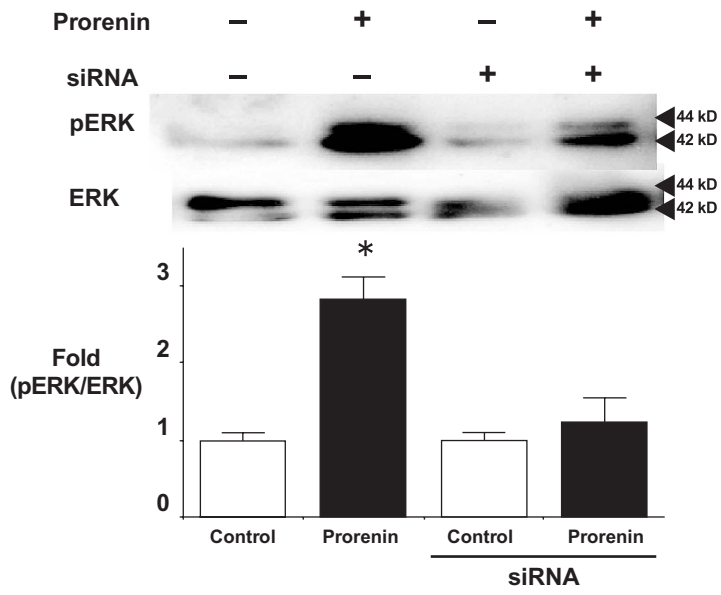

Fig. 6. *Effects of siRNA for human (pro)renin receptor mRNA on extracellular-signal–related protein kinase (ERK) activation at 20 min after the addition of 2 nmol/L human prorenin (n = 5). Data are presented as a representative Western blots. The relative density of phosphorylated ERK (pERK) to ERK is given in the bar graph. *p < 0.05 vs. the control. The siRNA, which reduced (pro)renin receptor expression to the control level, markedly inhibited ERK activation to an insignificant level.*

Results

Effects of Human Prorenin on MAPK Activation and Proliferation of Human VSMCs

Human recombinant prorenin did not affect the activation of JNK or p38 in human VSMCs ($n=5$) at any doses or incubation periods used in the present study (Fig. 1). However, human recombinant prorenin-treated human VSMCs ($n=4$) showed dose-dependent progressive increases in ERK phosphorylation (Fig. 2a). A 2.7-fold increase in ERK activation was observed 20 min following the addition of 2 nmol/L prorenin, which was the highest concentration of prorenin used in the present study. A time-dependent increase in ERK activation was also seen, with a 2.3-fold maximum increase at the 20 min interval ($n=4$; Fig. 2b). Significantly greater proliferation was seen in VSMCs treated with 2 nmol/L prorenin than in untreated VSMCs ($n=3$ in each; Fig. 2c). After 7 days in culture, the average number of VSMCs in the culture treated with prorenin was 1.6-fold greater than that in untreated VSMCs. The human prorenin–induced ERK activation was completely blocked by treatment with either the MEK inhibitor U0126 or the tyrosine kinase inhibitor genistein ($n=5$ in each; Fig. 3).

The Effects of Prorenin Were Independent of Angiotensin II Generation

We examined whether angiotensin II generated by non-proteolytic activation of prorenin (*13*) contributes to the prorenin-induced activation of ERK in human VSMCs ($n=5$). In the presence of the ACE inhibitor imidaprilat, prorenin significantly increased the ERK activation in human VSMCs, and the increase (2.8-fold) was similar to the 2.4-fold increase in the absence of imidaprilat (Fig. 4a). Treatment with the ARB candesartan significantly inhibited the ERK activation caused by angiotensin II but had no effect on the prorenin-induced activation of ERK in human VSMCs ($n=5$; Fig. 4b). The presence or absence of candesartan did not significantly affect the prorenin-induced ERK activation, with the average increases being 1.8- and 2.2-fold, respectively. The results indicate that the prorenin-induced activation of ERK was independent of angiotensin II in human VSMCs.

Presence and Role of the (Pro)Renin Receptor in Human VSMCs

To determine whether prorenin-induced ERK activation is mediated by the (pro)renin receptor, we measured the presence of human (pro)renin receptor mRNA and protein in human VSMCs (Fig. 5). Further, we showed that transfection of an siRNA targeting the (pro)renin receptor induced a significant decrease in receptor mRNA ($n=5$). Treatment of cells with Dharmafect or non-targeting control siRNA did not

affect (pro)renin receptor mRNA expression. Human VSMCs transfected for 24 h with 100 nmol/L siRNA molecules had no detectable (pro)renin receptor mRNA and showed significantly reduced induction of ERK activation with prorenin treatment ($n=5$; Fig. 6), suggesting that the human VSMC (pro)renin receptor mediates prorenin-induced activation of ERK.

Discussion

Binding of prorenin to the (pro)renin receptor promotes two mechanisms: activation of the RAS by non-proteolytic activation of prorenin and stimulation of the RAS-independent pathways emanating from the (pro)renin receptor (*14*). The present study clearly showed that recombinant human prorenin stimulated activation of ERK, but not p38 or JNK, independently of angiotensin II in human VSMCs, though it is well known that activation of type 1 angiotensin II receptor leads to phosphorylation of ERK in a variety of cells (*15*, *16*). Nonetheless, the prorenin-induced activation of ERK was here found to be dependent on tyrosine kinase(s) and MEK.

The ERK cascade is involved in the regulation of cell differentiation and proliferation by G-protein–coupled receptors. Once activated, ERK can translocate to the nucleus, where it is thought to regulate the expression of transcription factors, and thereby regulate cell differentiation and proliferation (*17*). In the present study, the (pro)renin receptor was present in human VSMCs, and VSMC proliferation was enhanced in its presence. In addition, a recent study demonstrated that hypertension develops in transgenic rats overexpressing the human (pro)renin receptor specifically in smooth muscle cells (*18*). This finding suggests that the (pro)renin receptor–mediated ERK activation in VSMCs may be involved in a vascular remodeling which induces an increase in peripheral vascular resistance, leading to the development of hypertension. However, the vascular remodeling process is caused by an imbalance between growth and apoptosis of VSMCs. Thus, further *in vivo* studies will be needed to clarify whether the (pro)renin receptor–mediated, angiotensin II–independent ERK activation stimulates the growth, inhibits the apoptosis, or both.

In cultured mesangial cells, renin/prorenin has been reported to stimulate activation of ERK (*5*) and expression of transforming growth factor-β (*7*) through a RAS-independent mechanism. Since the angiotensinogen-deficient mice have high prorenin levels in the juxtaglomerular area of the kidneys and develop glomerulosclerosis (*19*), stimulation of the (pro)renin receptor by prorenin can contribute to the development of angiotensin II–independent glomerulosclerosis. Likewise, the transgenic rats overexpressing rat prorenin specifically in the liver had high plasma prorenin levels with no increase in plasma renin activity or plasma angiotensinogen levels and developed arterial wall thickening and aortic wall hypertrophy without hypertension (*20*). Since the present study found that prorenin stimulates the activation of ERK in VSMCs, high plasma prorenin levels can stimulate the VSMC (pro)renin receptor and thereby cause cell proliferation independently of the activity of angiotensin II.

In conclusion, human prorenin caused a phosphorylation of ERK through angiotensin II–independent, (pro)renin receptor–mediated activation of tyrosine kinase and subsequent MEK in human VSMCs. Although further *in vivo* studies are needed to determine whether the prorenin effects observed in the present study play a role in development and progression of atherosclerosis, the results of the present study raise the possibility that the RAP system is a novel therapeutic target for atherosclerosis.

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