ONLINE SUPPLEMENT

AT2 RECEPTOR-MEDIATED AND NITRIC OXIDE-DEPENDENT RENAL VASODILATOR RESPONSE TO COMPOUND 21 UNMASKED BY ACE-INHIBITION IN SPONTANEOUSLY HYPERTENSIVE RATS IN VIVO

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Short title: AT\textsubscript{2} receptors and renal vasodilation

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Expanded Materials and Methods

Animals were maintained on normal rat diet (UAR A03; SAFE, Augy, France) with free access to tap water, at room temperature and on 12h light-dark cycle.

Animals and surgical procedures

Adult male normotensive Wistar Kyoto rats (WKY) and adult male spontaneously hypertensive rats (SHR) (Charles River Laboratories, Lyon, France) weighing between 250-325g were anesthetized with an intraperitoneal (i.p.) injection of 60 mg/kg sodium pentobarbital (Nembutal®) (Ceva Sante Animal, Brussels, Belgium) and fixed in the supine position on a heating pat kept at 37°C. The surgical procedure was a modification of the methods previously described. Briefly, following intubation of the trachea, the right jugular vein was catheterized for fluid maintenance, drug administration and supplemental anesthesia. The right carotid artery was cannulated for continuous monitoring of Mean arterial pressor (MAP) with a pressure transducer (HP Hewlett Packard, Boebingen, Germany). An ultrasound probe was secured around the left renal artery and connected with a transonic flow meter (type T 106, Transonic Systems Inc., NY, USA) for measuring total renal blood flow (RBF). MAP and RBF were simultaneously registered on-line by the software program VI-Logger (National Instruments, Austin, Texas, USA). The experimental protocol started after a 30 minutes equilibration period following surgery. First the baseline values were recorded for 15 minutes before the sequential administration of pharmacological compounds. During local administration, anesthesia was maintained with 0.8-1µl/min intravenous (i.v.) infusion of sodium pentobarbital.

Immunoblotting

Protein extraction and western blotting for AT1 and AT2 were performed as previously described. One half a kidney from WKY, SHR and SHR 20 minutes after iv captopril injection (10mg/kg) (n = 5 per group) was homogenized on ice in 2 mL lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L ethyleneglycol tetraacetate (EGTA), 1 mmol/L NaF, 2 mmol/L Na2VO4, 1% Triton X-100, 10% glycerol, 0.5% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 10% protease inhibitor cocktail (Sigma-Aldrich)), followed by centrifugation at 15,000 g for 20 minutes. The supernatant was aliquoted and stored at –80°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (4-12% Bis-Tris gel; Bio-Rad Laboratories, Nazareth, Belgium) under reducing conditions and transferred to a polyvinylidene fluoride membrane (PVDF Membrane; Bio-Rad Laboratories) using a Criterion Blotter (AT1; Bio-Rad Laboratories) or a nitrocellulose membrane using an iBLOT module (AT2,
Non-specific binding was blocked by incubating the membrane for 1 h at room temperature in 5% ECL membrane blocking agent (GE Healthcare, Roosendaal, The Netherlands). Blots were incubated overnight at 4°C with the immunoaffinity purified rabbit polyclonal antibodies to AT₁ (diluted 1:2000) or AT₂ (diluted 1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in membrane blocking agent. The next day, after incubation with horseradish-peroxidase-conjugated anti-rabbit antiserum (1/5000, 30 min; Dako, Glostrup, Denmark), immunoreactive proteins were visualized in the ImageQuant LAS4000 system using enhanced chemiluminescence (ECL) (ECL Select kit, GE Healthcare). Next, the membranes were rinsed very thoroughly, stained with Sypro Ruby protein blot stain (Invitrogen) for 15 minutes and rinsed again. Proteins were visualized using UV epi-illumination. The MultiMark Multi-Colored Standard (Novex, Invitrogen) was used as molecular weight standard. Densitometric analysis of the immunoreactive bands was performed using the ImageJ software (National Institute of Health, USA). Densities of AT₁/₂ immunoreactive bands are normalized to the density of the whole lane after total protein stain, detected on the same membrane (Sypro Ruby protein stain, Invitrogen). All samples that were being compared were always loaded on 1 gel and experiments were repeated 3 times. WKY and SHR samples were compared for AT₂R expression, SHR and SHR 20 minutes after iv captopril injection (10mg/kg) were compared for AT₁R and AT₂R expression. An arbitrary chosen sample of the control/untreated group was set as reference (100%) and relative AT₁/₂ expression levels in all groups are expressed as a percentage of this reference. Expression levels are expressed as means ± standard error of the mean (SEM). Statistical analysis of data was performed using an unpaired Student’s t-test (α=0.05).

Drugs
Captopril, candesartan, PD123319, fenoldopam and icatibant were purchased from Sigma-Aldrich Co. (St. Louis, USA). Indomethacin and L-NMMA were purchased from Merck (Darmstadt, Germany) and Tocris Bioscience (Bristol, UK) respectively. C21 was provided by Vicore Pharma AB (Göteborg, Sweden). Doses were selected based on previous studies⁴⁻⁹.

Data analysis
Data are presented as mean±SEM. The renal vascular resistance (RVR) was calculated as MAP divided by RBF. Differences in baseline parameters between WKY and SHR were evaluated with a two-tailed Mann-Whitney test. Intra-group differences were analysed using a one-way ANOVA for
repeated measures followed by Tukey’s multiple comparison test. A one-way ANOVA followed by a Dunnett’s multiple comparison test was used to compare two different treatments. Immunoblot data were analysed using an unpaired Student’s t-test. Alpha was set at 0.05. All calculations and graphs were obtained by using GraphPad Prism 4.03 (GraphPad Software Inc., San Diego, CA, USA).


Figures and Legends

Figure S1: No response to C21 in untreated SHR that do respond to fenoldopam

Figure S1: Absolute changes in mean arterial pressure (MAP), renal blood flow (RBF) and renal vascular resistance (RVR) after cumulative doses of C21 (0.01 + 0.05 mg/kg) and fenoldopam (100 nmol/kg) in untreated SHR. Data are shown as mean±SEM (n=4). *P<0.05; **P<0.01; ***P<0.001 (one-way Anova for repeated measures analysis compared to saline). C21 0.05 is a cumulative result of the response to C21 0.01 and to C21 0.05.
Figure S2: Reproducibility of the effect of C21

Figure S2: Absolute changes in mean arterial pressure (MAP), renal blood flow (RBF) and renal vascular resistance (RVR) after saline, captopril (10 mg/kg), cumulative doses of C21 (0.01 + 0.05 mg/kg) and fenoldopam (100 nmol/kg) in SHR. Data are shown as mean±SEM (n=5). *P<0.05; **P<0.01; ***P<0.001 (one-way Anova for repeated measures analysis compared to saline). For C21 0.05 the cumulative result of the response to C21 0.01 and to C21 0.05 is given.
Figure S3: Absolute changes in mean arterial pressure (MAP), renal blood flow (RBF) and renal vascular resistance (RVR) after PD123319 (1 mg/kg) in untreated SHR. Data are shown as mean±SEM (n=5). *P<0.05; **P<0.01; ***P<0.001 (one-way Anova for repeated measures analysis compared to saline).
Figure S4: Effect of PD123319 and fenoldopam in SHR pretreated with candesartan

Figure S4: Absolute changes in mean arterial pressure (MAP), renal blood flow (RBF) and renal vascular resistance (RVR) after PD123319 (1 mg/kg) injection after candesartan (1 mg/kg) in SHR. Data are shown as mean±SEM (n=6). *P<0.05; **P<0.01; ***P<0.001 (one-way Anova for repeated measures analysis compared to saline).
Figure S5: Absolute changes in mean arterial pressure (MAP) after captopril (10mg/kg) by cumulative dose of C21 (0.01 + 0.05 mg/kg) after administration of respectively saline, PD123319 (1 mg/kg), indomethacin (10 mg/kg), L-NMMA (30 mg/kg) and icatibant (20 µg/kg) in hypertensive (SHR) rats. Data are shown as mean±SEM (n=6 per group). *P<0.05; **P<0.01; ***P<0.001 (one-way Anova for repeated measures analysis compared to saline). £P<0.05; ££P<0.01; £££P<0.001 (one-way analysis of variance compared to the respective dose of C21 after saline). For C21 0.05 the cumulative result of the response to C21 0.01 and to C21 0.05 is given.
Figure S6: Immunoblot result for AT₁ and AT₂ receptors

Figure S6:  
A. Relative AT₂ receptor protein expression in kidney tissue from WKY and SHR (both n=5).  
B. Relative AT₂ receptor protein expression in kidney tissue in SHR after saline injection and after captopril injection.  
C. Relative AT₁ receptor protein expression in kidney tissue in SHR after saline injection and after captopril injection.  

***P<0.001 (unpaired T-test).