Role of Prolylcarboxypeptidase in Angiotensin II Type 2 Receptor–Mediated Bradykinin Release in Mouse Coronary Artery Endothelial Cells

Liping Zhu, Oscar A. Carretero, Tang-Dong Liao, Pamela Harding, Hongwei Li, Colin Summers, Xiao-Ping Yang

Abstract—Activation of angiotensin II type 2 receptors (AT2R) causes the release of kinins, which have beneficial effects on the cardiovascular system. However, it is not clear how AT2R interact with the kallikrein-kinin system to generate kinins. Prolylcarboxypeptidase is an endothelial membrane-bound plasma prekallikrein activator that converts plasma prekallikrein to kallikrein, leading to generation of bradykinin from high-molecular-weight kininogen. We hypothesized that AT2R-induced bradykinin release is at least in part mediated by activation of prolylcarboxypeptidase. Cultures of mouse coronary artery endothelial cells were transfected with an adenoviral vector containing the AT2R gene (Ad-AT2R) or green fluorescent protein only (Ad-GFP) as control. We found that overexpression of AT2R increased prolylcarboxypeptidase mRNA by 1.7-fold and protein 2.5-fold compared with Ad-GFP controls. AT2R overexpression had no effect on angiotensin II type 1 receptor mRNA. Bradykinin release was increased 2.2-fold in AT2R-transfected cells. Activation of AT2R by CGP42112A, a specific AT2R agonist, increased bradykinin further in AT2R-transfected cells. These effects were diminished or abolished by AT2R blockade or a plasma kallikrein inhibitor. Furthermore, blocking prolylcarboxypeptidase with a small interfering RNA partially but significantly reduced bradykinin release by transfected AT2R cells either at the basal condition or when stimulated by the AT2R agonist CGP42112A. These findings suggest that overexpression of AT2R in mouse coronary artery endothelial cells increases expression of prolylcarboxypeptidase, which may contribute to kinin release. (Hypertension. 2010;56:384-390.)

Key Words: prolylcarboxypeptidase ■ angiotensin II type 2 receptor ■ bradykinin ■ coronary artery endothelial cells ■ plasma kallikrein-kinin system

Angiotensin II (Ang II) exerts important biological functions through 2 main subtypes of receptors: type 1 (AT1R) and type 2 (AT2R). The AT1R is ubiquitous and abundant in adult tissues, whereas the expression of AT2R is high in the fetus but low in adult tissues.1 AT2R appears to be upregulated in pathological conditions, such as myocardial infarction2 and vascular injury.3 It is well known that AT1R mediates the effects of Ang II on blood pressure, water and sodium intake, renal sodium retention, secretion of vasopressin and aldosterone, and cell growth and proliferation.4 However, the role of AT2R is less clear. Although it is currently thought that the actions of AT2R oppose those of AT1R,5 the exact mechanisms by which the AT2R elicits its cardioprotective effects are not well understood. There is evidence that some beneficial effects of AT2R are mediated by the bradykinin/NO system.6,7 Tsutsumi et al8 demonstrated that AT2R overexpression in the vasculature stimulates the kinin system and causes dilatation, presumably by activating kininogenase. In addition, cardiac AT2R overexpression attenuates Ang II- or myocardial infarction–induced fibrotic responses, which are reportedly mediated via a kinin/NO-dependent mechanism.9,10

Kinins are released from high- and low-molecular-weight kininogen by 2 key kininogenases, plasma kallikrein and tissue kallikrein.11 Prolylcarboxypeptidase (PRCP; also called angiotensinase C) was initially described as an Ang II–inactivating enzyme.12,13 More recently it has been recognized as a plasma prekallikrein activator in endothelial cells (ECs)14–17 and is important for maintenance of EC function.18 When the complex of high-molecular-weight kininogen and plasma prekallikrein binds to the EC membrane, plasma prekallikrein is rapidly converted to kallikrein by PRCP.19 Plasma kallikrein then cleaves high-molecular-weight kininogen to liberate kinins, which act on constitutive B2 and inducible B1 receptors to stimulate the release of NO and prostacyclin. Shariat-Madar et al20 and Zhao et al21 have shown that PRCP overexpression enhances plasma prekallikrein activation and release of kinins and NO by cultured
Chinese hamster ovary cells and ECs and that these effects can be blocked by a small interfering RNA (siRNA) to PRCP, confirming the role of PRCP in kinin release. In addition, upregulation of PRCP expression in ECs causes increased kallikrein generation and sustained production of bradykinin in lipopolysaccharide-induced inflammation.\(^{18}\) The AT\(_1\)R antagonist losartan reportedly increased PRCP expression in hypertensive rats.\(^{22}\) We have demonstrated that the cardio-protective effect of AT\(_1\)R antagonists is mediated in part by activation of AT\(_2\)R.\(^{23}\) Also, in B2 receptor knockout mice the therapeutic effect of AT\(_2\)R antagonists was diminished, indicating a link between AT\(_2\)R and kinins.\(^{24}\) However, the precise mechanism by which AT\(_2\)R mediates kinin release is not yet known, and the role of PRCP in AT\(_2\)R-stimulated kinins release has not been explored.

In the present study, we used an adenoviral vector system to overexpress AT\(_2\)R in mouse coronary artery ECs. Using this system, we tested the hypothesis that AT\(_2\)R-induced bradykinin release in ECs is mediated by PRCP.

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.\(^{25}\) The study was approved by the Henry Ford Health System Institutional Animal Care and Use Committee 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 \(\mu\)g/mL of Opti-MEM I, and 10% FBS (HyClone). All of the experimental protocols were as described below. Experimental protocols were as described below.

Identification of ECs

ECs were recognized by their uptake of acetylated low-density lipoprotein (Ac-LDL; red fluorescence); magnification \(\times 200\). B, Immunostaining with an antibody to von Willebrand factor (vWF; green fluorescence). 4',6-Diamidino-2-phenylindole was used to indicate the nuclei (blue fluorescence); magnification \(\times 400\).

First, to determine the effect of AT\(_2\)R overexpression on basal bradykinin release, Ad-AT\(_2\)R cells were either left untreated or treated with the AT\(_2\)R antagonist PD123319 (100 \(\mu\)mol/L) or the AT\(_2\)R antagonist valsartan (1 \(\mu\)mol/L) for 2.5 hours. Ad-GFP cells were treated with culture medium alone and served as controls. One milliliter of the medium was collected in a test tube containing the peptidase inhibitor mixture for bradykinin measurement.

Second, to determine the effect of AT\(_2\)R activation on bradykinin release, Ad-AT\(_2\)R cells were treated with the AT\(_2\)R agonist CGP42112A (0.1 \(\mu\)mol/L) for 2 hours. To determine the effect of blockade of AT\(_2\)R on AT\(_2\)R activation-induced bradykinin release, Ad-AT\(_2\)R cells were pretreated with AT\(_2\)R antagonist PD123319 (100 \(\mu\)mol/L) for 30 minutes and then stimulated with CGP42112A (0.1 \(\mu\)mol/L) for 2 additional hours. Medium was collected for bradykinin measurement as described above.

Third, to determine whether AT\(_2\)R-induced bradykinin release is mediated via PRCP-dependent prekallikrein activation, Ad-AT\(_2\)R cells were treated with 1 \(\mu\)mol/L of soybean trypsin inhibitor (SBTI; a plasma kallikrein inhibitor) for 30 minutes and then either left untreated or treated with the AT\(_2\)R agonist CGP42112A (0.1 \(\mu\)mol/L) for 2 hours. Medium was then collected for bradykinin measurement.

Last, to determine the effect of PRCP blockade on AT\(_2\)R-stimulated bradykinin release, Ad-AT\(_2\)R cells were treated with...
either a scrambled siRNA or PRCP siRNA for 48 hours as described above and then subjected to the same experiments as in protocol 2.

Statistical Analysis

Results are expressed as mean±SEM. Student 2-sample t test was used to compare the differences between 2 groups. Bonferroni adjustment was applied in multiple comparisons. A difference was considered significant if the adjusted P value is <0.05.

Results

Adenovirus-Mediated Overexpression of AT₂R

Because the Ad-AT₂R vector contains the GFP gene, the fact that cells expressed green fluorescence indicated that AT₂R was successfully introduced. Figure 2A illustrated AT₂R expression after Ad-AT₂R transfection at MOIs of 40, 80, and 160. Dose-dependent AT₂R mRNA expression was determined by real-time RT-PCR (Figure 2B) using GAPDH as an internal control. Endogenous AT₂R was undetectable in Ad-GFP–transfected cells at a cycle of 21 but could be seen when the cycle was increased to 35 and, conversely, in Ad-AT₂R–transfected cells AT₂R transgene mRNA was detected at a cycle of 21 and expression increased at higher MOIs: compared with Ad-GFP 40, AT₂R mRNA increased 3.53±0.16-fold at MOI 80 and 6.95±0.49-fold at MOI 160 (Figure 2B).

To find out whether AT₂R overexpression affects AT₁R gene expression, we compared AT₁R mRNA expression in Ad-GFP- and Ad-AT₂R–transfected cells. We found that at 40 or 80 MOIs there was no significant difference in AT₁R mRNA expression between Ad-AT₂R- and Ad-GFP–transfected cells (Figure 3), suggesting that AT₂R overexpression at these titers does not influence endogenous AT₁R expression. However, AT₁R mRNA was elevated at the highest MOI (160), and for this reason we used MOI 80 in subsequent experiments to study the role of AT₂R without the confounding effects of the AT₁R.

Effect of AT₂R Overexpression on PRCP mRNA and Protein Expression

Compared with Ad-GFP, Ad-AT₂R transfection increased PRCP protein expression 2.5±0.2-fold (Figure 4A and 4B). In addition, Ad-AT₂R cells had a 1.7±0.16-fold increase in PRCP mRNA compared with Ad-GFP cells (Figure 4C). Both PRCP mRNA and protein levels were normalized to GAPDH, which did not differ significantly between Ad-GFP- and Ad-AT₂R–transfected cells. These findings indicate that PRCP is upregulated by AT₂R overexpression.

Effect of AT₂R Overexpression on Bradykinin Release

Basal bradykinin release in GFP-transfected cells was 2.1±0.2 pg/μg; overexpression of AT₂R increased it to 4.6±0.3 pg/μg, which was 2.2-fold higher than GFP cells (Figure 5). Blocking AT₂R with PD123319 reduced bradykinin levels to 2.9±0.3 pg/μg, but blocking AT₁R with valsartan had no effect on bradykinin release in Ad-AT₂R–transfected cells (4.8±0.4 pg/μg).

We next studied the effect of AT₂R activation on bradykinin release. Stimulation with the AT₂R agonist CGP42112A increased bradykinin levels to 6.7±0.5 pg/μg in AT₂R cells (Figure 6), and this increase was significantly blocked by the AT₂R antagonist PD123319 (3.4±0.4 pg/μg). Taken together, these data provide strong evidence that AT₂R overexpression induces bradykinin release from ECs, and this increase is AT₂R specific, because the AT₂R agonist CGP42112A enhanced bradykinin release, whereas the AT₂R antagonist PD123319 suppressed it.

Effect of Plasma Kallikrein Inhibition on Bradykinin Release

SBTI has been reported to block plasma kallikrein.30 In GFP-transfected cells, SBTI reduced bradykinin to an undetectable level (data not shown). In Ad-AT₂R cells, SBTI significantly
reduced bradykinin release from 4.6±0.3 to 1.1±0.5 pg/µg. Activation of AT2R with CGP42112A enhanced bradykinin release, which was reduced from 6.7±0.5 to 1.8±1.2 pg/µg by SBTI (Figure 7). These data indicate that cleavage of high-molecular-weight kininogen by plasma kallikrein contributes to AT2R-induced bradykinin release by ECs.

### Effect of Blocking PRCP on Bradykinin Release

Currently no PRCP-specific inhibitor is available. We, therefore, used an siRNA to investigate whether blockade of PRCP in AT2R-transfected cells affects bradykinin release. We found that PRCP siRNA reduced PRCP protein expression by 84% compared with scrambled-siRNA controls (Figure 8A). Furthermore, blockade of PRCP using an siRNA reduced basal bradykinin release by 35% and CGP42112A-stimulated bradykinin release by 49% in AT2R-transfected cells compared with scrambled siRNA controls (Figure 8B). These data suggest that AT2R-induced bradykinin release is mediated at least in part by PRCP.

### Discussion

We found that overexpression of AT2R in mouse coronary artery ECs increased bradykinin generation and that activation of AT2R with a specific agonist increased bradykinin levels further. These effects were diminished or abolished by AT2R blockade or a plasma kallikrein inhibitor. Overexpression of AT2R also upregulated PRCP mRNA and protein expression. Importantly, blockade of PRCP using an siRNA diminished AT2R-induced bradykinin release. We believe that these results provide the first evidence that AT2R-induced bradykinin release is mediated at least in part by a PRCP-dependent mechanism.

Activation of AT2R has been considered cardioprotective, partially because of stimulation of kinins.6,7 In the present study, we transfected the AT2R gene into mouse coronary artery ECs.
artery ECs to see whether its overexpression would heighten bradykinin release. We observed that the amount of bradykinin in the medium of Ad-AT2R–transfected cells was significantly higher in the presence of 0.5% FBS and that when FBS was removed bradykinin levels were undetectable. In the absence of an exogenous AT2R agonist, AT2R-mediated bradykinin release could be attributed to the presence of 0.5% FBS, which is known to contain Ang II and/or Ang II fragments that could activate AT2R and thereby stimulate bradykinin release. However, when we measured Ang II in medium containing 0.5% FBS, Ang II was undetectable (<15 pmol/L). Because the concentration of Ang II needed to induce biological effects in cultured cells generally involves nanomoles per liter concentration, it is thus unlikely that the increased basal bradykinin release from Ad-AT2R cells is attributable to the presence of Ang II or its fragments in 0.5% FBS culture medium. Another possible explanation for increased basal bradykinin release in Ad-AT2R cells is ligand-independent activation AT2R. Jin et al31 reported that transfection of AT2R increased bradykinin and inducible NO synthase protein expression in vascular smooth muscle cells independent of Ang II. In addition, Li et al32 demonstrated that AT2R overexpression induced-apoptosis in prostate cancer cells is Ang II independent, and AT2R itself has constitutive activity to cause apoptosis. Miura et al33 further demonstrated that constitutive activation of AT2R induces cell signaling in a ligand-independent manner. Our data agree with these findings and demonstrate that overexpression of AT2R is able to stimulate bradykinin release independent of a specific ligand. Moreover, this bradykinin-stimulated action is AT2R specific, because this effect was blocked by the AT2R antagonist PD123319 but unaffected by AT1R blockade, and activation of AT2R by a specific agonist or Ang II in the presence of valsartan (Figure S1 in the online Data Supplement at http://hyper.ahajournals.org) increased bradykinin release further in AT2R-transfected ECs, and this effect was blocked by the AT2R antagonist PD123319. We also demonstrated that overexpression of AT2R increased NO release from cultured ECs. This increase was further enhanced by CGP42112A but blocked by PD123319 or bradykinin B2 receptor antagonist HOE-140 (Figure S2), indicating that the effect of AT2R may be mediated by the bradykinin/NO pathway.

The precise mechanism by which AT2R increases kinin release is not known. Tsutsumi et al8 reported that in the mouse aorta AT2R activation lowered cellular pH associated with increased kininogenase activity, suggesting that an acid-optimal kininogenase may be responsible for vascular kinin release. However, it is questionable whether this enzyme is tissue kallikrein, because the optimum pH for tissue kallikrein is ~8.5. It is possible that AT2R activates an acidic protease(s) that converts tissue prekallikrein to active tissue kallikrein, or perhaps it is able to activate acidic kininogenases. Interestingly, PRCP enzyme activity appears to occur at acidic pH levels.15 However, the role of PRCP in AT2R-induced kinin release has not been explored to our knowledge.

Prekallikrein is one of the physiological substrates of PRCP. Recently, PRCP has been described as a novel plasma prekallikrein activator that is responsible for kinin release from high-molecular-weight kininogen in the endothelium.16 In the present study we found that overexpression of AT2R
increased PRCP mRNA 1.7-fold and protein expression 2.5-fold in mouse coronary artery ECs, coupled with increased bradykinin release. Importantly, blockade of PRCP with an siRNA significantly diminished the basal and AT$_2$R-stimulated bradykinin release in Ad-AT$_2$R cells, indicating that activation of PRCP, which activates plasma prekallikrein, might play a crucial role in AT$_2$R-induced bradykinin release. To confirm this, we incubated cells in serum-free medium with or without exogenous high-molecular-weight kininogen and prekallikrein. We found that, in the absence of high-molecular-weight kininogen and prekallikrein, bradykinin levels were undetectable, whereas adding them to serum-free medium, bradykinin release was significantly increased in GFP-transfected cells or even more so in AT$_2$R-transfected cells (Figure S3). Furthermore, inhibition of plasma kallikrein with SBTI abolished the basal or AT$_2$R agonist-stimulated bradykinin release. Taken together, our data support the hypothesis that AT$_2$R-induced bradykinin release is mediated at least in part by activation of plasma prekallikrein via PRCP and cleavage of high-molecular-weight kininogen by kallikrein.

There is conflicting evidence regarding the effects of overexpression of AT$_2$R on endogenous AT$_1$R. Although Jin et al reported that overexpression of AT$_2$R downregulated the AT$_1$R in vascular smooth muscle cells, others have stated that AT$_2$R overexpression has no effect on AT$_1$R expression in the heart or blood vessels. In the present study we examined the effect of overexpression of AT$_2$R on AT$_1$R mRNA in coronary artery ECs and found that low titers of Ad-AT$_2$R transfection that increased bradykinin release did not affect endogenous AT$_1$R mRNA, whereas at higher titers overexpression of AT$_2$R increased AT$_1$R expression. Thus, our data suggest that whether AT$_2$R overexpression affects AT$_1$R expression is largely dependent on the level of AT$_2$R gene transfection.

**Limitations**

The present study has some limitations. First, we demonstrated that overexpression of AT$_2$R increased PRCP protein expression and bradykinin release. Activation of AT$_2$R with an agonist increased bradykinin release further but did not alter PRCP protein levels (data not shown). Although this may argue against a role for PRCP in AT$_1$R-induced bradykinin release, it is possible that 2-hour stimulation with an AT$_2$R agonist is not long enough to alter protein expression. In addition, we cannot exclude the possibility that PRCP activity or its translocation to the membrane is increased by AT$_2$R stimulation. Furthermore, it is possible that another unknown mechanism of enzymatic activation of PRCP is involved in AT$_2$R-stimulated bradykinin release. Second, the signaling and molecular mechanism(s) by which AT$_2$R activates PRCP have not been explored, although this is not the focus of our current study. Third, SBTI is not a specific inhibitor for plasma kallikrein. Other proteases, such as trypsin and elastase, are also able to cleave high-molecular-weight kininogen and liberate kinins, and these enzymes could also be inhibited by SBTI. Thus, we cannot exclude the possibility that other proteases also contribute to AT$_2$R-stimulated bradykinin release. All of these limitations warrant further investigation. Nevertheless, these limitations do not negate the evidence that PRCP plays an important role in AT$_2$R-induced bradykinin release from ECs.

In summary, we have shown that overexpression of AT$_2$R increases bradykinin release from mouse coronary artery ECs. This effect was blocked by the AT$_2$R antagonist PD123319 but was not influenced by the AT$_1$R antagonist valsartan. Activation of AT$_2$R by a specific agonist increased bradykinin release further. PRCP expression was increased by AT$_2$R overexpression, whereas downregulation of PRCP using an siRNA reduced bradykinin release in AT$_2$R-transfected cells. Therefore, we conclude that overexpression of AT$_2$R increases expression of PRCP, which may contribute to AT$_2$R-induced bradykinin release.

**Perspectives**

Cardiovascular disease is the leading cause of death in the United States. Angiotensin receptor blockers reduce morbidity and mortality in patients with cardiovascular disease. The effects of angiotensin receptor blockers are mediated in part by activation of the AT$_2$R, which leads to the release of kinins. However, the precise mechanism(s) by which AT$_2$R stimulates kinin release are not fully understood. We believe that our findings provide the first evidence that AT$_2$R-induced bradykinin release is mediated in part by a PRCP-dependent mechanism in mouse coronary artery ECs. These data will enhance our understanding of the role of the AT$_2$R and how it interacts with the kallikrein-kinin system to provide cardioprotection. Moreover, we believe that they will facilitate the development of better therapeutic targets for hypertension and cardiovascular disease.

**Sources of Funding**

This work was supported by National Institutes of Health grants HL-028982 Project II and HL-078951 (to X.-P.Y.).

**Disclosures**

None.

**References**


Role of Prolylcarboxypeptidase in Angiotensin II Type 2 Receptor–Mediated Bradykinin Release in Mouse Coronary Artery Endothelial Cells

Liping Zhu, Oscar A. Carretero, Tang-Dong Liao, Pamela Harding, Hongwei Li, Colin Sumners and Xiao-Ping Yang

_Hypertension_. 2010;56:384-390; originally published online July 6, 2010; doi: 10.1161/HYPERTENSIONAHA.110.155051

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/56/3/384

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2010/07/02/HYPERTENSIONAHA.110.155051.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/
Online Data Supplement

Role of prolylcarboxypeptidase in angiotensin II type 2 receptor-mediated bradykinin release in mouse coronary artery endothelial cells

Running title: Role of PRCP in AT2R-mediated bradykinin release

Liping Zhu¹, Oscar A. Carretero¹, Tang-Dong Liao¹, Pamela Harding¹, Hongwei Li², Colin Sumners², Xiao-Ping Yang¹

¹Hypertension & Vascular Research Division, Department of Internal Medicine
Henry Ford Hospital, Detroit, Michigan
²Department of Physiology and Functional Genomics, University of Florida, Gainesville, Florida

Correspondence to:
Xiao-Ping Yang, M.D., F.A.H.A.
Senior Staff Investigator
Associate Professor of Medicine
Hypertension and Vascular Research Division
Department of Internal Medicine
Henry Ford Hospital
2799 West Grand Blvd.
Detroit, Michigan 48202-2689
Tel. (313) 916-7058
Fax: (313) 916-1479
E-mail: xpyang1@hfhs.org
MATERIALS AND METHODS

Identification of ECs. To measure uptake of Dil-Ac-LDL (red fluorescence), ECs were grown on cover slides and incubated with 10 µg/ml Dil-Ac-LDL in growth medium containing serum for 4 hours at 37°C. Then they were washed three times with PBS, fixed in 3% formaldehyde/PBS for 20 min at room temperature, rinsed with PBS and examined under a fluorescence microscope (Fig. 1A).

For vWF immunostaining, cells were grown on cover slides, fixed in ice-cold acetone-methanol (1:1) for 10 min at -20°C, rinsed with PBS and incubated with: (1) 1% BSA in PBS for 30 min at room temperature, (2) a primary anti-vWF polyclonal antibody (Santa Cruz) overnight at 4°C, (3) a secondary anti-rabbit IgG antibody (Santa Cruz) for 45 min at room temperature, (4) fluorescein streptavidin (Vector Laboratories) for 10 min and (5) 4',6-diamidino-2-phenylindole (DAPI) for 2 min to stain the nuclei. Cells were washed with PBS between incubations. Slides were mounted using a fluorescent medium from Dako Cytomation, examined under an inverted microscope (IX81, Olympus, America, Center Valley) and photographed with a digital camera (DP70, Olympus America). Only cultures > 90% positive for vWF were used (Fig. 1B).

Reverse-Transcription Polymerase Chain Reaction. Total mRNA levels of AT2R, AT1R and PRCP were determined by real-time PCR using ABI 7500. Gene expression was quantified and analyzed using the comparative cycle threshold (C_T) method as described in the Applied Biosystems user bulletin. All data were normalized to GAPDH as an internal control. Samples were run in triplicate, and C_T was averaged for each sample. Primer sequences are shown in Table S1.

Western Blot. Cells were harvested in cell lysis buffer with protease inhibitors. Lysates were centrifuged at 14,000g for 10 min to remove insoluble material. Following SDS-PAGE. Proteins were transferred onto a polyvinylidene fluoride membrane (Millipore), which was blocked with 5% non-fat powdered milk in Tris-buffered saline–Tween solution and probed with a polyclonal antibody to PRCP (Santa Cruz). Horseradish peroxidase–conjugated IgG (Cell Signaling Technology) was used to visualize proteins by a chemiluminescence reaction. Protein expression was normalized to GAPDH.

Measurement of Bradykinin. Bradykinin production was assessed by measuring the amount of bradykinin released into the incubation medium. For this, cell culture medium (1 ml) was collected in a test tube containing a peptidase inhibitor cocktail (aprotinin, 7.69 TIU/ml; soybean trypsin inhibitor, 1,10 phenanthroline, 2 mg/ml; EDTA 20 mg/ml; polybrene, 4 mg/ml). To purify-bradykinin, samples were applied to a pre-activated C18 Bond Elut cartridge (3 ml/200 mg; Varian), washed with 4 ml 0.1% trifluoroacetic acid in ultrapure water (wash buffer) and eluted with 3 ml 60% acetonitrile combined with 40% wash buffer. The elutes were collected in polypropylene tubes and vacuum-dried overnight. Bradykinin was measured with an EIA kit (Peninsula). Bradykinin levels were undetectable in medium 199-F-12 (1:1) with 0.5% FBS in the absence of cells, which was used as a blank. For normalization, cells were harvested and total protein content determined by BCA protein assay (BioRad). Bradykinin concentration was calculated according to a calibration curve, normalized to total protein and expressed as pg/µg protein.
Measurement of Nitric Oxide (NO). NO production was assessed by measuring the amount of NO released into the incubation medium by the ECs. For this, cells were incubated overnight in 6-well plates with medium 199-F-12 (1:1) containing 0.5% FBS and switched to the same medium without phenol red containing 100 µM arginine and 10 µM captopril for 2 hrs before the experiments. NO was measured with a colorimetric assay kit (Oxford Biomedical Research). For normalization, cells were harvested and total protein content determined by BCA protein assay (BioRad). NO concentration was calculated according to a calibration curve, normalized to total protein and expressed as nM/µg protein.

RESULTS

Effect of AT2R Overexpression on Ang II-stimulated Bradykinin Release. To confirm that activation of AT2R increases bradykinin release as demonstrated in Fig. 6, Ad-AT2R cells were stimulated with Ang II in the presence of the AT1R antagonist valsartan. We found that Ang II in the presence of valsartan enhanced bradykinin release by 2.4-fold compared to Ad-GFP cells; and this increase was significantly blocked by the AT2R antagonist PD123319 (Fig. S1). Valsartan by itself had no effect on bradykinin release in Ad-AT2R cells (Fig. 5). These data provide evidence that activation of AT2R by either a specific AT2R agonist or Ang II stimulates bradykinin release from ECs and this effect is AT2R-specific, since it was suppressed by an AT2R antagonist.

Effect of AT2R Overexpression on NO Release. Because it has been suggested that the beneficial effects of AT2R are mediated by the bradykinin/NO pathway, we measured NO release in Ad-AT2R cells and found that overexpression of AT2R increased NO release by 33% compared to Ad-GFP cells. NO release was enhanced further by the AT2R agonist CGP42112A (Fig. S2). This increase was significantly blocked by both the AT2R antagonist PD123319 and the bradykinin B2 receptor antagonist HOE-140 (Fig. S2), suggesting that the effects of AT2R are mediated by the bradykinin/NO pathway.

Effect of AT2R Overexpression on Bradykinin Release in Serum-Free Culture Medium in the Absence or Presence of HK and PK. We measured bradykinin release by ECs in serum-free culture medium with or without high-molecular-weight kininogen (HK) and prekallikrein (PK). Bradykinin was undetectable in the absence of PK and HK in both Ad-GFP and Ad-AT2R cells. When HK and PK were added to the culture medium, bradykinin release increased significantly in Ad-AT2R cells (9.56 ± 0.53 pg/µg; n = 3) compared with Ad-GFP cells (3.52 ± 0.39 pg/µg; n = 3). Taken together with our finding that overexpression of AT2R upregulated PRCP mRNA and protein expression (Fig. 4), these data suggest that AT2R activates a PRCP-dependent PK pathway, thereby enhancing liberation of bradykinin.
<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT2R</td>
<td>5'-CTCTGACCTGGATGGGTATCA-3'</td>
<td>5'-AACACAGCTGTTGGTGAATCC-3'</td>
</tr>
<tr>
<td>AT1R</td>
<td>5'-TCGCTACCTGCCATTGTC-3'</td>
<td>5'-TGACTTTGGCCACCAGCAT-3'</td>
</tr>
<tr>
<td>PRCP</td>
<td>5'-ATCTGAGACCTGATGGTATG-3'</td>
<td>5'-CTGCTGAACCTGAGAGTTTG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TGGAGAAACCTGCAAGATG-3'</td>
<td>5'-GTTGAAGTCGAGGAGACAAC-3'</td>
</tr>
</tbody>
</table>
Figure S1. Effect of AT2R overexpression on Ang II-stimulated bradykinin release. Mouse coronary artery endothelial cells transfected with adenovirus-induced Ang II type 2 receptors (Ad-AT2R at MOI 80) were treated with Ang II (0.1 µM) in the presence of the AT1R antagonist valsartan (Val, 1 µM) with or without the AT2R antagonist PD123319 (PD, 100 µM). Ad-GFP cells served as controls. Data are expressed as fold change relative to Ad-GFP cells. n = 3-6.
**Figure S2. Effect of AT2R overexpression on nitric oxide (NO) release.** Ad-AT2R cells were treated with the AT2R agonist CGP42112A (CGP, 0.1 µM) in the presence or absence of the AT2R antagonist PD123319 (PD, 100 µM) or the bradykinin B2 receptor antagonist HOE-140 (HOE, 1 µM). Ad-GFP cells served as controls. Data are expressed as fold change relative to Ad-GFP cells. *n* = 3-6.
Figure S3. Effect of AT2R overexpression on bradykinin release in serum-free culture medium in the absence or presence of HK and PK. Ad-AT2R cells were first incubated with 2 nM high-molecular-weight kininogen (HK) in serum-free medium for 1 hr; then 2 nM prekallikrein (PK) was added and the cells were incubated for another hour. Ad-GFP cells served as controls. Bradykinin concentration was normalized to total protein and is expressed as pg/µg protein. \( n = 3 \).