

Role of (pro)renin receptor in Ang II-mediated EGF receptor transactivation

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1. ABSTRACT

Prorenin-induced intracellular signaling pathway is not fully elucidated. We investigated whether the (pro)renin receptor mediates epidermal growth factor (EGF) receptor transactivation through angiotensin (Ang) II-dependent and -independent pathways in human embryo kidney 293 cells. Prorenin (2 nmol/L) caused biphasic phosphorylation of EGF receptor (Tyr992) and extracellular signal-regulated kinase (ERK) 1/2, peaking at 5 minutes followed by a decrease and a second peak at 60-120 minutes, whereas EGF receptor (Tyr1068) and Src were phosphorylated at only 120 minutes. These prorenin-induced phosphorylation processes were inhibited by (pro)renin receptor siRNA. Similarly, Ang II type 1 (AT₁) receptor blocker (ARB) or AT₁ receptor siRNA completely inhibited prorenin-induced phosphorylation of EGF receptor (Tyr1068) and Src, as well as the late peaks of EGF receptor (Tyr992) and ERK 1/2. However, early peaks of EGF receptor (Tyr992) and ERK 1/2 at 5 minutes were not effectively blocked by ARB or AT₁ receptor siRNA. Incubation with prorenin significantly increased Ang II levels of cell lysate. These data indicate that the (pro)renin receptor mediates EGF receptor transactivation in both Ang II-dependent and -independent pathways.

2. INTRODUCTION

Prorenin is generally known as an inactive precursor of mature renin; it is activated by proteolytic cleavage of a 43-amino acid prosegment. Binding of prorenin to the (pro)renin receptor promotes two distinctive mechanisms, the angiotensin (Ang) II-dependent and -independent pathways (1). (Pro)renin receptor-bound prorenin induces cleavage of angiotensinogen into Ang I without its proteolytic conversion to renin (nonproteolytic activation) (2-4). It has also been demonstrated that binding of prorenin to (pro)renin receptor directly triggers activation of intracellular signaling pathways, such as mitogen-activated protein (MAP) kinase, independent of Ang II generation (5-13).

We recently showed that prorenin-(pro)renin receptor binding directly activates extracellular signal-regulated kinase (ERK) 1/2 via epidermal growth factor (EGF) receptor-dependent signal transduction in rat vascular smooth muscle cells (VSMCs) (12). However, EGF receptor is reportedly not activated by prorenin in U937 monocytes (5). Thus, phosphorylation of EGF receptor by prorenin is controversial. In this regard, activation of the intracellular signaling pathway by

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prorenin-induced Ang II by nonproteolytic activation has also not yet been fully elucidated. Several tyrosine residues in EGF receptor play a regulatory role in mediating the intracellular signaling pathway (14). In VSMCs, Ang II phosphorylates at both Tyr992 and Tyr1068 sites of EGF receptor which activates c-Src and ERK 1/2 (15, 16). Different phosphorylation sites of EGF receptor may induce activation of different downstream signaling pathways.

Previous studies showed that Ang II levels in cell lysates were significantly increased by prorenin in cultured human podocytes and human embryo kidney (HEK) 293 cells (17, 18). However, our previous data showed that prorenin did not produce detectable Ang II in rat VSMCs (12). It is possible that the basal expression of (pro)renin receptor and other components of renin-angiotensin system (RAS) in rat VSMCs were insufficient to produce enough Ang II by prorenin to activate the downstream signals, such as EGF receptor.

In the present study, we hypothesized that prorenin activates intracellular signaling pathways via both Ang II-dependent and -independent mechanisms. To test this hypothesis, we evaluated the effects of prorenin on transactivation of EGF receptor in HEK293 cells which reportedly expressed all RAS components (17, 19-22). We also investigated the effects of prorenin on the downstream signaling pathway of EGF receptor, such as ERK 1/2 and Src phosphorylation.

3. MATERIALS AND METHODS

3.1. Materials

An active form of candesartan (CV11974), the Ang II type 1 (AT₁) receptor blocker (ARB), was kindly provided by Takeda Pharmaceutical Co. Ltd. (Osaka, Japan). Recombinant human prorenin was prepared from Chinese hamster ovary cell lines harboring human prorenin cDNA (23).

3.2. Cell culture

HEK293 cells were grown in Dubecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (100 U/mL; Life Technologies, Carlsbad, CA), streptomycin (100 µg/mL; Life Technologies) and maintained at 37°C under 5% CO₂/95% air in a humidified incubator.

3.3. Experimental protocols

Cells were treated with 2 nmol/L human recombinant prorenin or Ang II (100 nmol/L), and cells were harvested for analyses. Experiments were repeated in cells pretreated with candesartan (100 nmol/L), 60 minutes before prorenin treatment. In another series of experiments, we performed a transient transfection of HEK293 cells with siRNA of AT₁ receptor or (pro)renin receptor (ATP6AP2). The Stealth Select RNAi pre-designed against AT₁ receptor or (pro)renin receptor was synthesized by Life Technologies. HEK293 cells were transfected with Lipofectamine 2000 (Life Technologies)

and 100 pmol of siRNA for 48 hours, according to the manufacturer's protocol. As a negative control, a non-targeting scrambled siRNA (control siRNA) was purchased from Life Technologies. After 24 hours transfection, cells were washed and incubated with or without prorenin.

3.4. Western blot analysis

Following HEK293 cells incubation with prorenin or Ang II, protein was extracted as previously described (24). The protein concentration was determined by Bio-Rad Protein Assay using bovine serum albumin as the standard. Total protein extracts (30 µg) were electrophoretically separated using 10% SDS-polyacrylamide gels at 150 V for 90 minutes. Proteins were subsequently transferred electrophoretically onto a Hybond-ECL polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK). Blots were blocked for 60 minutes at room temperature with 5% skimmed milk in PBS with Tween 20. The sample protein was immunoblotted with appropriate anti-phospho-EGF receptor (Tyr992 and Tyr1068), anti-phospho-Src (Tyr416), anti-phospho-ERK 1/2 (Cell Signaling Technology, Danvers, MA), anti-(pro)renin receptor or anti-AT₁ receptor (N-10) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). To confirm equal protein loading, each membrane was reprobed with an antibody against -actin (Sigma-Aldrich), anti-EGF receptor (Millipore, Temecula, CA), anti-Src and anti-ERK 1/2 (Cell Signaling Technology). The protein bands were visualized with an ECL plus system (GE Healthcare). Band intensities were quantified by densitometry of the immunoblots using NIH ImageJ software.

3.5. Gene expression of the RAS components

Total RNA was extracted by ISOGEN (Nippon Gene, Tokyo, Japan) from HEK293 cells. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed applying a SYBR Green reaction mix, and was run on Applied Biosystems 7300 Real-Time PCR System (Life Technologies). The primers used for the RT-PCR analyses are summarized in Table 1. RT-PCR amplification was performed as follows: 35 cycles of denaturation for 2 minutes at 94°C, annealing for 1 minute at 58°C, and extension for 1 minute and 15 seconds at 72°C. The PCR products were size fractionated on 2% agarose gel, and visualized with ethidium bromide staining and ultraviolet (UV) transillumination. -actin was used as a housekeeping gene control. Human proximal tubular (HPT) cells were applied to positive controls, because HPT cells are known to have all the RAS components (25-27).

3.6. Ang II content of cell lysate

The cell lysate of HEK293 cells was collected from dishes with or without prorenin stimulation. After a treatment with human recombinant prorenin, culture medium was removed, and cells were washed twice with ice-cold PBS. Cells were extracted in 450 µL/well lysis buffer containing 20 mmol/L Tris-HCl, 10 mmol/L EDTA, 10 µmol/L captopril, 5 mmol/L EGTA, 5 mmol/L mercaptoethanol, 50 µg/mL phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin and 1 µg/mL pepstatin A. Each cell sample was scraped, collected into a microcentrifuge tube

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Table 1. The sequence of sense and antisense primers for RT-PCR of target gene mRNA

Gene	Sense Primer sequence (5'-3')	Antisense Primer sequence (5'-3')	GenBank accession number
AGT	ACTGCACCTCCCGCTGGAT	TGAACACGCCCACCACCGTG	NM000029
Renin	CCACCCCAAACCTTCAAAGTCG	TGCCACAACCCCATCAAATC	NM000537
ACE	GCAGCCCGGCAACTTTTCTGC	ATGTTGGTGTCTGCGCCCA	NM00789
AT ₁ R	TGATGCCATCCCAGAAAGTCGGC	CCTCCTCTGAGCTGTGGAGCCTT	NM031850
-actin	CACAGAGCCTCGCCTTTGCCGATC	ACGAGCGCGCGATATCATCATC	NM001101

AGT: angiotensinogen, ACE: angiotensin-converting enzyme, AT₁R: angiotensin II type 1 receptor

and centrifuged at 4°C, 13,000 rpm for 15 minutes. The supernatant was filtered on Amicon Ultra devices (10 kDa cut-off; Millipore). We measured Ang II concentration by radioimmunoassay using specific antibody for Ang II, monoiodinated ¹²⁵I-labeled Ang II, and Ang II standard as previously described (15, 28). In brief, each lyophilized antiserum was diluted sufficiently to yield a specific binding rate after incubation with a 9,000 cpm label for 48 hours at 4°C. Bound and free Ang II were separated with dextran-coated charcoal (10 mg of charcoal and 1 mg of dextran in 1 mL of assay buffer/tube). After centrifugation, the supernatants were decanted and counted 3 minutes in an automated gamma counter.

3.7. Statistical analysis

All results are expressed as mean ± S.E. Statistical significance was assessed using one-way ANOVA and Bonferroni's post-hoc test. A value of *P*<0.05 was considered to be statistically significant.

4. RESULTS

4.1. Effect of prorenin on EGF receptor, Src and ERK phosphorylation

Recombinant human prorenin (2 nmol/L) significantly increased phosphorylation of two different tyrosine sites (Tyr992 and Tyr1068) of EGF receptor, as well as Src and ERK 1/2 in a time-dependent manner (Figure 1, *n*=4, *P*<0.05). Prorenin augmented EGF receptor phosphorylation at Tyr992 not only at 5 minutes, but also at 60 and 120 minutes (Figure 1A, *n*=4, *P*<0.05). On the other hand, EGF receptor phosphorylation at Tyr1068 was increased at 120 minutes (Figure 1B, *n*=4, *P*<0.05). Phosphorylation of Src was increased at 60 and 120 minutes (Figure 1C, *n*=4, *P*<0.05), whereas ERK 1/2 phosphorylation was biphasic, similarly to Tyr992 on EGF receptor (Figure 1D, *n*=4, *P*<0.05). Ang II (100 nmol/L) increased phosphorylations of both EGF receptor (Tyr992) at 120 and 240 minutes, and ERK 1/2 at 240 minutes (Figures 2A and 2B).

4.2. Role of (pro)renin receptor in EGF receptor transactivation

To determine whether EGF receptor transactivation was mediated by the (pro)renin receptor, we measured the effect of (pro)renin receptor knockdown using siRNA on EGF receptor phosphorylation. The transfection of siRNA targeting the (pro)renin receptor decreased protein expression of (pro)renin receptor (Figure 3A), while scrambled siRNA did not affect protein expression. The depletion of (pro)renin receptor significantly reduced prorenin-induced EGF receptor (Tyr992) phosphorylation at both 5 and 120 minutes (Figure 3B, *n*=4, *P*<0.05). Prorenin-induced increase in

EGF receptor (Tyr1068) and Src phosphorylation were also blocked by siRNA of (pro)renin receptor (Figures 3C and 3D, *n*=4, *P*<0.05). Similarly, ERK 1/2 phosphorylation induced by prorenin was completely suppressed by (pro)renin receptor knockdown (Figure 3E, *n*=4, *P*<0.05).

4.3. EGF receptor transactivation induced by Ang II generation

To examine whether Ang II generated by nonproteolytic activation of prorenin was involved in EGF receptor transactivation, we examined the effect of an ARB (candesartan; CV11974) and siRNA of AT₁ receptor on EGF receptor transactivation. Pretreatment with candesartan (100 nmol/L) inhibited late-phase prorenin-induced phosphorylation of EGF receptor (Tyr992) (Figure 4A, *n*=4, *P*<0.05). Candesartan treatment did not affect EGF receptor phosphorylation at 5, 10 or 20 minutes. Prorenin-induced phosphorylation of EGF receptor (Tyr1068) and Src were completely suppressed by treatment with candesartan (Figures 4B and 4C, *n*=4). Candesartan did not affect ERK 1/2 at 5 minutes, but suppressed its phosphorylation at late phase (Figure 4D, *n*=4, *P*<0.05). The transfection of siRNA targeting AT₁ receptor decreased AT₁ receptor protein expression (Figure 5A). The AT₁ receptor siRNA failed to inhibit EGF receptor (Tyr992) phosphorylation at 5 minutes, and also reduced prorenin-induced EGF receptor transactivation at 120 minutes (Figures 5B, 5C, 5D and 5E, *n*=4, *P*<0.05). The ERK 1/2 phosphorylation was increased, in the same manner as EGF receptor (Tyr992) (Figure 5E, *n*=4, *p*<0.05).

We measured Ang II concentration in cell lysate to investigate whether Ang II levels were increased by prorenin. First, we confirmed mRNA expression of all RAS components by RT-PCR in HEK293 cells (Figure 6A). Prorenin significantly increased Ang II concentrations in cell lysates (Figure 6B, *n*=6, *p*<0.05).

5. DISCUSSION

We confirmed that HEK293 cells express all RAS components in agreement with previous studies (17, 19-22). The present study also showed that human recombinant prorenin binds to the (pro)renin receptor and activates intracellular signaling pathways via both Ang II-dependent and -independent mechanisms in HEK293 cells (Figure 7). Prorenin triggers transactivation of two different EGF receptor tyrosine sites (Tyr992 and Tyr1068). Early-peak prorenin-induced ERK 1/2 phosphorylation is associated with EGF receptor (Tyr992) phosphorylation. Consistent with our previous results in VSMCs (12), we could not detect the early-peak EGF receptor (Tyr1068) phosphorylation in

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HEK293

cells.

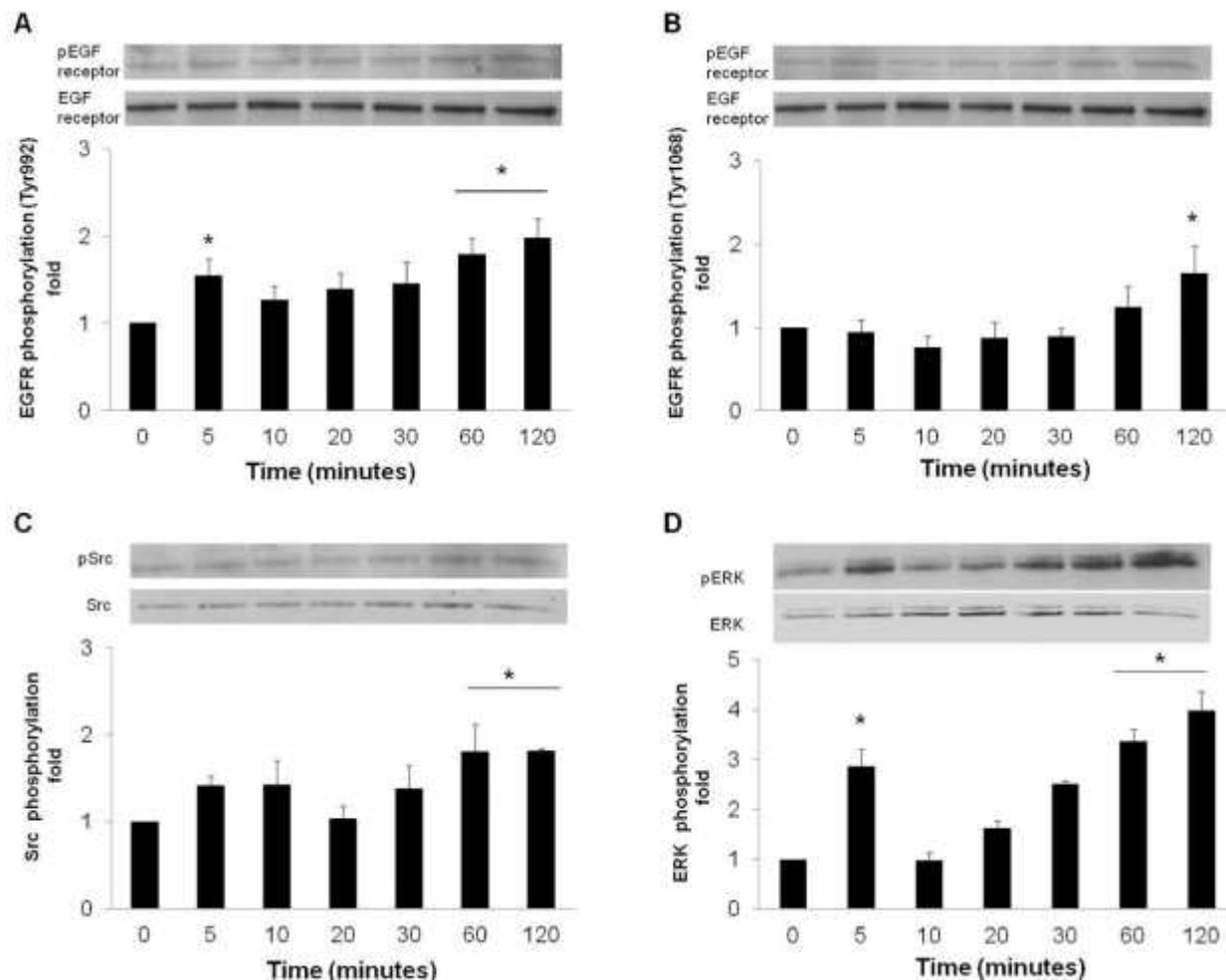


Figure 1. Effect of prorenin on phosphorylation of EGF receptor at Tyr992 (A) and Tyr1068 (B), Src (C) and ERK 1/2 (D) in HEK293 cells. Bar graphs represent mean \pm SE (n=4), expressed as fold change compared with vehicle-treated cells. * P <0.05 vs. vehicle-treated cells.

Thus, EGF receptor transactivation was induced differently by prorenin stimulation depending on which of its tyrosine sites were phosphorylated, as suggested by other investigators (5). The present study also showed that EGF receptor Tyr992 and Tyr1068 sites, as well as Src and ERK 1/2 phosphorylation, are significantly activated by prorenin at the late phase. Previous studies indicated that transactivations of EGF receptor Tyr992 and Tyr1068 play a role in the activation of ERK/MAPK cascade (29). In addition, EGF receptor (Tyr992) induces PKC-dependent ERK 1/2 phosphorylation via PLC (30); EGF receptor (Tyr1068) associates with the adaptor Grb2, which is the main step in EGF receptor-dependent induction of RAS/MAPK (30). Further studies with site-directed mutation of each EGF receptor tyrosine residue are needed to clarify which tyrosine residues are associated with phosphorylation of Src and ERK 1/2. Since (pro)renin receptor siRNA attenuated both early- and

late-phase EGF receptor transactivation, both phases are mediated by (pro)renin receptor.

Another novel finding in the present study is that prorenin-induced EGF receptor, Src and ERK 1/2 phosphorylation at 120 minutes are blocked by an ARB or AT₁ receptor siRNA, whereas early-peak EGF receptor (Tyr992) and ERK 1/2 phosphorylation are not inhibited. We also confirmed that Ang II levels of cell lysate were significantly increased after prorenin treatment. These results indicate that prorenin increases Ang II production, which induces EGF receptor transactivation and is associated with Src and ERK 1/2 phosphorylation. However, Ang II levels in cell medium were under detectable range. Although Ang II levels in cell lysate may reflect Ang II generation in cells, further evaluation of Ang II levels in cell medium will be needed. The siRNAs of both (pro)renin receptor and AT₁ receptor inhibited prorenin-induced EGF receptor transactivation at 120 minutes. Therefore, Ang II generated by nonproteolytic activation of prorenin might induce EGF receptor

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transactivation via the activation of AT₁ receptor. Previous studies showed that Ang II levels in the cell lysate are elevated in human podocytes and HEK293 cells by

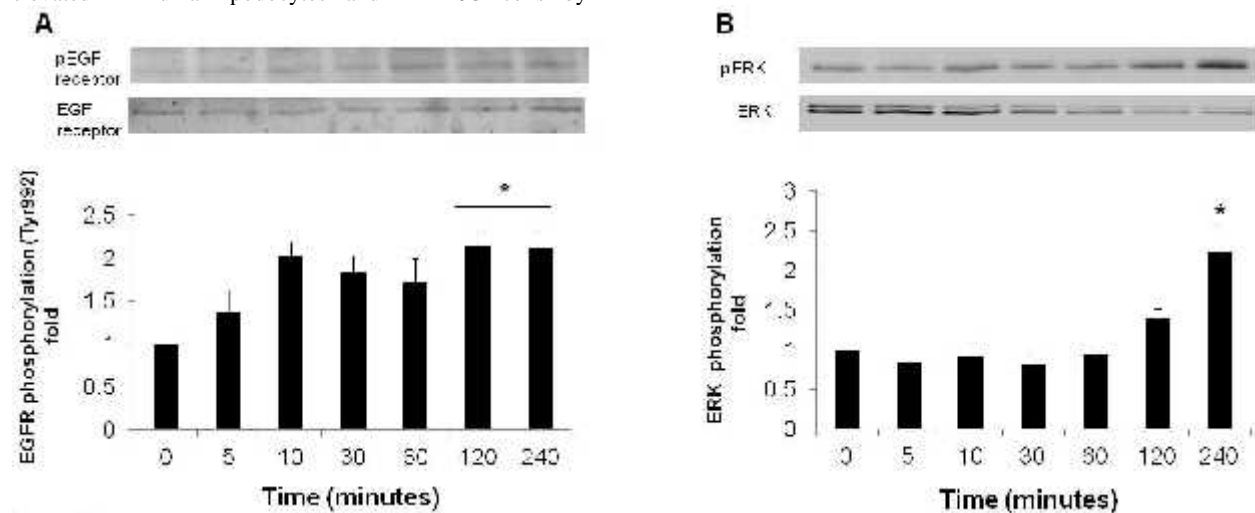


Figure 2. Effect of Ang II on phosphorylation of EGF receptor at Tyr992 (A) and ERK 1/2 (B) in HEK293 cells. Bar graphs represent mean \pm SE (n=4), expressed as fold change compared with vehicle-treated cells. * P <0.05 vs. vehicle-treated cells.

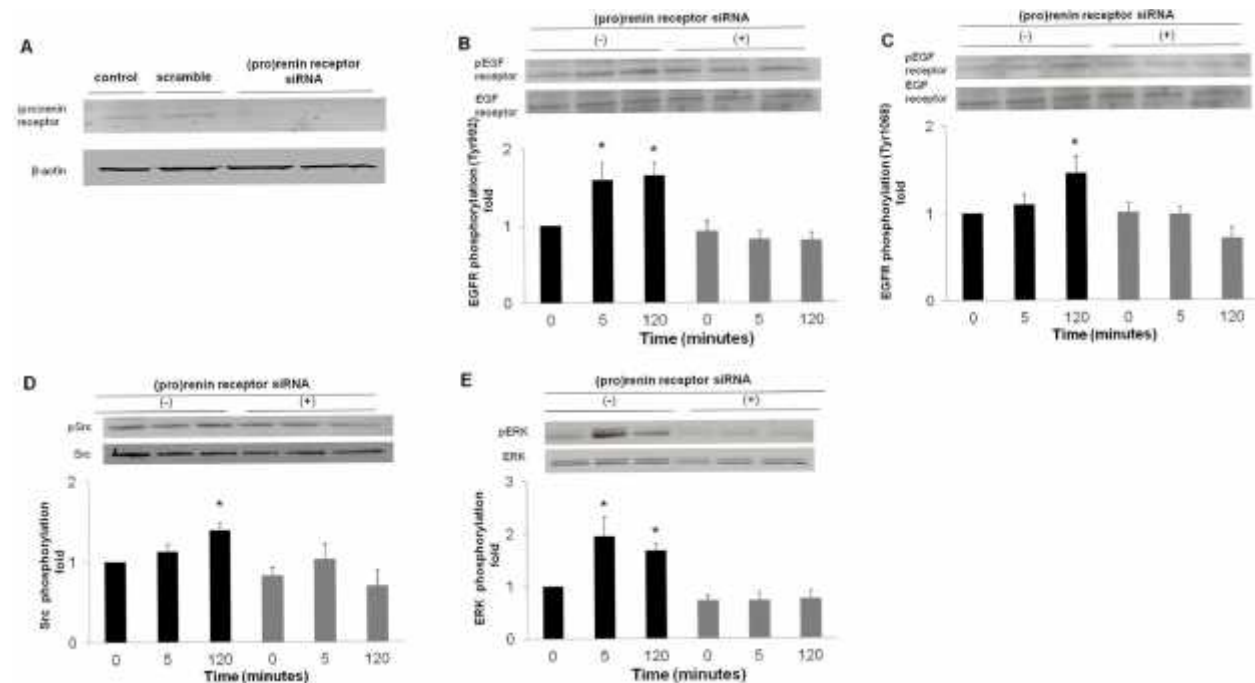


Figure 3. Effect of (pro)renin receptor siRNA on (pro)renin receptor protein expression (A), and prorenin-induced phosphorylation of EGF receptor at Tyr992 (B) and Tyr1068 (C), Src (D) and ERK 1/2 (E) in HEK293 cells. Bar graphs represent mean \pm SE (n=4), expressed as fold change compared with vehicle-treated cells. * P <0.05 vs. vehicle-treated cells.

prorenin stimulation (17, 18). Furthermore, EGF receptor is transactivated via the activation of AT₁ receptor induced by Ang II in VSMCs and HEK293 cells (15, 16, 31).

As shown in previous studies using mesangial cells (3, 32), VSMCs (9, 12), vascular endothelial cells (13)

and U937 monocytes (5), the present results indicate that prorenin-induced early-peak EGF receptor (Tyr992) and ERK 1/2 phosphorylation is mediated through mechanisms that are independent of Ang II generation (12). On the other hand, our results also indicate that prorenin-induced late-phase EGF receptor, Src and ERK 1/2 phosphorylation

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are mediated by Ang II-dependent pathways. Previous studies failed to show Ang II-dependent EGF receptor and ERK 1/2 phosphorylation. A possible reason may be due to the observation time. In the present study, Ang II levels of cell lysate increased at 120 minutes after prorenin stimulation in HEK293 cells, while the period for prorenin

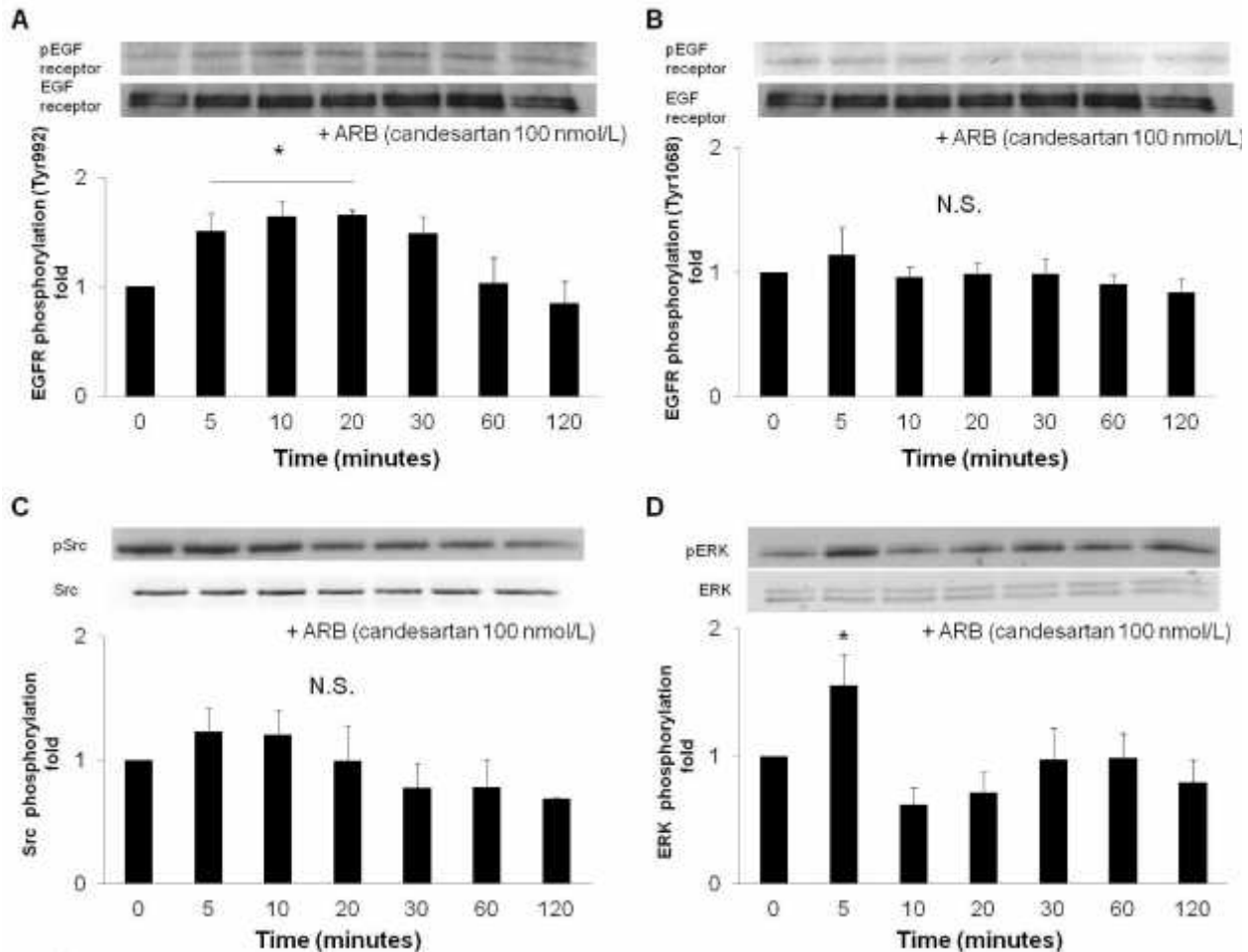


Figure 4. Effect of an ARB on prorenin-induced phosphorylation of EGF receptor at Tyr992 (A) and Tyr1068 (B), Src (C) and ERK 1/2 (D) in HEK293 cells. Bar graphs represent mean \pm SE (n=4), expressed as fold change compared with vehicle-treated cells. * P <0.05 vs. vehicle-treated cells

stimulation was limited to less than 60 minutes in previous studies. Another possibility is the basal expression of RAS components in cells. We and others showed that HEK293 cells express all the RAS components (17, 19-22), which could generate endogenous Ang II, leading to activation of the intracellular signaling pathway in the end. However, it is unclear whether previously used cultured cells actually express all RAS components.

Basically, biphasic phosphorylation is an important issue, because there are two independent mechanisms of phosphorylation. Early phosphorylation occurred in an Ang II-independent manner, and later phosphorylation in an Ang II-dependent manner. Further experiments are needed to know the clinical implications

of biphasic response, focusing on which phase of phosphorylation contributes to a proliferating signaling pathway. In addition, conventional RAS blockade such as ARB failed to inhibit early intracellular signaling activation, although ARB completely attenuated late-phase of activation. Therefore, only depletion of prorenin and renin, or inhibition of (pro)renin receptor bindings may attenuate Ang II-independent proliferative signaling pathways. These findings suggest that prorenin-(pro)renin receptor activation may be a new target to prevent proliferative changes in cardiovascular and renal disease. Further studies are required to elucidate the clinical benefits of inhibiting Ang II-independent activation of proliferative signaling pathway induced by (pro)renin receptor upon attenuating cardiovascular and renal pathogenesis.

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In conclusion, the present study demonstrates that both binding of prorenin to (pro)renin receptor, and binding of Ang II generated by nonproteolytic activation to AT₁ receptor, lead to activation of the intracellular signaling pathway, including EGF receptor, Src and ERK 1/2.

6. ACKNOWLEDGMENTS

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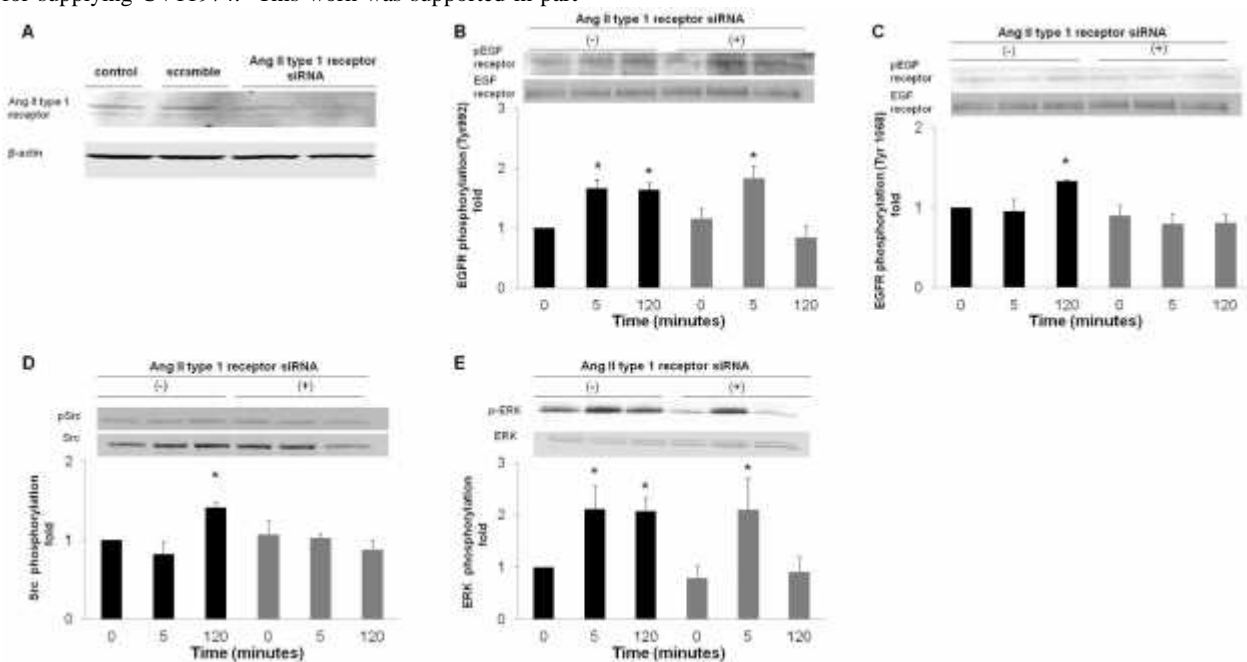
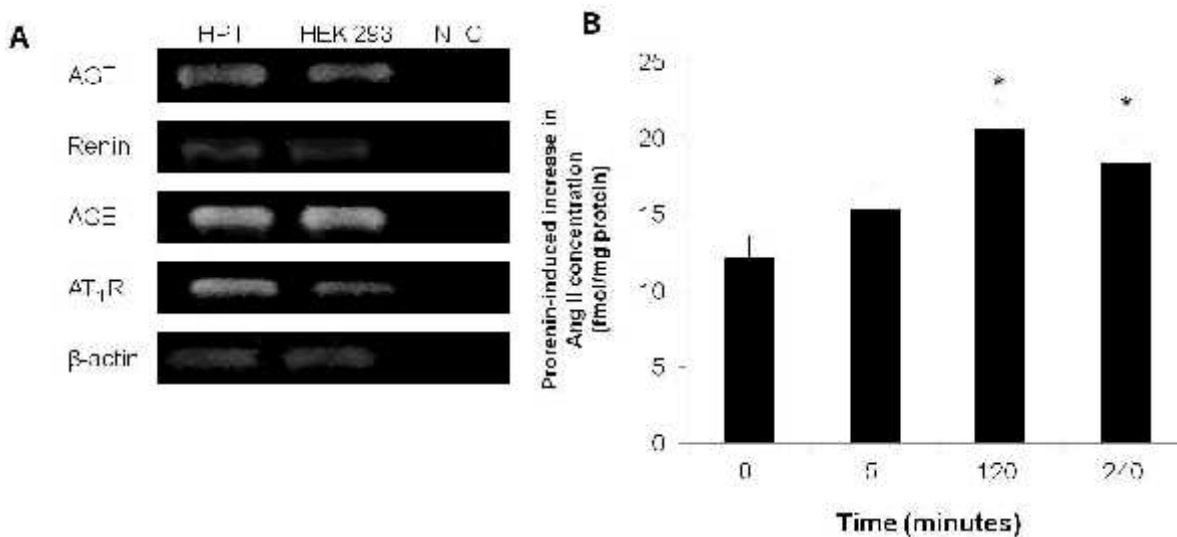


Figure 5. Effect of AT₁ receptor siRNA on AT₁ receptor protein expression (A), and prorenin-induced phosphorylation of EGF receptor at Tyr992 (B) and Tyr1068 (C), Src (D) and ERK 1/2 (E) in HEK293 cells. Bar graphs represent mean \pm SE (n=4), expressed as fold change compared with vehicle-treated cells. * $P < 0.05$ vs. vehicle-treated cells.



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Figure 6. RT-PCR expression analysis of all the RAS components. HPT and HEK293 cells were analyzed by RT-PCR. AGT: angiotensinogen; ACE: angiotensin-converting enzyme. β -actin served as positive control. A reaction without a non-template control served as negative control (NTC). Intracellular Ang II concentration in HEK293 cells (B). Bar graphs represent mean \pm SE (n=6), expressed as fold change compared with vehicle-treated cells. * P <0.05 vs. vehicle-treated cells.

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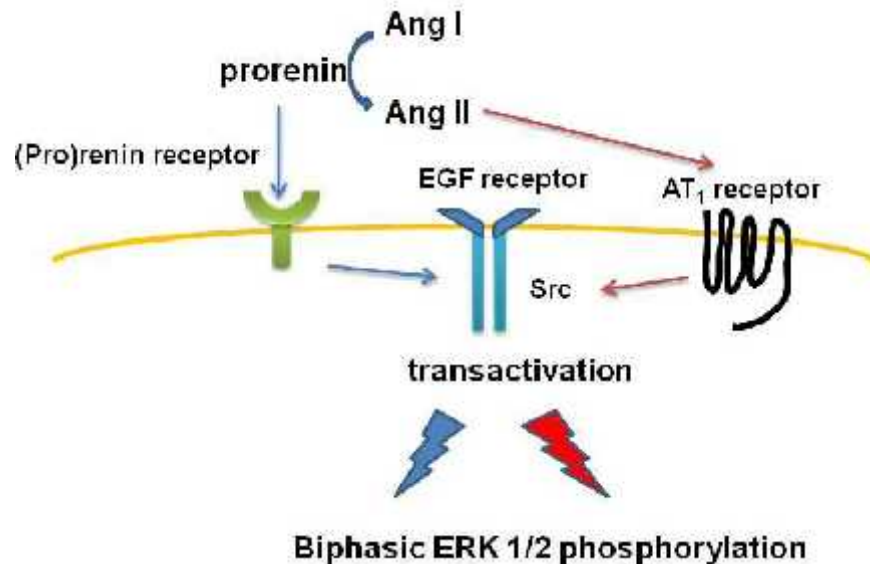


Figure 7. Proposed molecular mechanisms of prorenin-induced biphasic activation of intracellular signaling. See Discussion for details.

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