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

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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^{o}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^{o}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^{o}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, 800 μg/ml; 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 CGP 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 S1. Primer sequence

Target	Forward	Reverse
AT2R	5'-CTCTGACCTGGATGGGTATCA-3'	5'-AACACAGCTGTTGGTGAATCC-3‘
AT1R	5'-TCGCTACCTGGCCATTGTC-3‘	5'-TGACTTTGGCCACCAGCAT-3'
PRCP	5'-ATCTGGCAGCTTGATGGTATG-3'	5'-CTGCCTGAACCTGAGAGTTTG-3'
GAPDH	5'-TGGAGAAACCTGCCAAGTATG-3'	5'-GTTGAAGTCGCAGGAGACAAC-3'

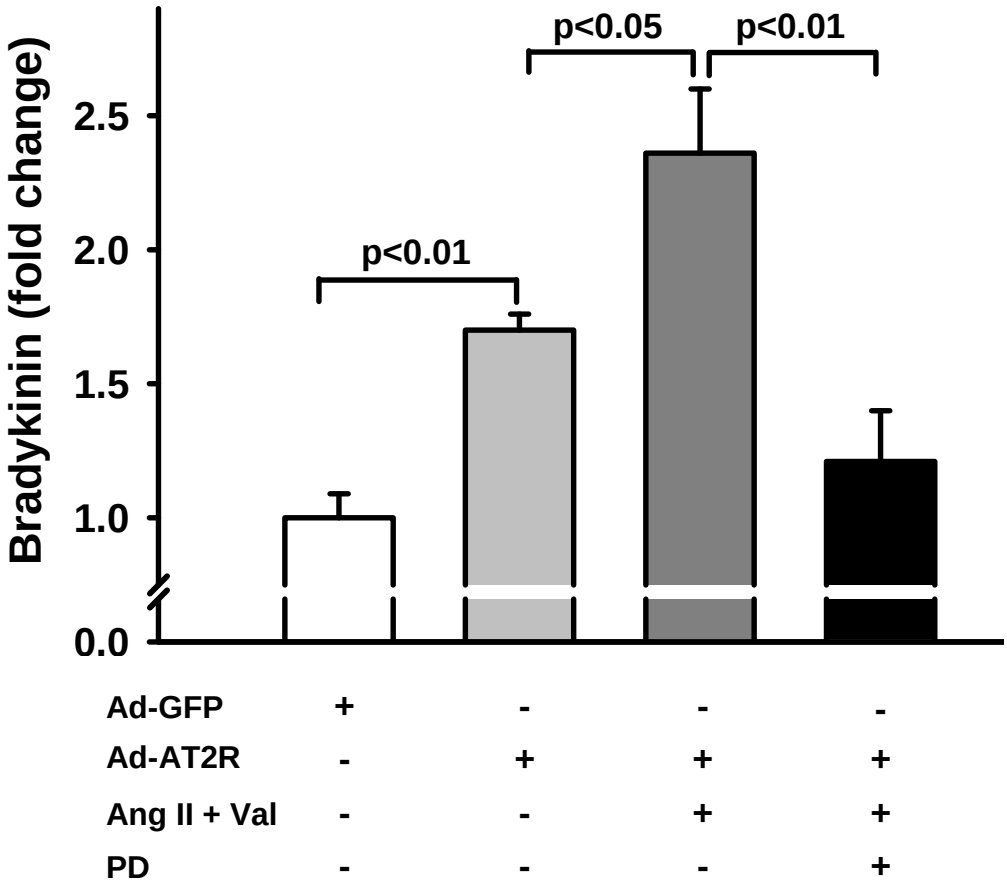

Figure S1. **Effect of AT2R overexpression on Ang II-stimulated bradykinin release**. Mouse coronary artery endothelial cells transfected with adenovirus-induced Ang II type 2 receptors (Ad-AT2R at MOI 80) were treated with Ang II (0.1 μM) in the presence of the AT1R antagonist valsartan (Val, 1 μM) 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.

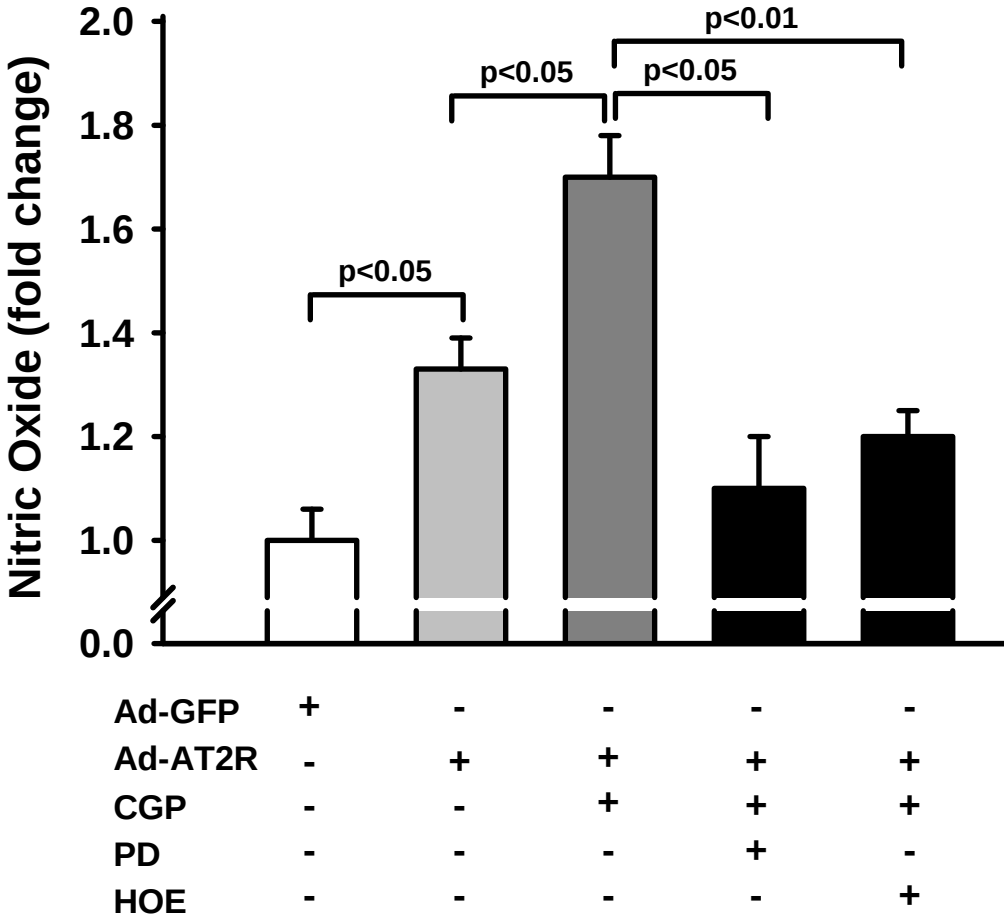

Figure S2. **Effect of AT2R overexpression on nitric oxide (NO) release**. Ad-AT2R cells were treated with the AT2R agonist CGP42112A (CGP, 0.1 μM) in the presence or absence of the AT2R antagonist PD123319 (PD, 100 μM) or the bradykinin B2 receptor antagonist HOE-140 (HOE, 1 μM). Ad-GFP cells served as controls. Data are expressed as fold change relative to Ad-GFP cells. *n* = 3-6.

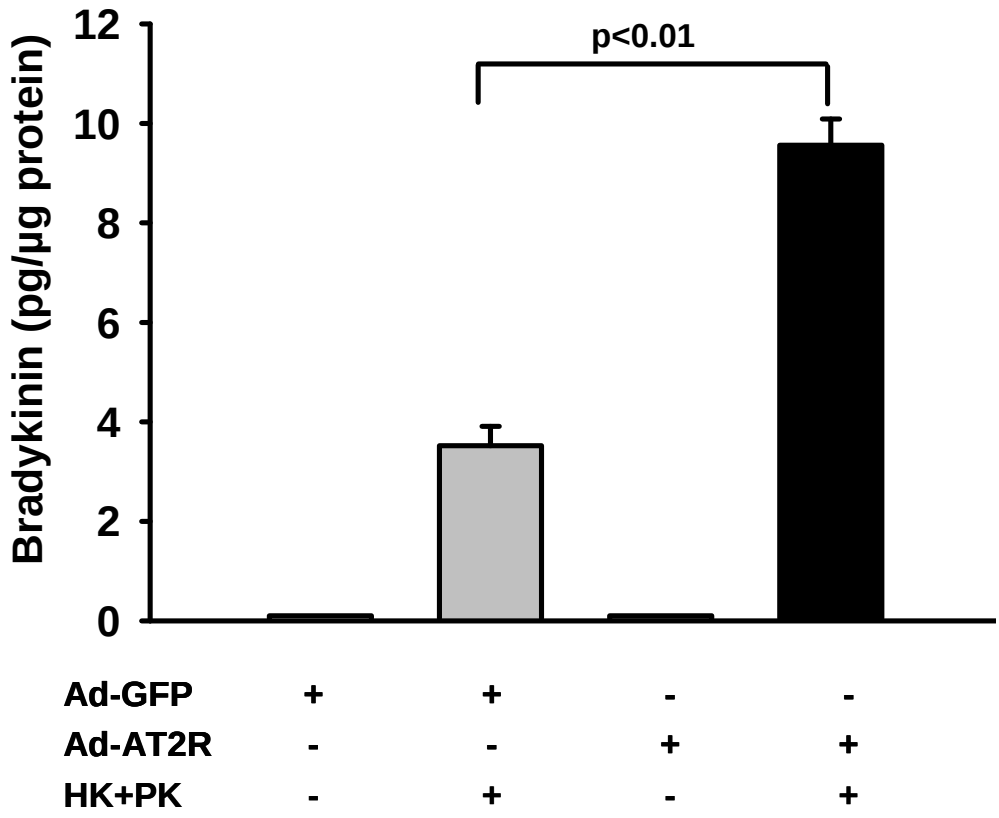

Figure S3. **Effect of AT2R overexpression on bradykinin release in serum-free culture medium in the absence or presence of HK and PK.** Ad-AT2R cells were first incubated with 2 nM high-molecular-weight kininogen (HK) in serum-free medium for 1 hr; then 2 nM prekallikrein (PK) was added and the cells 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. $n = 3$.