Angiotensin II type 2 receptor-stimulated activation of plasma prekallikrein and bradykinin release: role of SHP-1

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Zhu L, Carretero OA, Xu J, Wang L, Harding P, Rhaleb NE, Yang JJ, Sumners C, Yang XP. Angiotensin II type 2 receptor-stimulated activation of plasma prekallikrein and bradykinin release: role of SHP-1. Am J Physiol Heart Circ Physiol 302: H2553–H2559, 2012. First published April 20, 2012; doi:10.1152/ajpheart.01157.2011.—ANG II type 2 receptors (AT2R) elicit cardioprotective effects in part by stimulating the release of kinins; however, the mechanism(s) responsible have not been fully explored. We demonstrated previously that overexpression of AT2R increases expression of prolylcarboxypeptidase (PRCP; a plasma prekallikrein activator) and release of bradykinin by mouse coronary artery endothelial cells (ECs). In the present study we hypothesized that the AT2R-stimulated increase in PRCP is mediated by the tyrosine phosphatase SHP-1, which in turn activates the PRCP-dependent prekallikrein–kallikrein pathway and releases bradykinin. We found that activation of AT2R using the specific agonist CGP42112A increased SHP-1 activity in ECs, which was blocked by the AT2R antagonist PD123319. Activation of AT2R also enhanced conversion of plasma prekallikrein to kallikrein, and this effect was blunted by a small interfering RNA (siRNA) to SHP-1 and abolished by the tyrosine phosphatase inhibitor sodium orthovanadate. Treating cells with a siRNA to PRCP also blunted AT2R-stimulated prekallikrein activation and bradykinin release. Furthermore, blocking plasma kallikrein with soybean trypsin inhibitor (SBTI) abolished AT2R-stimulated bradykinin release. These findings support our hypothesis that stimulation of AT2R activates a PRCP-dependent plasma prekallikrein pathway, releasing bradykinin. Activation of SHP-1 may also play an important role in AT2R-induced PRCP activation.

Prolylcarboxypeptidase (PRCP) is a serine carboxypeptidase that cleaves a variety of COOH-terminal amino acids adjacent to proline and has been linked to hypertension and preeclampsia (1, 32) as well as obesity (23). Dysfunction of PRCP is associated with adverse cardiovascular events such as hypertension and inflammation (18). In recent years PRCP-dependent plasma prekallikrein activation on endothelial cells (ECs) has received much attention. When the complex of high-molecular-weight kininogen (HMWK) and plasma prekallikrein binds to the endothelial membrane, PRCP converts plasma prekallikrein to kallikrein, which then cleaves HMWK, generating bradykinin, which leads to NO and prostacyclin formation as well as vasodilatation, probably by activating constitutive kinin B2 and inducible B1 receptors (15, 24, 25). Overexpression of PRCP in cultured Chinese hamster ovary cells and ECs enhanced plasma prekallikrein activation and release of kinins and NO, and these effects can be blocked by a small interfering RNA (siRNA) to PRCP (18, 26, 37). These data confirm the role of PRCP-dependent plasma prekallikrein activation in bradykinin release. It is suggested that PRCP constitutively regulates bradykinin production, thereby maintaining the integrity of the endothelium (20). Because PRCP activates prekallikrein to kallikrein, leading to formation of bradykinin and NO, it might be considered a negative regulator of the pressor actions of the renin-angiotensin system (12) as well as a means of maintaining stores of NO (23).

The role of PRCP in AT2R-mediated bradykinin release is not fully understood. According to one study, the AT2R antagonist losartan increased PRCP expression in hypertensive rats (22). Recently we demonstrated that overexpression of AT2R in ECs increased expression of PRCP and release of bradykinin, whereas stimulating AT2R with the agonist CGP42112A increased bradykinin release further (38). However, the signaling and molecular mechanism(s) by which AT2R activates PRCP have not been explored to our knowledge. SHP-1, a Src homology region 2 domain-containing protein tyrosine phosphatase-1, has been implicated in the AT2R intracellular pathway, which negatively interacts with AT2R signaling (2, 27). We hypothesized that activation of AT2R enhances tyrosine phosphatase SHP-1 activity, which stimulates PRCP and thereby increases conversion of plasma prekallikrein to kallikrein, liberating bradykinin. To test this possibility, we conducted studies in mouse coronary artery ECs transfected with the AT2R gene and examined 1) the effect of AT2R activation on SHP-1 activity, 2) whether inhibition of SHP-1 or
protein tyrosine phosphatase diminishes or blocks AT2R-induced plasma prekallikrein activation, 3) the effect of PRCP inhibition on AT2R-stimulated plasma prekallikrein activation and bradykinin release, and 4) the effect of blocking plasma kallikrein with soybean trypsin inhibitor (SBTI) on AT2R-stimulated bradykinin release.

**MATERIALS AND METHODS**

**EC cultures.** Coronary artery ECs were isolated from 10-week-old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) as described previously (30) and identified as ECs by uptake of acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3',3'-tetramethyindocarbocyanine perchlorate and immunostaining with an antibody to von Willebrand factor (38). The study was approved by the Henry Ford Health System Institutional Animal Care and Use Committee (IACUC) in accord with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. ECs were maintained in medium 199-F-12 (1:1), 1% penicillin-streptomycin (Invitrogen), 100 μg/ml EC growth supplement (Sigma), and 10% FBS (HyClone). All experiments were conducted with cells between passages 3 and 5.

**AT2R gene transfer to ECs.** An adenoviral vector containing genomic AT-R DNA and a green fluorescent protein gene driven by a cytomegalovirus promoter was used to induce overexpression of AT2R in ECs (8). Cells were transfected with Ad-AT2R at a multiplicity of infection of 80 for 24 h.

**Immunoprecipitation and SHP-1 activity.** SHP-1 activity was determined by immunoprecipitation of SHP-1 followed by a colorimetric assay using a tyrosine phosphatase assay kit from Promega. Cells seeded in a 100-mm tissue culture dish were incubated overnight with medium 199-F-12 (1:1) containing 0.5% FBS, then washed with PBS and incubated for 1 h in serum free medium containing 20 nM HMWK. Prekallikrein (PK; 20 nm) was then added, and the mixture incubated for 1 h in the absence or presence of the AT2R antagonist PD123319 (100 μM). Cells were then treated with or without (as control) the AT2R agonist CGP42112A (0.1 μM) for 2 h. After the incubation, cells were solubilized for 30 min at 4°C in lysis buffer containing 25 mM Tris-HCl (pH 7.2), 2 mM EDTA, 0.4% Triton X-100, and a cocktail of protease inhibitors. After centrifugation at 10,000 g for 10 min at 4°C, the supernatant (200 μg proteins) was incubated with anti-SHP-1 antibodies (BD Transduction Laboratories) overnight at 4°C and then precipitated with protein A/G PLUS-agarose (Santa Cruz) according to the manufacturer’s protocol. After extensive washing, the immunocomplexes were resuspended in 42 μl phosphatase buffer containing (in mM) 25 Tris-HCl (pH 7.2) and 2 EDTA, and phosphatase activity was measured using a tyrosine phosphatase assay kit as instructed by Promega. After 40 min incubation at 37°C, the reaction was terminated by adding molybdate dye, and color induced by free phosphate released from the phosphotyrosine peptide was measured in an enzyme-linked immunosorbent assay reader at 620 nm.

**Small interfering RNA.** The SHP-1 and PRCP siRNAs were purchased from Santa Cruz, and the control scrambled siRNA was purchased from Qiagen. The siRNA was transfected into ECs using an enzyme immunoassay kit as described previously (38). Cells were incubated overnight in 6-well plates with medium 199-F-12 (1:1) containing 0.5% FBS, then washed with PBS and incubated for 1 h in serum free medium containing 2 nM HMWK, Prekallikrein (PK; 2 nM) and 10 μM captopril were then added, and the mixture incubated for 1 h in the absence or presence of the AT2R antagonist PD123319 (100 μM), SBTI (1 μM), or the tyrosine phosphatase inhibitor sodium orthovanadate (100 μM). The AT2R agonist CGP42112A (0.1 μM) was then added, and the mixture incubated for 2 h. Finally, 1 ml of the medium was collected in a test tube containing the peptidease inhibitor cocktail and bradykinin measured as described previously (38). Bradykinin concentration was calculated according to a calibration curve, normalized to total protein, and expressed as picograms per microgram protein.

**Statistical analysis.** Results are expressed as means ± SE. Student’s two-sample t-test was used to compare differences between two groups. Bonferroni’s adjustment was applied in multiple comparisons. A difference was considered significant if the adjusted P value was less than 0.05.

**RESULTS**

**Effects of AT2R activation on SHP-1 activity.** We first examined the effect of AT2R activation on tyrosine phosphatase SHP-1 activity and found that activating AT2R using its agonist CGP42112A significantly increased SHP-1 activity, and this effect was blocked by the AT2R antagonist PD123319 (Fig. 1). We then examined the effect of AT2R activation on

![Fig. 1. Effect of ANG II type 2 receptor (AT2R) stimulation with the agonist CGP42112A (CGP) on SHP-1 activity. AT2R-transfected cells were activated with CGP (0.1 μM) in the presence or absence of the AT2R antagonist PD123319 (PD; 100 μM; n = 4 to 5).](http://ajpheart.physiology.org/)
prekallikrein activation by measuring kallikrein activity. Kal-
likrein activity is assayed by measuring liberation of parani-
troanilide from S2302, a synthetic substrate for plasma kal-
likrein. We found that kallikrein activity was undetectable in
the absence of HMWK and/or prekallikrein in AT2R-trans-
ected EC cells; however, when HMWK and prekallikrein
were added, stimulating AT2R with CGP42112A significantly
increased conversion of plasma prekallikrein to kallikrein com-
pared with AT2R-transfected cells. This effect was diminished
by the AT2R antagonist PD123319 (Fig. 2). These data confirm
that activating AT2R increased plasma prekallikrein activation,
and this effect was AT2R specific.

Effect of blocking SHP-1 or protein tyrosine phosphatase on
AT2R-stimulated prekallikrein activation. We next studied
whether knockdown of SHP-1 with a siRNA or inhibition of
tyrosine phosphatase with sodium orthovanadate (vanadate)
blocks AT2R-mediated prekallikrein activation. We found that
the SHP-1 siRNA reduced SHP-1 protein expression by 75%
compared with the control, a scrambled siRNA (Fig. 3A); moreover, the increase in plasma kallikrein activity stimulated
by the AT2R agonist CGP42112A was significantly diminished
by the SHP-1 siRNA compared with the scrambled control
(Fig. 3B). In addition, the tyrosine phosphatase inhibitor so-
dium orthovanadate abolished both AT2R-stimulated plasma
callikrein activity (Fig. 4A) and the AT2R-stimulated increase
in bradykinin release (Fig. 4B). These data suggest that SHP-1
could be an important upstream component in the AT2R
intracellular cascade and that tyrosine phosphatase activity
could be a critical regulator of AT2R-stimulated prekallikrein
activation and bradykinin release.

Effect of PRCP blockade on AT2R-stimulated plasma pre-
kalikrein activation. We reported previously that overexpres-
sion of AT2R increased PRCP mRNA and protein expression
and blocking PRCP with a siRNA reduced basal and AT2R-
stimulated release of bradykinin, suggesting that PRCP plays
an important role in AT2R-mediated bradykinin release (38).
We now tested whether the effect of PRCP on AT2R-stimu-
lated bradykinin release is mediated via activation of plasma
prekallikrein. Because a specific PRCP inhibitor is not com-
mmercially available, we again used a PRCP siRNA to deter-
mine the effect of PRCP blockade on kallikrein activity and
bradykinin release in AT2R-transfected cells stimulated with
the AT2R agonist CGP42112A. We found that PRCP siRNA pre-
vented CGP42112A-stimulated prekallikrein activation (Fig. 5A)
and bradykinin release (Fig. 5B), which were not observed with
the scrambled siRNA controls. These data confirm that AT2R-
stimulated prekallikrein activation is PRCP-dependent and
contributes to AT2R-stimulated bradykinin release.

Effect of plasma kallikrein inhibition on AT2R-stimulated
bradykinin release. SBTI reportedly inhibits plasma kallikrein
(16). To confirm the role of plasma kallikrein in AT2R-
stimulated bradykinin release, we blocked plasma kallikrein
with SBTI and found that this abolished both endogenous
callikrein activity and AT2R-stimulated prekallikrein activation
or formed plasma kallikrein activity (Fig. 6A). Likewise

Fig. 2. Effect of AT2R stimulation on plasma kallikrein activity. AT2R-
transfected cells were incubated with HEPES-NaHCO3 buffer in the presence
or absence of 1) high-molecular-weight kininogen (HMWK) and/or plasma
prekallikrein (PK), 2) the AT2R agonist CGP42112A (CGP, 0.1 μM), or 3) the
AT2R antagonist PD123319 (PD; 100 μM; n = 12–15).

Fig. 3. Effect of SHP-1 blockade on AT2R-stimulated plasma pre-
kalikrein activation. AT2R-transfected cells were treated with a scram-
bled siRNA (Sc-siRNA) or SHP-1 small interfering RNA (SHP-1-siRNA). A: repre-
sentative Western blot showing SHP-1 protein expression (top) and quantitative data
(bottom). B: cells activated with the AT2R agonist CGP42112A (CGP; 0.1 μM; n = 12–30).
bradykinin release by AT2R-transfected cells or stimulated by the AT2R agonist was also abolished by SBTI (Fig. 6B), confirming that the increased plasma kallikrein activity is responsible for AT2R-stimulated bradykinin release.

**DISCUSSION**

We found that activation of AT2R in mouse coronary artery ECs increased both tyrosine phosphatase SHP-1 activity and plasma prekallikrein activation. Knockdown of SHP-1 or inhibition of tyrosine phosphatase reduced or prevented AT2R-induced plasma prekallikrein activation. Blocking the plasma prekallikrein activator PRCP using a siRNA diminished AT2R-stimulated plasma kallikrein activity and bradykinin release. We believe these results provide the first evidence that activation of AT2R increases plasma kallikrein activity, which may involve SHP-1-dependent activation of PRCP. In turn PRCP transforms plasma prekallikrein to kallikrein, thereby liberating bradykinin from HMWK.

We and others have demonstrated a cardioprotective role of AT2R, partially due to stimulation of kinins (5, 10, 11, 34–36). However, the mechanism by which AT2R increases kinins is not fully understood. Tsutsumi et al. (31) reported that activation of AT2R decreased cellular pH associated with increased kininogenase activity in the mouse aorta (31), suggesting the existence of an acid-optimal kininogenase or kininogenase-like acidic protease that is responsible for release of kinins from the vasculature. Interestingly, PRCP has an optimal acid pH (19) and has been described as a novel plasma prekallikrein activator. Shariat-Madar et al. (25) reported that when plasma prekallikrein binds to HMWK on ECs, PRCP rapidly converts prekallikrein to kallikrein, which cleaves HMWK and liberates bradykinin. We recently reported that overexpression of AT2R in mouse coronary artery ECs increased PRCP mRNA and protein expression as well as bradykinin release (38). In the present study we further demonstrated that stimulation of AT2R increased plasma kallikrein activity, whereas in the absence of HMWK and/or prekallikrein such kallikrein activity was undetectable. Pretreatment with SBTI completely abolished both kallikrein activity and bradykinin release. However, the SBTI data must be interpreted with caution, since this
compound is not a specific inhibitor for plasma kallikrein. Importantly, blockade of PRCP using a siRNA blunted AT_{2}R-stimulated plasma prekallikrein activation and bradykinin release. Taken together, our data suggest that PRCP-dependent plasma prekallikrein activation is at least partially responsible for AT_{2}R-stimulated kinin release. We confirmed that activation of plasma prekallikrein by the AT_{2}R agonist CGF42112A or ANG II in the presence of the AT_{1}R blocker valsartan is AT_{2}R-specific, since it was blocked by the AT_{2}R antagonist PD123319 (data not shown).

The precise signaling and molecular mechanism(s) by which AT_{2}R activates PRCP has not been explored to our knowledge. AT_{2}R reportedly enhanced activity of protein tyrosine phosphatase SHP-1 (a tyrosine phosphatase containing Src homology 2 domains) in various cells and tissues, including rat fetal vascular smooth muscle cells (VSMC), rat neurons, and fetal rat tissues (2, 4, 9, 29, 33). Sohn et al. (28) reported that treating human umbilical vein ECs with ANG II for 15 min markedly increased SHP-1 activity, whereas Min et al. (13) reported that ANG II significantly increased SHP-1 activity in vascular smooth muscle cells, reaching a maximum at 4 h. Because we found that AT_{2}R activated plasma prekallikrein and increased bradykinin within 2 h, we wanted to know whether SHP-1 activity was altered at the same time. We observed that AT_{2}R stimulation activated SHP-1 in mouse coronary artery ECs, and this effect was blocked by the AT_{2}R antagonist PD123319, confirming that the role of AT_{2}R in activating protein tyrosine phosphatases. Activation of SHP-1 has been known to negatively regulate cell proliferation and growth via dephosphorylation of critical molecules involved in proliferative pathways (2, 4, 17), which may contribute to the antihypertrophic and antigrowth effects of AT_{2}R. However, we know of no study addressing whether AT_{2}R-induced activation of PRCP and/or kinin release involves SHP-1 or any other tyrosine phosphatases. Using a siRNA to knock down SHP-1, we found that SHP-1 siRNA partly but significantly reduced the AT_{2}R-induced increase in plasma kallikrein activity. Moreover, inhibition of tyrosine phosphatase with sodium orthovanadate abolished AT_{2}R-stimulated plasma prekallikrein activation and bradykinin release. Because plasma prekallikrein activation is an indirect measure of PRCP activity (18, 25) and PRCP is known to have several tyrosine phosphorylation sites, our data suggest that AT_{2}R-stimulated PRCP-dependent plasma prekallikrein activation is mediated at least in part via SHP-1.

In summary, we have shown that activation of AT_{2}R by a specific agonist increases plasma prekallikrein activation and bradykinin release from mouse coronary artery ECs. These effects were blocked by the AT_{2}R antagonist PD123319 or knockdown of PRCP. Furthermore, knockdown of SHP-1 or inhibition of the tyrosine phosphatase significantly reduced PRCP activity as indicated by decreased plasma prekallikrein activation. Therefore, we conclude that AT_{2}R-stimulated bradykinin release is mediated in part by a PRCP-dependent plasma prekallikrein activation pathway in mouse coronary artery ECs, while SHP-1 may play an important role in AT_{2}R-stimulated bradykinin release in endothelial cells. Stimulation of AT_{2}R with an AT_{2}R agonist or ANG II in the presence of an AT_{1}R blocker (ARB) activates the protein tyrosine phosphatase SHP-1, which may positively regulate activation of PRCP; in turn, PRCP activates PK to plasma kallikrein, which liberates bradykinin from HMWK. Arrows (↑) indicate an increase in factors.
stimulated PRCP activation. (The schematic flow chart of the possible signaling mechanism in ECs is shown in Fig. 7.)

**Perspectives**

A large body of evidence has suggested that AT2R expressed in the vasculature may have vasodilator properties via release of kinins and NO, counteracting AT1R and leading to cardiovascular protection. However, the precise mechanism(s) by which AT2R stimulates kinins and NO is not fully understood. The present study demonstrates the signaling cascade involved in AT2R-mediated bradykinin release in ECs, which could help to clarify the mechanisms responsible for the cardioprotective role of AT2R. Because AT2R stimulation could be a potential therapeutic option in the treatment of cardiovascular disease, a study of this signaling cascade is of great clinical relevance.

**REFERENCES**


