ONLINE SUPPLEMENT

Angiotensin II-independent upregulation of Cyclooxygenase-2 by activation of the (pro)renin receptor in rat renal inner medullary cells.

EXPANDED MATERIALS AND METHODS

Animal samples

Kidneys were obtained from routinely euthanized Sprague Dawley rats by decapitation which is extensively used for accurate measurements of renal renin mRNA expression, kidney renin content and plasma renin activity. This method of euthanasia in rats and mice has been used in our laboratory to avoid the sympathetic-dependent stimulation of the RAS during harvesting of blood and kidney samples. By using this method, major alterations of plasma renin activity due to stress or anesthesia are avoided. This animal protocol is approved by the Tulane IACUC.

Primary cultures of rat IM cells

Primary cultures of IM cells were prepared as described previously. [1] Briefly, after kidney excision, inner medullary tissues were minced under sterile conditions and digested in 10 ml of DMEM-Ham's F-12, 20 mg of collagenase B, 7 mg of hyaluronidase, supplemented with 80 mM urea and 130 mM NaCl, and incubated at 37°C under continuous agitation for 90 min. After centrifugation, the pellet was washed in pre-warmed culture media without enzymes [DMEM-Ham's F-12, 80 mM urea, 130 mM NaCl, 10 mM HEPES, 2 mM L-glutamine, penicillin-streptomycin (10,000 U/ml), 50 nM hydrocortisone, 5 pM 3,3,5-triiodothyronine, 1 nM sodium selenate, 5 mg/l transferrin, without FBS (pH 7.4, 640 mosmol/kgH2O)]. The resulting IM cell suspension was seeded in 3 mm petri-dishes. At 70% confluence (4-6 days), IM cells were either transfected with plasmids containing shRNA-GFP against PRR (36 hours before treatments), or incubated with AngII at 100 nmol/L (Sigma Chemical Co, St. Louis, MO), or rat recombinant prorenin at 100 nmol/L (rrPR; Innovative Research, Novi, MI) for 6 hours. ERK1/2 inhibitor U0126 (Cell Signaling Technology, Beverly, MA) was used at 10 µM and added 30 minutes prior to AngII or rrPR treatments.

Immunofluorescence in rat kidney sections and primary cultures of IM cells

Methanol-fixed cultured rat IM cells were stained with specific antibodies: a rabbit anti-AT1R at 1:200 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), a goat anti-COX-2 (Cat. # sc-1747, 1:400 dilutions, Santa Cruz Biotechnology Inc, Santa Cruz, CA), a rabbit anti-PRR (Cat. # HPA003156, 1:100 dilutions, Sigma Chemical Co, St. Louis, MO), and cells specific immunomarkers of principal cells (anti-aquaporin-2, AQP-2, Cat. # 178612, at 1:400 dilutions, Calbiochem, San Diego, CA); intercalated cells (anti-anion exchanger type 1, AE1, Cat. # AE11-A, at 1:200 dilutions, Apha Diagnostic Intl, San Antonio, TX) and interstitial cells (anti-tenascin C, Cat. # Ab3970, 1:200 dilutions, Abcam, Cambridge, MA). Secondary antibodies Alexa Fluor (Invitrogen, Carlsbad, CA) were used. Samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad CA). Kidney sections (3 µm) were costained with an anti-anion exchanger type 1 antibody at 1:500 dilution (AE1, Cat. # AE11-A; Alpha Diagnostic Intl; San Antonio, TX) and anti AQP-2 and a rabbit anti-PRR (Cat. # HPA003156, Sigma Chemical Co, St. Louis, MO) at 1:200 dilutions and a goat anti-COX-2 (Cat. # sc-1747, Santa Cruz Biotechnology Inc, Santa Cruz, CA) followed by the incubation of the corresponding immunofluorescent secondary antibodies (1:1000, Alexa Fluor®, Invitrogen, Carlsbad, CA). Some kidney sections were stained with anti-COX-2 antibody followed by peroxidase-diaminobenzidine Reaction. Negative controls were obtained by omission of the specific primary antibody.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed to evaluate rat COX-2 mRNA expression levels in long term primary cultures of rat IMCD cells using the TaqMan PCR system. For total RNA isolation, treated cells (n=6, per group) were washed with phosphate buffered solution before RNA isolation. Total RNA was isolated from the cells using RNeasy Mini Kit

(Qiagen). Total RNA (20 ng) was used to amplify COX-2 using the following primers: 5'-TCCGTAGAAGAACCTTTTCC -3' (sense); 5'- GGAGTCTGGAACATTGTGAA -3' (antisense) and 5'-6-FAM- GGAAATAAGGAGCTTCCTGA -BHQ1-3' (fluorogenic probe). Data were normalized against β-actin mRNA as previously described [1] and expressed as percentage of control.

Western blot analysis

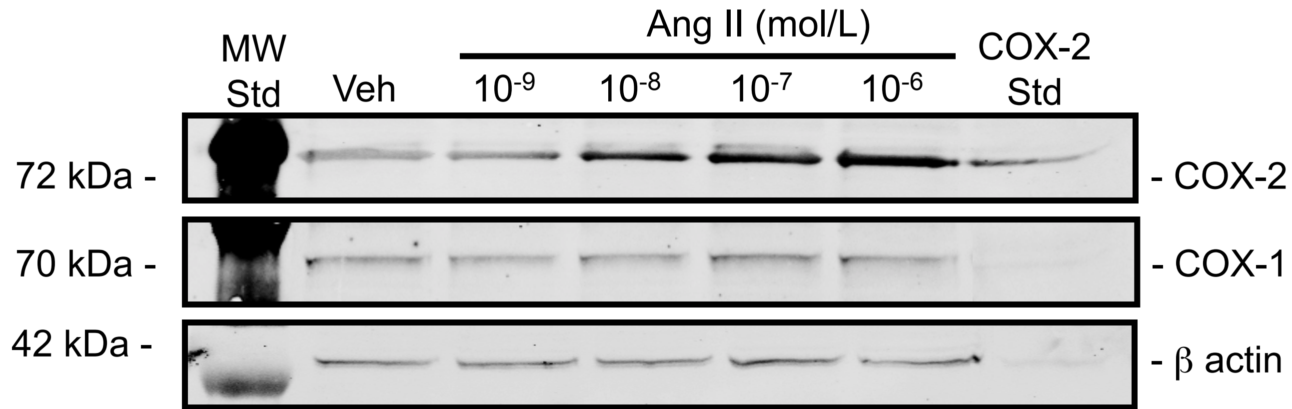
For protein levels quantification, a 1:200 dilution of a rabbit COX-2 antibody (Cayman, Ann Arbor, MI), a mouse anti-phospho-p44/42 ERK1/2 (Thr202/Tyr204) antibody (Cat. # 91065, Cell Signaling Technology, Beverly, MA), and a rabbit anti total ERK (Cat. # 9122, Cell Signaling Technology, Beverly, MA) was used. Primary antibodies were followed by incubations with either donkey anti-mouse or anti-rabbit IgG IRDye® 800 CW (Li-cor Biosciences, Lincoln, NE) at a 1:30,000 dilution. Densitometric analyses were performed by normalization against β-actin (Santa Cruz Biotechnology; Santa Cruz, CA).

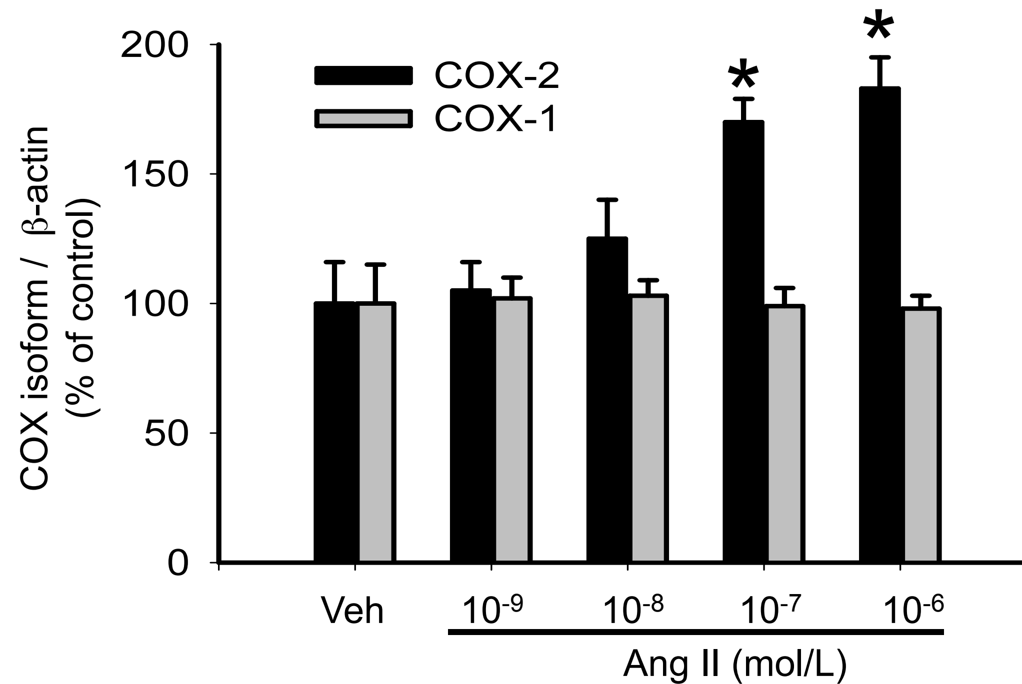
PRR knockdown in primary cultures of IMCD cells using short hairpin RNA (shRNA)

Plasmids (pGeneClipTM hMGFP Vector) containing four different sequences (SureSilencing shRNA Plasmids, QIAGEN®) were tested previously and the one that most effectively decreased PRR expression was selected. IMCD cells were transfected with 1 µg of DNA using Lipofectamine LTX (Invitrogen, Carlsbad, CA) for 36 hours. Efficiency of transfection was confirmed by green fluorescent protein (GFP) detection (Supplementary data 4). Scramble shRNA sequence containing GFP was used as a negative control.

Data Supplements

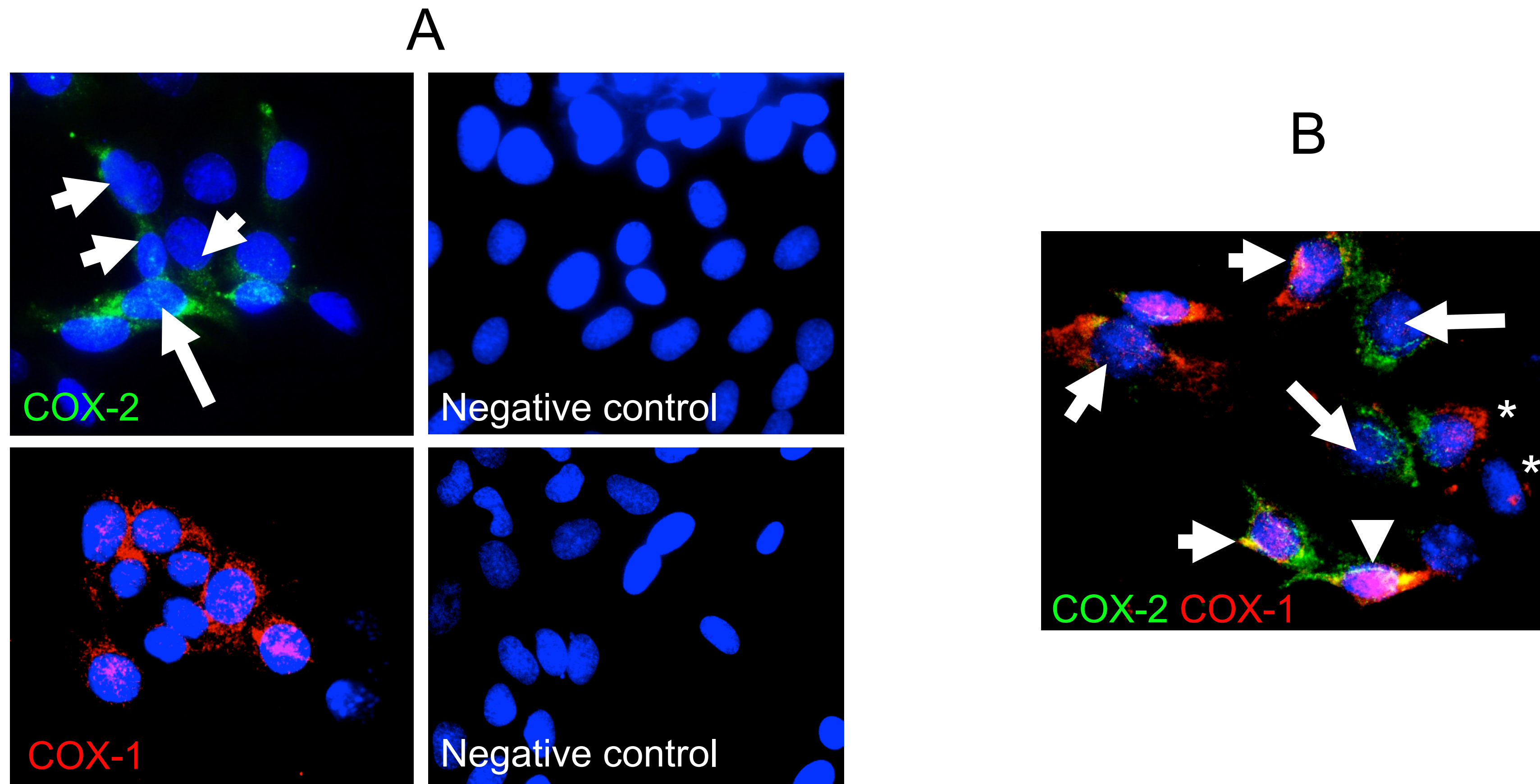
S1





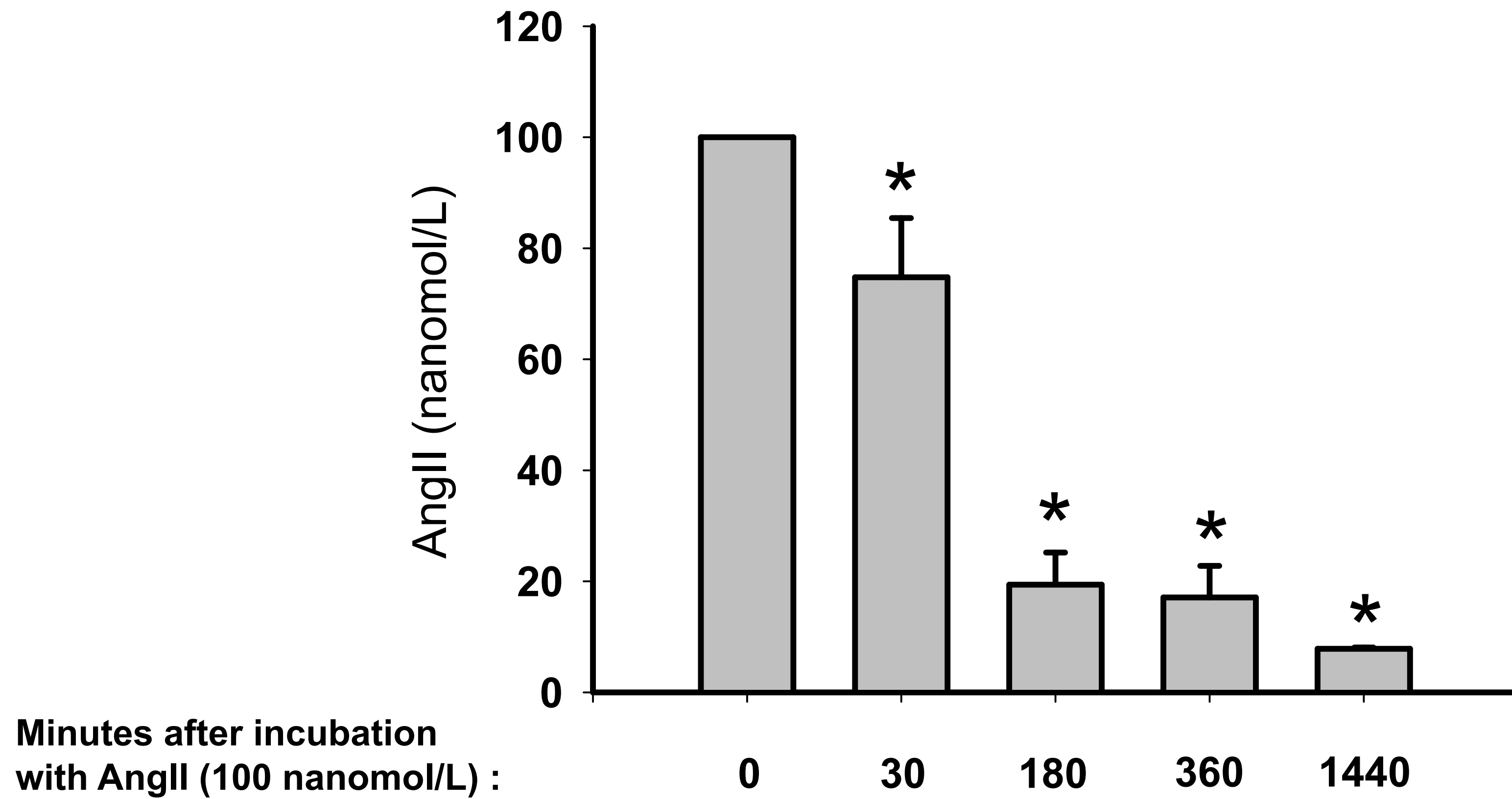
Supplementary data 1. Dose response effect of AngII on COX-2 and COX-1 protein levels. A COX-2 positive standard (COX-2 Std; Cayman, Ann Arbor, MI) confirmed the specificity of the antibody used. COX-2 was significantly higher than vehicle (Veh) at doses of 10^{-6} and 10^{-7} mol/L. COX-1 protein levels did not change in response to the different doses of AngII tested. Protein levels were evaluated after 6 hours of treatment by normalization against β-actin protein levels. *$P<0.05$, n=6.

S2



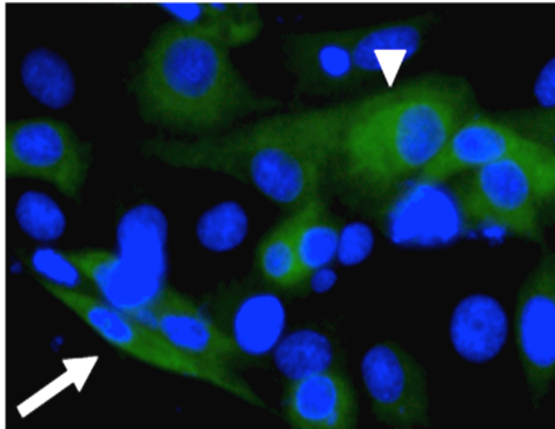
Supplementary data 2. Immunolocalization of COX-2 and COX-1 in rat renal inner medulla fresh isolated cultured cells. **A.** COX-2 (left upper panel) was detected mainly in interstitial cells with typical elongated shape and strong signal (arrow), but also in epithelial cells (arrowheads). COX-1 (left bottom panel) was widely expressed. Unspecific background was ruled out by omission of the primary COX-2 and COX-1 antibodies and incubation with secondary antibodies (right panels) . **B.** Double staining of COX-2 and COX-1 showed that both proteins are co-localized in intercalated cells (arrowheads and yellow merged color). [2] Cells stained only for COX-2 (arrows) or COX-1 (asterisks) were also observed corresponding to interstitial cells and principal cells respectively as described. [3]

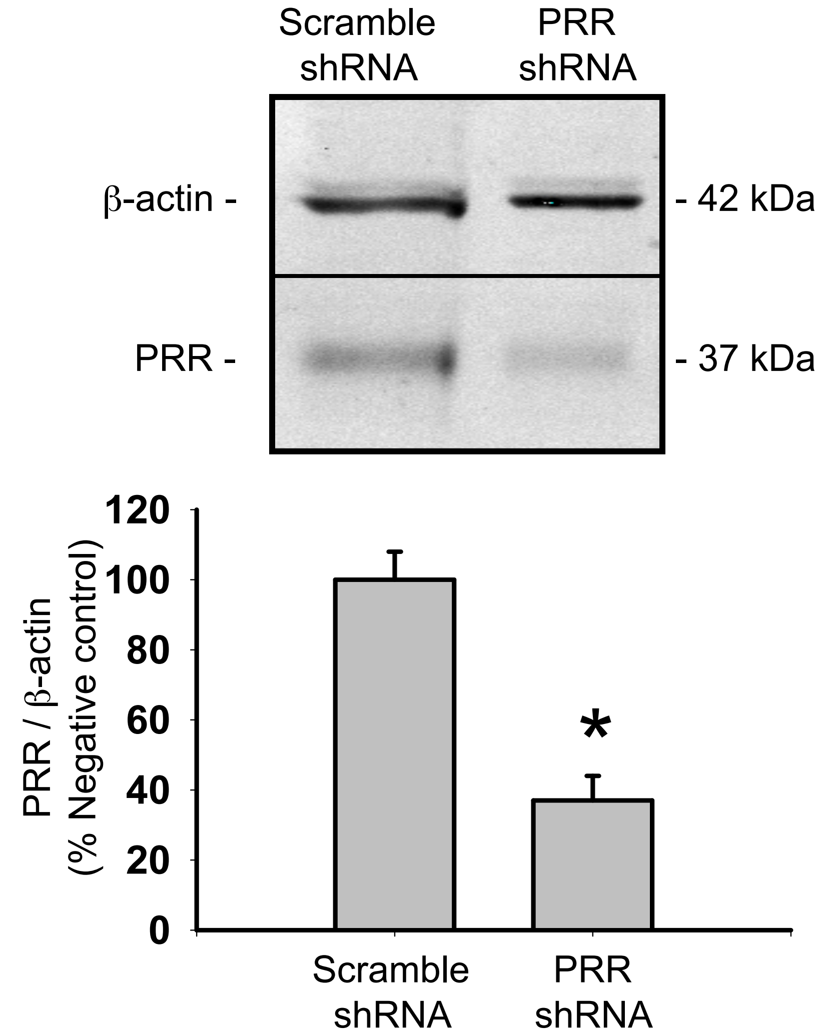
S3



Supplementary data 3. AngII levels assayed with different incubation times in cell culture media in the presence of 70% confluent cells. AngII content was measured as described previously. [1] The experiment demonstrates that AngII is stable in cell culture media after adding a single dose of 10 nanomol/L. (n=3).

S4





Supplementary data 4. A. Representative picture showing GFP positive cells. Transfected targeted cells were epithelilal tubular cells (arrowhead) and interstitial cells (arrow) **B.** IM cells transfected with PRR-shRNA showed a decrease in PRR protein levels.

REFERENCES ONLINE SUPPLEMENT

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3. Campean V, Theilig F, Paliege A, Breyer M, Bachmann S. Key enzymes for renal prostaglandin synthesis: site-specific expression in rodent kidney (rat, mouse). *Am J Physiol Renal Physiol.* 2003;285:F19-F32.