Supplemental Material

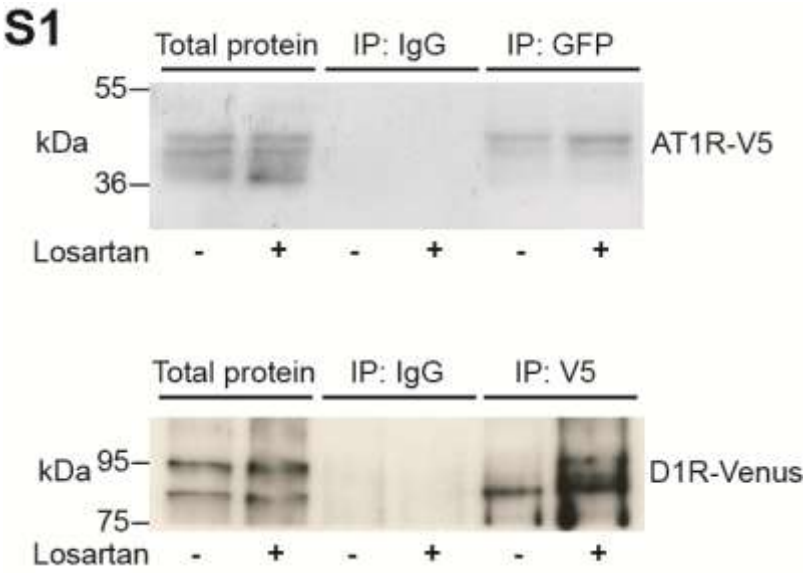

Figure S1 Losartan increases the strength of interaction between D1R and AT1R. Effect of Losartan (10^{-5}M, 15 minutes) on co-immunoprecipitation from HEK cells transfected with D1R-Venus and AT1R-V5 using GFP and V5 antibodies. For total protein 10µg of protein was loaded.

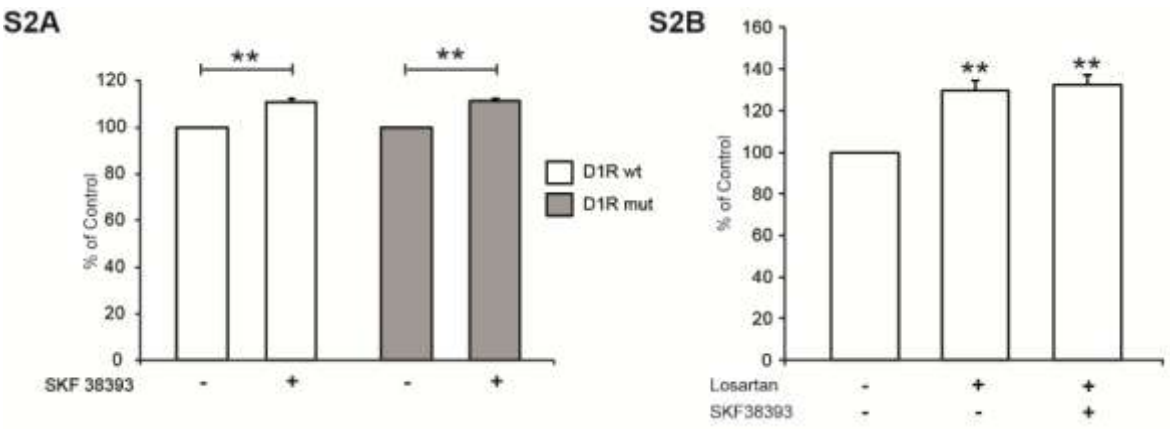

Figure S2A To confirm the functionality of the mutant D1R, transfection of HEK cells with wild type D1R (white bars) or mutant D1R (grey bars) alone was used for cAMP measurement. Treatment with D1R agonist SKF-38393 (10^{-5}M, 10 min) significantly increases the amount of cAMP in cells transfected with both wild type D1R (n=6, **p < 0.01 vs. control) and mutant D1R (n=6, **p < 0.01 vs. control). **S2B)** Losartan (10^{-5}M, 20 min) significantly increases the amount of cAMP in HEK cells transfected with AT1R and wild type D1R (n=7, **p < 0.01 vs. control).) Exposure to a D1R agonist, SKF38393 (10^{-5}M, 5 min) did not further increase the level of cAMP in cells treated with Losartan (10^{-5}M, 20 min, n=7, **p < 0.01 vs. control).) cAMP level of control (vehicle treated) was set to 100%. Statistical analysis was performed using the Mann-Whitney U-test. Values are means ± SEM

Complete material and methods

Animals: Male Sprague-Dawley rats aged 4-8 weeks were used in this study. The studies performed in Sweden followed the Karolinska Institutet regulations concerning care and use of laboratory animals, and were approved by the Stockholm North ethical evaluation board for animal research. The studies performed in Argentina followed the Faculty of Medicine, University of Buenos Aires regulations concerning care and use of laboratory animals, and were approved by the Comité Institucional Para el Cuidado y Uso de Animales de Laboratorio (CICUAL) of the Faculty of Medicine, University of Buenos Aires.

Tissue and Cells: Renal cortical slices were used for co-immunoprecipitation studies and for preparation of primary cell cultures. Immediately after animals were sacrificed, 250 µm slices were taken from the outer renal cortex using a microtome. The outer 250 µm region of rat renal cortex was previously shown to contain more than 90 % proximal tubule cells.[1]

Primary cultures of renal proximal tubule cells were prepared as described previously.[2] Briefly, cells were cultured in supplemented Dulbecco's modified Eagle's medium; 20 mM Hepes, 24 mM $NaHCO_3$, 10 µg/ml penicillin, 10 µg/ml streptomycin, 10% FBS, on Petri dishes or 25 mm glass cover slips for 48h in 5 % CO_2 at 37°C. HEK 293a (HEK) cells, human embryonic kidney cell line QBI293A were obtained from Qbiogene Inc. Studies was performed on HEK 293a cells, passage 3-10, cultured for 24-48 hours in Dulbecco´s modified eagle medium (Gibco 41966) supplemented with 10% FBS, at an approximate humidity of 95–98% with 5% CO_2. Cells were used within a maximum of four days in all studies.

Immunoprecipitation and Western Blot: Renal cortical slices or HEK cells were incubated for 15 min at 20^{o}C with losartan (10^{-5}M) or vehicle in treatment buffer containing (mM): 124 NaCl, 4 K, 26 $NaHCO_3$, 10 D-Glucose, 1.5 $MgSO_4$, 1 n-butyric acid, 1.5 $CaCl_2$ and 0.25 KH_2PO, pH 7.4 and rapidly frozen (only for slices). Homogenization was performed in a buffer containing (mM): 65 Tris-base, 154 NaCl, 6 sodiumdeoxycholate, 1 ethylenediamine tetra acid (EDTA), 10% NP-40, 1 DTT, pH 7.4 with complete protease inhibitors (Roche Diagnostics, Mannheim, Germany). Samples were centrifuged, supernatants collected and protein concentrations adjusted to equal amount. To exclude unspecific binding, homogenates were incubated with sepharose-G (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for one hour at 4^{o}C using end to end mixing. Samples were incubated with precipitating antibody against AT1R or D1R for one hour at 4°C, and incubated with sepharose-G over night at 4^{o}C using end to end mixing. Samples were centrifuged, washed three times, and sepharose-G was collected. The samples were eluted with Laemmli sample buffer, heated for 15 min at 70^{o}C and subjected to SDS-PAGE electrophoresis together with homogenates of renal cortical slices and species specific IgG as controls (Sigma Aldrich, St.Louis, MO, USA). Proteins were transferred to a polyvinylidine difluoride (PVDF) membrane (Hybond-P, Amersham Biosciences). Membranes were blotted with antibodies against D1R and AT1R, and visualized by secondary horseradish peroxidase conjugated antibodies (GE Healthcare). Immunoreactivity was detected using enhanced chemiluminescence (ECL plus Western blotting detection system, Amersham Biosiences). Molecular sizes were assessed using prestained SDS-PAGE standards (Bio-Rad Laboratories). Western blot films were scanned and densitometric quantification of films was done for all blots. Membranes were stained with amidoblack. Correction for total protein was made and control values were set to 100%. Immunoprecipitation for AT1R and blot for D1R was repeated four times and immunoprecipitation for D1R and blot for AT1R was repeated five times.

Cell Surface Biotinylation: Biotinylation was performed using RPTC or HEK cells. Cells were treated in presence of vehicle or losartan (10^{-5}M) in a Krebs buffer containing (mM) 110 NaCl, 4 KCl, 1 NaH_2PO_4 x $2H_2O$, 25 $NaHCO_3$, 1.5 $CaCl_2$ x $2H_2O$, 1.2 $MgCl_2$ x $6H_2O$, 10 glucose, 20 hepes, pH 7.4. After 20 minutes of treatment cell surface proteins were biotinylated in PBS using EZ linked Sulfo-NHS-SS Biotin (Pierce), at a final concentration of 1 mg/ml at 4°C, during gently shaking. Cells were rinsed and homogenized in a buffer containing (mM) 320 Sucrose, 25 imidazole, 1 EDTA using a teflon homogenizer and finally passed through a syringe. Homogenates were centrifuged at 800 x g for 5 min, and supernatants were collected. Protein concentrations were adjusted to equal amount. Supernatants were subjected to centrifugation at 10.000 x g for 20 min. The resulting pellets were resuspended in a buffer containing (mM) 20 Tris, 150 NaCl, 1% TritonX 100, pH 7.5. Proteins were adjusted to equal concentration. Biotinylated proteins were captured with 100 µl streptavidin-agarose beads (Pierce Immobilized Streptavidin, 6% beaded agarose) for 2 hours at 4°C with end to end mixing. Proteins were recovered by centrifugation, washed and eluted with 2x Laemmli sample buffer, heated to 70°C for 15 minutes and resolved by Western blot. Western blot films were scanned and densitometric quantification of bands was done for all blots, assessment of equal total protein loaded was made and control values were set to 100%. All experiments were repeated four times.

Transfection and constructs: Cloning of dopamine receptor 1 expressing constructs was described previously.[3] Rat AT1a receptor cDNA[4] was amplified using total rat brain cDNA and following primers: sense 5'- CACCaaaATGGCCCTTAACTCTTCTG -3' and antisense 5'- CTCCACCTCAAAACAAGAC -3' (GenBank X62295). The AT1a receptor PCR fragment was cloned into pENTR/D/TOPO using pENTR Directional TOPO Cloning Kit (Invitrogen). Construct pDEST-rAT1aR-Venus for expression of rat AT1a receptor fused with Venus at COOH-terminus (rAT1AR-Venus) was cloned using pDEST-N-Venus as destination vector and pENTR-rAT1aR as entry construct in Gateway recombination reaction (Invitrogen). Similar strategy was used to clone construct pDEST-rAT1aR-CFP expressing rAT1aR-CFP fusion protein. Structure of all constructs was verified using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Transfection of RPTC was performed using Lipofectamine 2000 with a total of 4 µg DNA for a 60mm petri dish. Transfection of HEK cells was performed using Exgene 500 with a total amount of 3 or 1.5 µg DNA for a 60 or 35 mm petri dish respectively. All transfection protocols were performed according to the manufacturer's suggestion. Transfection with two plasmids was performed at 1:1 ratio. Cells were cultured an additional 24h following transfection before studies were performed.

cAMP Assay: Two hours before treatment, RPTC or HEK cells were exposed to a serum free medium. The cells were then incubated with losartan (10^{-5}M) or vehicle in an oxygenated buffer containing (mM): 124 NaCl, 4 K, 26 $NaHCO_3$, 10 D-Glucose, 1.5 $MgSO_4$, 1 n-butyric acid, 1.5 $CaCl_2$ and 0.25 KH_2PO and 0.5 3-isobutyl-1-methylxanthine (IBMX) for 20 minutes at 37°C. We had in pilot studies found that effects of losartan on cAMP generation reached a plateau after 15-20 minutes. Cells were rinsed with PBS and lysed in 0.1 M HCl and protein concentration was measured. cAMP was determined using the Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Designs) according to manufacturer's protocol. Results from each experiment were normalized to the control (vehicle) value that was set to 100%, in order to

compare the relative increase in cAMP level minimizing any influence of cells confluence and transfection efficiency differences between experiments.

Live Cell Imaging: Experiments were performed using a Zeiss LSM 510 laser scanning confocal microscope, using a 40x (1.2 NA, water) objective. Emission was induced with laser lines at 514 nm and 485 nm, for detection LP 530 and BP 47-500 filters were used for Venus and CFP labels, respectively. Cells with low to medium intensity fluorescence were selected for further studies.

Cells were continuously perfused with Krebs buffer, for the duration of the treatment perfusion was switched to a Krebs buffer with losartan (10^{-5}M). Images were collected before treatment, after 5 and 10 minutes or 20 and 30 minutes of treatment. For each time point at least 16 cells were analyzed. Images were analyzed by using Image J (National Institutes of Health). The membrane to cytosol ratio was calculated by dividing the average intensity of an area in the region of the plasma membrane with the average intensity of an identical area of the cytosolic region immediately subjacent to the chosen region of the plasma membrane.

Antibodies and Chemicals: All chemicals used were from Sigma Aldrich, Stockholm, Sweden, unless otherwise stated. Chemicals used in cell cultures were all from Invitrogen. Losartan potassium was from Fluka analytical (Sigma Aldrich, Stockholm, Sweden). The following antibodies were used; AT1R: SC Ab 1173, rabbit and SC Ab 57036, mouse (Santa Cruz Biotechnology Inc. Heidelberg, Germany). D1R: Chemicon Ab 1765, rabbit and Chemicon MAB 5290, mouse (Millipore AB, Solna, Sweden), Anti V5 R960-25 (Invitrogen), Anti GFP A-11122 (Invitrogen) and 632375 (Clontech).

Blood pressure recording in rats with experimental hypertension: Seven week old rats were subjected to ether anesthesia in an ad hoc chamber. The abdominal aorta, proximal to renal arteries was visualized through abdominal left flank incision. The vagus nerve was separated and a silk ligature wet in glycerol was placed around the aorta. A probe was placed over the aorta and the ligature was tied around the probe and the aorta. Once the tight was secured, the probe was removed. The procedure lasted for less than 30 seconds. The kidney turned pale during ligation and regained normal color when the probe was removed.[5]

One week after surgery rats were randomly divided into control (n=5), SCH23390 only (n=5), Losartan (n=8) and Losartan + SCH23390 (n=9) groups. The rats in the losartan treatment groups received losartan (20 mg/kg/day) in the drinking water. One week later the rats receiving SCH23390 was randomly selected for co-treatment with SCH 23390 (1mg/kg BW/day). SCH 23390 was administered subcutaneous, divided in two doses per day for four days. Control rats and rats treated with losartan only received vehicle subcutaneous. After four days, rats were anesthetized with thiopental intraperitoneal, placed on a heated stage at 37°C, and catheters were inserted into the carotid and right femoral arteries for recording of mean arterial pressure above and below the aortic constriction. A Statham transducer was used for recording. The depth of anesthesia was controlled by testing the lack of response to stimulation of posterior limbs and by visual observation of a stable and regular breathing.

Statistical analysis: Statistical analysis of data obtained from immunoprecipitation, biotinylation and cAMP studies were performed using the Mann-Whitney U-test. Statistical analysis of results from live cell imaging studies were performed using ANOVA repeated measurement and multiple t-test comparison. Statistical analysis of data from the in vivo study was performed using ANOVA and Students-Newman-Keuls multiple comparison test. Data are given as mean ± SEM. $p < 0.05$, 0.01 or 0.001 was used as the level of significance.

Supplement references

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